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COMMENTARY

Vacuolar deposition of recombinant proteins in plant vegetative organs as a strategy to increase yields

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ABSTRACT

Delivery of recombinant proteins to vegetative tissue vacuoles was considered inconvenient since this compartment was expected to be hydrolytic; nevertheless there is growing evidence that certain foreign proteins accumulate at high yields in vacuoles. For example avidin, cellulolytic enzymes, endolysin, and transglutaminases were produced at high yields when were sorted to leaf central vacuole avoiding the detrimental effect of these proteins on plant growth. Also, several secretory mammalian proteins such as collagen, α1-proteinase inhibitor, complement-5a, interleukin-6 and immunoglobulins accumulated at higher yields in leaf vacuoles than in the apoplast or cytosol. To reach this final destination, fusions to sequence specific vacuolar sorting signals (ssVSS) typical of proteases or proteinase inhibitors and/or Ct-VSS representative of storage proteins or plant lectins were used and both types of motifs were capable to increase accumulation. Importantly, the type of VSSs or position, either the N or C-terminus, did not alter protein stability, levels or pos-translational modifications. Vacuolar sorted glycoproteins had different type of oligosaccharides indicating that foreign proteins reached the vacuole by 2 different pathways: direct transport from the ER, bypassing the Golgi (high mannose oligosaccharides decorated proteins) or trafficking through the Golgi (Complex oligosaccharide containing proteins). In addition, some glycoproteins lacked of paucimannosidic oligosaccharides suggesting that vacuolar trimming of glycans did not occur. Enhanced accumulation of foreign proteins fused to VSS occurred in several plant species such as tobacco, *Nicotiana benthamiana*, sugarcane, tomato and in carrot and the obtained results were influenced by plant physiological state. Ten different foreign proteins fused to vacuolar sorting accumulated at higher levels than their apoplastic or cytosolic counterparts. For proteins with cytotoxic effects vacuolar sorted forms yields were superior than ER retained variants, but for other proteins the results were the opposite an there were also examples of similar levels for ER and vacuolar variants. In conclusion vacuolar sorting in vegetative tissues is a satisfactory strategy to enhance protein yields that can be used in several plant species.

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The production of high-value proteins in plant has become a reality with numerous products on the market.[1] Plant based platforms have several advantages such as low upstream costs, no risk of contamination with human or animal pathogens, absence of bacterial toxins, easy and rapidly scale up with low investment cost, availability of different technology to reduce downstream process cost, etc. Foreign proteins can be produced by using either transient expression in leaves or transgenic expression systems in whole plants or plant cell culture.[1] Both transient and stable systems are fully scalable and several large scale manufacture facilities are available, including those that satisfy good manufacturing practice (GMP).[1] Recombinant protein yields are widely variable and depend on numerous factors such as plant species, promoter, enhancers, incorporation of intron sequences, mRNA stability, 5′ and 3′ untranslated regions, codon usage, protein folding and stability, etc.[2,3] Different technologies have been developed to increase transcription efficiency, mRNA stability and translation effectiveness and to improve protein folding and stability.[3,4] Among the post-translational factors, subcellular localization is of particular interest as it has a profound impact on

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protein yields.[2] In leaves, some complex proteins are usually targeted to the apoplastic space where proteolysis can occur[5-7]; alternatively if Golgi post-translational modifications are not necessary to obtain a fully active molecule, they can be retained on the endoplasmic reticulum (ER).[8] In leaves, an alternative destination is to target the recombinant protein to vacuoles. Plant vacuoles are multifunctional organelles, essential to plant life, which share some of their properties with the lysosomes in animal cells. Although plant vacuoles are lytic compartments, they also have unique functions such as reservoirs for ions and metabolites, plant defense, detoxification processes, general cell homeostasis, etc.[9] In seeds and specialized tissues that evolve to store high amounts of proteins, special storage compartments called protein storage vacuole (PSV) or ER derived protein bodies (PB) are found.[10] Foreign proteins sorted to these special compartments accumulate in large amounts and in stable forms for long periods of time.[3,11] Deposition of recombinant proteins in central vacuoles of vegetative tissues has initially been considered inadequate since this compartment was expected to be hostile. For example, green fluorescent protein is unstable in the central vacuole of *Arabidopsis thaliana* leaves or cultured cells since light triggers vacuolar acidification and proteolysis by cysteine proteases.[12] Despite the lytic characteristics of central vacuole, when seed storage proteins are ectopically expressed in leaf tissue, they are located in neutral vacuoles that resemble seed PSV.[13] In addition, different types of vacuoles can be generated from existing vacuoles as a consequence of environmental changes[14] or stage of development.[15] Taking all these facts into consideration, vacuoles of vegetative tissue are highly dynamic structures whose characteristics are affected by environmental conditions, development programs and even ectopic deposition of proteins. Herein we discuss the current status of the employment of vacuolar delivery in vegetative tissues as a strategy to enhance heterologous protein yields. Accumulation of foreign protein in reproductive seed storage compartments has been reviewed elsewhere.[3,11] Selected examples of vacuolar sorted recombinant proteins in vegetative tissue are presented in Table 1, and we shall highlight their particularities.

Production of egg white avidin or streptavidin in plants is of interest since they are efficient biocontrol agents.[16] Avidin binds with very high affinity to biotin, which impairs the activity of carboxylases, enzymes that are essential for cellular metabolism in different organisms including insects and plants. Taking into account that 80% of biotin pools of plant cells are located in the cytoplasm and the rest in the mitochondria and chloroplast, delivery of avidin to vacuole was hypothesized as a safe strategy to avoid detrimental effects caused by biotin sequestration. Avidin and streptavidin were expressed in transgenic *Nicotiana tabacum* fused to the NH2 terminus (Nt) vacuolar sorting signal (VSS) MESKFAHIIVFFLLATPFETLLARKESDGPE of potato proteinase inhibitor I (PPI-I) that is sufficient to target to vacuoles that have δ-TIP on their tonoplast defined as ΔV.[14] ΔV–sorted avidin yields in leaves were around 1.5 % TSP and remained relatively constant throughout leaf lifetime. Avidin was detected in protein body-like structures within the vacuole. Plants had a normal phenotype and produced fertile pollen and seeds.[16] Furthermore, avidin was also fused to a different type of VSS: sugarcane legumain sequence specific (ssVSS) that targets to lytic vacuole (LV). The expression was analyzed in transgenic sugarcane.[17] The highest avidin levels in leaves, stem and roots were found for the ΔV sorted version, compared to the LV, ER, apoplast or cytosol targeted variants, but these plants developed a biotin deficient phenotype. In contrast, sugarcane plants that expressed LV-avidin had a normal phenotype but avidin suffered a site-specific limited proteolysis.[17] Therefore, sugarcane ΔV was shown to be a stable environment for recombinant protein accumulation. It is worth noticing that the co-existence of 2 different types of vacuoles in the same cells has been described in a limited number of cell types[18]; sugarcane has the unusual capacity to accumulate sucrose in stem cell vacuoles and contains several types of vacuoles that differ in their pH and capacity to hydrolyze different substrates.[19] Unlike sugarcane, *N. tabacum* leaves does not specialize in storage. However, they were able to accumulate ΔV–sorted avidin in a stable form.

A further example of stable deposition of proteins in sugarcane vacuoles is the cellulolytic enzyme that also needs to be compartmentalized to avoid interference with the cell wall structure. The production of this enzyme is of interest to make cost-competitive cellulosic ethanol. Fungal cellobiohydrolase (CBH) I and II and bacterial endoglucanase (EG) accumulated to higher levels when fused to the barley polyamine oxidase Ct VSS compared to the fusion to an ER-retention signal.[20] Yields of CBH I, CBH II and EG

Table 1. Impact of vacuolar targeting on vegetative tissue on recombinant protein yields.

Protein	Production Host	Transformation Method	Plant Organ	Vacuolar Signal	Yields of proteins sorted to different subcellular Localizations				Level, stability or other relevant information	References
					ER	Vacuole	Apoplast	Cytosol		
Egg white Avidin Streptavidin	*Nicotiana tabacum* (tobacco)	Stable	Leaves	Nt VSS (MESKFAHIIVFFLLATPF ETLLARKESDGPE) potato proteinase inhibitor I (PPI-I)		1.5%			Avidin was found in protein body-like structures within the vacuole	Murray et al., 2002 [16]
Egg white Avidin	*Saccharum officinarum* (sugarcane)	Stable	Stems (cane)	Nt ssVSS s legumain (LV) Nt VSS (PPI-I) (ΔV)	NR	NR			ΔV - avidin yield was higher than ER-avidin and LV-avidin ones.	Jackson et al., 2010 [17]
Cellobiohydrolase I CBH I Cellobiohydrolase II CBH II Endoglucanase (EG)	*Saccharum officinarum* (sugarcane)	Stable	Green leaves	Ct VSS (DELKAEAK) barley polyamine oxidase	2.38 AU/mg 5.92 AU/mg 210.1 ng/mg	11.18 AU/mg 7.33 AU/mg 281.36 ng/mg			vacCBH II yield was 2- fold higher than ER-CBH II.CBH I and CBH II yields were reduced in senescent leaves and EG was not detected	Harrison et al., 2011 [20]Harrison et al., 2014 [21]
Bacteriophage CP933 endolysin (EL)	*Nicotiana benthamiana*	Transient (PVX)	Leaves	Nt VSS potato proteinase inhibitor I (PPI-I) (ΔV)		NR	0.6mg/g	NR	Cyto- EL produced severe growth detriment. Vac-EL had no toxic effect	Kovalskaya et al., 2016 [22]
Human tissue trans-glutaminase (TG2)	*Nicotiana benthamiana*	Transient	Leaves	Ct VSS (KISIA) Amaranth 11S globulin	9.5 μg/g	9.9 μg/g	0.6 μg /g	1.1 μg /g	ER-TG2 and vac-TG2 yields were 9- to 16-fold higher cyto-TG2 and sec-TG2	Marin Viegas et al., 2015 [23]
Glucocerebrosidase	*Daucus carota subsp. sativus* (carrot)	Stable	suspension culture cells	Ct VSS (DLLVDTM) Tobacco chitinase A	—	NR	—		Vacuolar glucocerebrosidase was stable	Shaaltiel et al., 2007 [24]
Collagen (rhCOL1)	*Nicotiana tabacum* (tobacco)	Stable	Leaves	Nt ssVSS (NPIRL) barley aleurain	—	2%	NS	NS	vac- rhCOL1 yield was 10-fold higher than apo- and cyto- rhCOL1 ones.	Stein et al., 2009 [25]
Silk-like protein (DP1B)	*Arabidopsis thaliana*	Stable	Leaves	Nt ssVSS (NPIRL) sweet potato sporamin	6.7%	NS	8.5%		Vacuolar DP1B was unstable	Yang et al 2005[26]
human α1-proteinase inhibitor (α1-PI)	*Solanum lycopersicum* (tomato)	Stable	Leaves	Nt ssVSS (NPIRL) sweet potato sporamin	3.05%	1.89%	1.40%	0.458%	ER- α1-PtdIns yield was 1.6-fold-higher than vac- α1-PI yield	Jha et al., 2012 [27]
Human complement 5a C5a	*Nicotiana tabacum* (tobacco)	Stable	Leaves	Ct VSS (AFVY) Phaseolin 7S globulin	0.0003%	0.001%	0.0002%		Vac C5a yield was 3 to 5-fold higher in ER-C5a and Apo-C5a	Nausch et al., 2012 [28]
Human Complement 5a C5a	*Nicotiana benthamiana*	Transient	Leaves	Ct VSS (AFVY) Phaseolin 7S globulin	0.2%	0.7% 558 μg/g			Vac C5a yield was 3.5-fold higher than ER-C5a yield	Nausch et al., 2012 [28]
Interleukin 6 (IL6)	*Nicotiana tabacum* (tobacco)	Stable	Leaves	Ct VSS (AFVY) Phaseolin 7S globulin	0.005%	0.0008%	0.0005%		ER-IL6 yield was 625 fold higher than vac-IL6 yield	Nausch et al., 2012 [29]
Human Ig G1 and G4	*Nicotiana tabacum* (tobacco)	Stable	BY2 cells	Ct VSS (DLLVDTM) Tobacco chitinase A	NR	NR	NR		Apo-IgG yield was higher than ER- and vac-IgG ones.	Shaaltiel et al., 2012 [30]Tekoah et al.2015 [31]
Mouse IgG	*Nicotiana tabacum* (tobacco)	Stable	BY2 cells	Nt ssVSS (NPIRL) sweet potato sporamin	—	28.5-80 ng/g	—	—	IgG exhibit paucimannose glycan structure	Misaki et al., 2011 [32]
Mouse Ig G1	*Nicotiana benthamiana*	Transient	Leaves	Ct VSS (KISIA) and ssVSS (NIFRGF) Amaranth 11S globulin	1.7%	1.6%	0.13%	NR	ER- and vac-Abs yields were 10-15-fold higher than sec-Ab	Ocampo et al., 2016 [33]

Foreign proteins were grouped based on their characteristics in: I) cytotoxic or with detrimental effect on plant growth (light blue), II) fibrous proteins (yellow). III) secretory mammalian protein (pink). Ct, COOH terminus, Nt, NH2 terminus, VSS: vacuolar sorting signal, LV: lytic vacuole, ΔV: delta vacuole, %: g. protein of interest per 100 g total soluble protein, NR no reporter, NS: no stable, AU: arbitrary units.

were reduced in senescent leaves probably due to endo- and exo-peptidases released during leaf senescence.[20,21] These results emphasize the importance of the development stage for a foreign protein deposition in leaves.

Another toxic protein that was successfully expressed in *Nicotiana benthamiana* leaves is the bacteriophage CP933 endolysin (EL), an enzyme that hydrolyzes peptidoglycan. This feature makes EL a promising antimicrobial agent for antibiotic-resistant microorganism. EL was targeted to ΔV by fusion to Nt VSS of PPI-I. Plants producing the ΔV-EL did not exhibit the severe detrimental effects on growth found in cytosolic-EL plants. This result suggests that sequestration of EL in the vacuole reduces its toxicity.[22]

Transglutaminases 2 (TG2) are also challenging proteins for the different expression systems since their cross-linking activity has toxic effects on cell growth and development.[34] Attempts to produce transgenic BY-2 expressing cytosolic-TG2 were unsuccessful, probably due to the toxic effect of this enzyme.[35] We have recently shown, by using transient expression in *Nicotiana benthamiana* leaves, that ER-TG2 and vac-TG2 yields are 9 to 16-fold higher than cytosolic and secretory versions.[23] Therefore, compartmentalization of TG within the endomembrane systems avoids cytosolic toxicity and also apoplastic proteolysis.

Glucocerebrosidase is an acid-β-glucosidase used in enzyme replacement therapy for Gaucher's disease, a rare lysosomal storage disorder. The manufacture cost of this enzyme in other expression systems was very high; therefore, Protalix Biotherapeutics developed a technology to produce it in carrot suspension culture. Two variants were produced by fusion to the Ct-VSS from tobacco chitinase A (DLLVDTM) and also to an ER retention sequence. Vacuolar glucocerebrosidase yields were higher than ER variants. In addition, paucimannose glycan structures in vacuolar glucocerebrosidase favored mannose receptor-mediated uptake by macrophages which made this variant more effective therapeutically than the ER version.[24]

Deposition of proteins with the ability to produce fibers on vacuoles of vegetative tissues has also been assayed. For example, human collagen type I (rhCOL1) is a heterotrimeric protein that requires essential posttranslational modifications to self-organize into fibers. These modifications are performed by human prolyl-4-hydroxylases (P4H) and lysyl hydroxylase 3 (LH3). The genes encoding for rhPCOL1 α 1 and α 2 chains, P4H α, P4H β, and LH3 were expressed in transgenic tobacco plants using different targeting signals to sort to vacuoles (barley aleurain Nt ssVSS MAHARVLLLALAVLATAAVA-VASSSSFADSNPIRPVTDRAASTLA), apoplast or cytosol.[25] Cytoplasm sorted rhCOL1 was not detectable, while apoplast-targeted rhCOL1 yields were very low. Vac-rhCol1 yields were the highest, and molecules were able to form stable triple helical structures that were fully functional in inducing proliferation of human cells.[25] These results highlight that leaf vacuoles are a suitable compartment to store rhCOL1. Another fibrous protein: the spider dragkine silk (DP18) was also fused to a ssVSS: the NPIRL from sporamine. Different sorted version were expressed in transgenic *A. thaliana*. However, in this case only the ER variant accumulated at high levels while vac-DP18 was not stable.[26]

Moreover, different biopharmaceuticals proteins have been produced successfully in vacuoles of vegetative tissues. Human α 1-proteinase inhibitor (α1-PI), also known as α1- antitrypsin, is a serine protease inhibitor essential to keep lung elasticity. The production of a glycosylated biologically active α1-PI has been assayed in different systems, but none of them could fulfill the requirements of cost-effective production, clinical safety and biological activity. Consequently, this protein was expressed in *Solanum lycopersicum* (tomato) by using different sorting signal to target to cytosol, apoplast, ER and vacuole [Nt ssVSS (NPIRL) sweet potato sporamine]. The highest average yields in T1 progeny were 3.05 % TSP for ER, 1.89% for vacuolar, 1.40% for apoplast and 0.08 % for cytosolic forms. Although vacuolar α1-PI was produced in tomato leaves with comparable yields respect to the ER form, the enzyme exhibit lower specific activity.[27]

Another example of therapeutic protein sorted to vacuoles is human complement factor 5a (C5a) that was expressed in leaves and seeds of transgenic *N. tabacum*. The Ct VSS AFVY of phaseolin 7S storage protein was used to target C5a to the vacuole. Vac-C5a yields were 3 to 5-fold higher than ER- or apo-C5a. These C5a versions were also transiently expressed in *Nicotiana benthamiana* leaves using an hybrid binary vector (MagnICON) based on tobacco mosaic virus (TMV) that contains viral sequences required for RNA replication leading to amplification of RNA transcript, and also the highest yields were

detected for vac-C5a variants (3.5-fold higher than ER).[28] Therefore vacuoles were found as the most suitable compartment to produce C5a, and the higher yields were attributed to the selection of the AFVY Ct, which is considered a PSV-specific targeting signal.[28] The authors argue that although lytic vacuoles are expected to be prevalent in vegetative tissues, the expression of storage protein derived sequence could induce the formation of storage organelles in vegetative tissue.[28] Unexpected transient overexpression of ER-C5a and vac-C5a in *Nicotiana benthamiana* was accompanied by cytotoxic effects and a rapid decrease of recombinant C5a even though it is not anticipated that this protein could interfere with plant metabolism.[28] Due to toxic effect, yields for vac-C5a (0,7%TSP) were higher than ER-C5a (0.2% TSP) but for non toxic protein using MagnICON system around 10% TSP were expected. Using the same sorting strategy, human interleukin (IL) 6 was expressed in stable transgenic *N. tabacum* and temporally in *Nicotiana benthamiana*, but ER sorted-IL6 produced yields 6.25 higher than vac-IL6, although IL6 was also fused to AFVY Ct VSS.[29] ER-targeted IL6 in leaves using the MagnICON system resulted in yields of up to 7% TSP and none cytotoxic effect were observed.[29]

Deposition of antibodies (Abs) in vacuoles of vegetative tissues had also been studied and information about trafficking and modifications in different compartments was obtained based on its N-glycosylation pattern. The N-glycosylation of proteins starts in the ER with the transfer of the Glc3Man9GlcNAc2 oligosaccharide to a specific Asn residues on the nascent polypeptide followed of a limited trimming in both the ER and Golgi and sequential addition of monosaccharides, as the protein travel through the Golgi complex, to yield complex N-glycans, typically GlcNAc2Man3FucXylGlcNAc2 structures (Fig. 1).[36] Secretory plant N-glycans contain galactose β 1,3 and fucose α 1,4 linked to the terminal GlcNAc forming the called Lewis A oligosaccharide structure.[36] In addition, paucimannosidic, that derives from the removal of terminal GlcNAc residues from

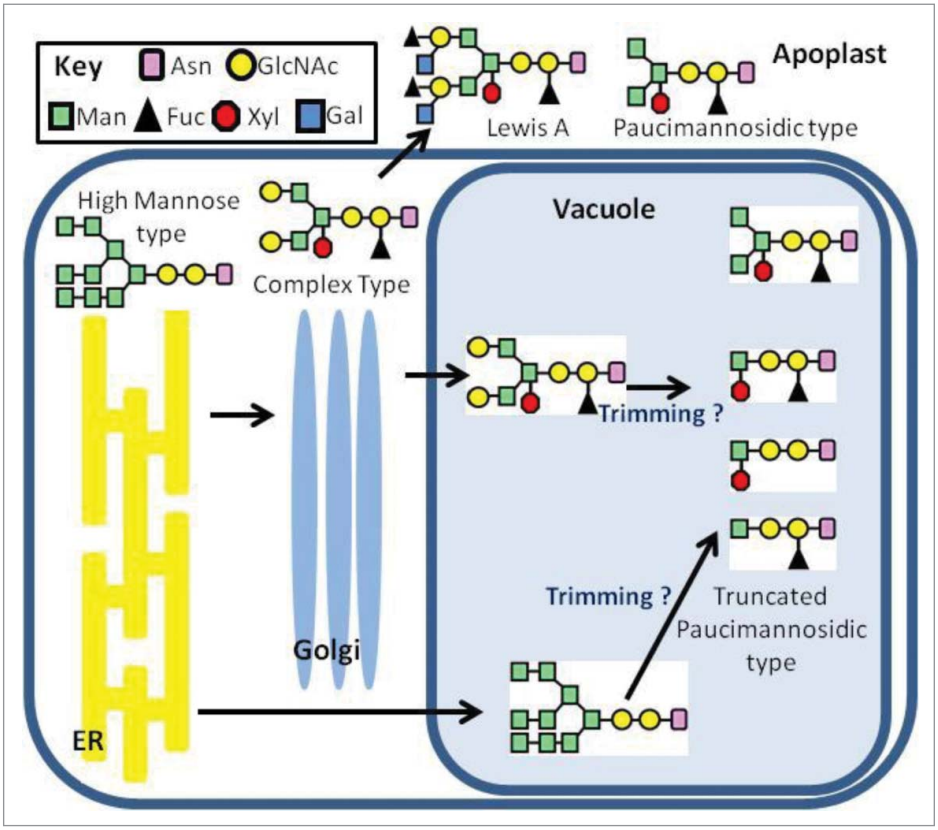

Figure 1. Schematic representation of the plant N-glycans processing pathway. The arrows indicate the trafficking pathways. N-glycosylation of vacuolar proteins suggests a direct ER-vacuole transport route bypassing the Golgi apparatus, and also the classical Golgi-dependent pathway. ER, endoplasmic reticulum; Asn, asparagine; GlcNAc, N-acetylglucosamine Man, mannose; Fuc, fucose; Xyl, xylose ; Gal, galactose. **High-mannose type**: Man 9 Man9GlcNAc2; Man 8: Man8GlcNAc2. Man 7: Man7GlcNAc2 oligosaccharides. **Complex type**: GlcNAc2Man3GlcNAc2 and GlcNAc2Man3XylFucGlcNAc2 oligosaccharides; **Lewis** (**A**) GalFucGlcNAc2Man3XylFucGlcNAc2 oligosaccharide. **Paucimannosidic type**: Man3XylFucGlcNAc2; ManXylFucGlcNAc2, ManXylGlcNAc2, ManFucGlcNAc2 oligosaccharides.

complex N-glycans, are present in vacuolar and secreted proteins.[36-38] Humans IgG1 and IgG4 were expressed, in transgenic suspension-cultured of tobacco BY2, sorted to different compartments, resulting in secretory versions producing higher yields than ER and vacuolar versions.[30,31] In addition, a mouse IgG fused to the sporamin Nt ssVSS (NPIRL) was produced also in transgenic BY2 cells, in intact form at levels of 8.5-80 ng/g and paucimannose Man3FucXylGlcNAc2 as main N-glycan structure.[32] We had also produced a vacuolar mouse IgG1 by transient expression in *Nicotiana benthamiana* leaves. To target the Ab to vacuoles, the heavy chain was fused to 2 sequences derived from amaranth 11S storage protein: KISIA Ct VSS (vac1-Ab) and NIFRGF ssVSS (vac2-Ab), and as control ER-Ab and sec-Ab variants were produced. ER-Ab and vac-Abs accumulations levels were 10-15-fold higher than sec-Ab.[33] Although NPIRL motif is typical of lytic vacuole proteins and the short and hydrophobic C terminus are distinctive of storage proteins[39], no significant differences were found between vac1-Ab and vac2-Abs yields. Another important finding of our work, was the presence of oligomannosidic (Man 7-9) as the major glycoform in vac-Abs (75%), what suggests a direct transport from the ER to vacuoles bypassing the Golgi apparatus.[33] Furthermore vac-Abs have 25% of GlcNAc2Man3XylFucGlcNAc2 therefore removal of terminal GlcNAc residues in the vacuole did not occur.[33]

Ability of plants cells to accumulate toxic proteins in vacuoles is not surprising since variety of natural and synthetic chemicals are inactivated and transported to vacuole by different detoxification mechanisms.[40] For example, some xenobiotic compounds are conjugated to glutathione in the cytosol and then transported to vacuole by an ATP-dependent tonoplast transporter.[40] Plant secondary metabolites, such as flavonoids are also delivery to vacuoles using tonoplast transporters, but for anthocyanins a transport mediated by vesicle trafficking has also been described.[41] Anthocyanins are uploaded into the ER compartment by membrane translocators, followed by an ER to vacuole transport either by a direct route (bypassing Golgi) or by Golgi dependent pathway.[41]

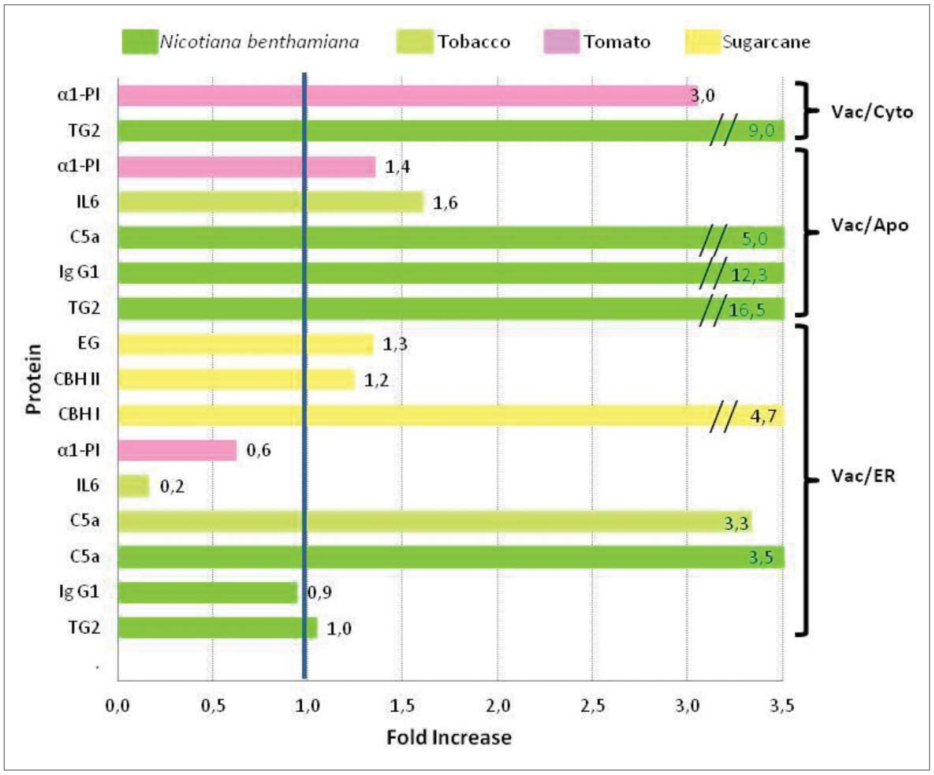

Figure 2. Comparison of yields obtained when proteins were sorted to different compartments in the secretory pathway. X axis represents the ratio of yields obtained for vacuolar-/ER-versions (Vac/ER), vacuolar-/secreted-forms (Vac/apo) or vacuolar-/cytosolic-variants (Vac/cyto). The obtained value are shown in each bar. The X axis has a maximum value of 3.5 and higher values are not to at scale. The bar color represents the plant species used to express the different proteins showed in the Y axis. α1-PI: human α1-proteinase inhibitor, TG2: Human tissue transglutaminase, IL6: Interleukin 6, C5a: Human complement 5a, IgG1: Immunoglobulin G1, EG: Endoglucanase, CBH I: Cellobiohydrolase I , CBH II: Cellobiohydrolase II.

From the 15 vegetative vacuole-sorted proteins listed in Table 1, accumulation levels of variants fused to different targeting signals, were informed only for 8 and the results are summarized in Fig. 2. Vacuolar sorted variants had yields 3,0-9,0 and 1,2-16,5-fold-times higher than their cytosolic or apoplastic counterparts, respectively. Although these values are based on a reduced number of proteins (5), other proteins in Table 1 such as rhCol1, EL, CBH I, CBH II and EG had the same behavior, but apoplast or cytosolic yields were not reported due to instability or low levels (Table 1). Therefore for 10 proteins the fusion to vacuolar sorting signals enhanced the production of recombinant proteins. The impact of vacuolar versus ER location on foreign protein accumulation was variable. For proteins that produced detrimental effect in cellular metabolism, such as CBH II, EG, C5a and CBH I, vacuolar sorted forms yields were 1,2-4,7 higher than ER retained variants. In contrast, ER-IL6- and ER-α1-PI had higher accumulation levels than their vacuolar counterparts. Vacuolar- and ER- sorted forms of mouse IgG1- and TG2 had equal protein yields. These results indicate that a vacuolar sorting strategy is superior to apoplastic or cytosolic targeting, and could be also better that ER retention.

To target foreign proteins to vacuoles, different signals have been used located either in the N or C terminus, including a NPIR/NPIXL sequence specific motif typical of protease inhibitors or vacuolar proteases [Nt-ssVSS of barley aleurain, legumain, and sweet potato sporamin] or short-hydrophobic Ct characteristic of chitinases, cereal lectins or storage proteins (Ct-VSS of phaseolin 7S globulin, amaranth 11S protein, barley polyamine oxidase and tobacco chitinase A).[42] Both types of VSSs were demonstrated to be useful to maximize recombinant protein levels. Although Nt-VSS and Ct-VSS were supposed to target proteins to lytic and storage vacuoles, respectively, both type of motif targeted proteins to central vacuole of vegetative tissue by a molecular mechanism that is currently unclear.[43] The N-glycosylation pattern of the foreign exemplified differences in vacuolar sorting mechanism, for example glucocerebrosidase-Ct-VSS exhibited paucimannose structures and complex glycan added in the trans Golgi; supporting a Golgi dependent transport[24] while a mouse IgG1 fused to a ssVSS and Ct-VSS of amaranth storage proteins is decorated with Man 7 and Man 8 glycans supporting a direct transport bypassing the Golgi[33] (Fig. 1) These glycosylation patterns maybe adequate for some foreign proteins such as glucocerebrosidase whose vacuolar variant is easily internalized by human cells, but it is no convenient for therapeutic antibodies since effectors' functions are dependent of heavy chain glycosylation. Nevertheless vacuolar sorted antibodies could be useful for diagnostic, purification and other research applications.

Table 1 and Fig. 2 also showed also that vacuolar targeting is an effective strategy to produce high yields of intact and fully active proteins in several plant species such as *Nicotiana benthamiana*, tobacco, tomato, sugarcane and carrot. The only species that showed unsatisfactory results was arabidopsis. Other important conclusion is that the accumulation levels of vacuolar sorted foreign proteins were dependent of the developmental stage and physiological condition of leaves, therefore to achieve high yields samples should be collected prior senescence.[20,21]

In conclusion, vacuolar sorting in vegetative plant tissues is a satisfactory strategy to enhance protein yields and the obtained results are superior than targeting to cytosol or to apoplast an could be also better than ER retention for cytotoxic proteins. For recombinant glycosylated proteins will be desirable to have a better understanding of the mechanism that control vacuolar delivery by the different targeting routes in order to predict glycosylation pattern.

Abbreviations

Ab	monoclonal antibody
CBH I	Cellobiohydrolase I
CBH II	Cellobiohydrolase II
Ct	COOH terminus
C5a	human complement 5a
DP1B	silk-like protein
EG	endoglucanase
EL	bacteriophage CP933 endolysin
ER	endoplasmic reticulum
GMP	good manufacturing practice
IL6	interleukin 6
IgG	immunoglobulin
LV	lytic vacuole
Nt	NH2 terminus
PB	protein bodies
PSV	protein storage vacuole
PPI-I	potato proteinase inhibitor I
rhCOL1	human collagen type I
ssVSS	sequence specific VSS
TG2	human tissue transglutaminase
TIP	tonoplast intrinsic proteins

TSP	total soluble protein
VSS	vacuolar sorting signal
α1-PI	human α1-proteinase inhibitor
ΔV	delta vacuole

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

VSMV; CGO and SP wrote the paper. Authors contributed equally to this work.

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