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Letter

Response to Gomord *et al.*: Golgi-bypassing: delivery of biopharmaceutical proteins to protein storage vacuoles in plant bioreactors

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The recent article by Gomord *et al.* [1] summarized and discussed the current attempts and potential problems in exploiting plants as bioreactors to produce pharmaceutical proteins. Because the high-mannose-type N-glycans are structurally identical in plants and mammals [1], potentially, plants are excellent economic and pathogen-free bioreactors to produce glycosylated biopharmaceutical proteins such as glycan-specific antibodies for oral administration. However, the plant-unique, Golgi-derived complex N-glycan modifications made to these plant-made biopharmaceutical proteins are potential immunogens when they are delivered, parenterally, into the human body; therefore, several strategies have been developed and tested to reduce the potential immunogenicity of these complex N-glycans. For example, the expressed recombinant proteins were specifically retained in the endoplasmic reticulum (ER) by adding an ER retention signal, thus avoiding Golgi trafficking and complex glycan modifications to these pharmaceutical proteins in transgenic plants [1,2]. Alternatively, transgenic plants with knockout or knockdown of glycosyltransferases – enzymes that are responsible for Golgi-specific complex glycan modifications in plants – have been used as the starting materials for transferring and producing pharmaceutical proteins [3]. As a result, no complex glycan modifications occur to these plant-made biopharmaceutical proteins, even although they are trafficked through the Golgi apparatus [1,3].

Here, we offer an alternative approach that would direct the biopharmaceutical proteins from the ER to a native storage compartment of plants, the protein storage vacuoles (PSVs), thereby avoiding the plant Golgi-specific complex glycan modifications and reducing their potential immunogenicity. Such an approach relies on our understanding of the molecular mechanisms of protein trafficking and protein transport in the plant secretory pathway leading to the PSVs.

As shown in Figure 1, the plant secretory system consists of several membrane-bound organelles, such as the ER, Golgi apparatus, prevacuolar compartments (PVCs) and vacuoles, including the transport vesicles that mediate protein traffic [4,5]. Plant cells contain both lytic vacuoles and protein storage vacuoles – lytic vacuoles are the equivalent of mammalian lysosomes or yeast vacuoles [6]. During seed development, plants accumulate proteins in their abundance in PSVs, making these

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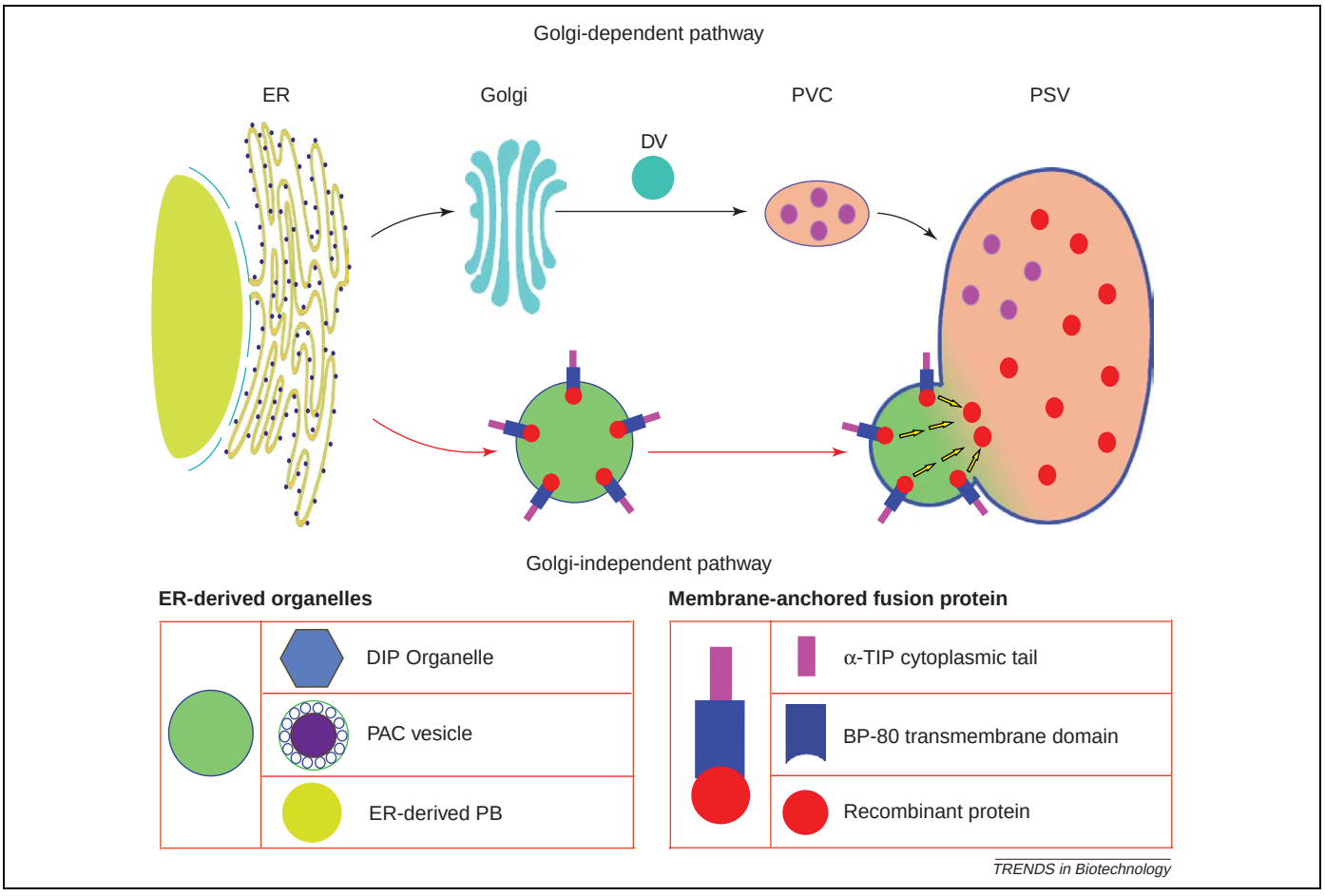

Figure 1. Delivery of pharmaceutical proteins to protein storage vacuoles by ER-derived vesicles and/or organelles and membrane-anchored fusion in plant bioreactors. Proteins reach protein storage vacuoles (PSVs) by either Golgi-dependent or Golgi-independent pathways. The Golgi-dependent pathway is represented by the trafficking of a storage protein destined for PSVs from the endoplasmic reticulum (ER) to the Golgi apparatus and then to a multivesicular prevacuolar compartment (PVC), mediated by Golgi-derived dense vesicles (DV) in pea cotyledons [8]. The transport of proteins to PSVs by the Golgi-independent pathway uses ER-derived organelles, including the DIP (dark intrinsic protein) organelle in tobacco seed [9], the PAC (precursor accumulating) vesicles in pumpkin seed [11] and ER-derived PB (protein body) in rice and wheat [12,13]. Membrane-anchored fusion proteins containing the BP-80 transmembrane domain and the cytoplasmic tail of α-TIP (tonoplast intrinsic protein) were targeted to PSVs in transgenic tobacco seed, where the recombinant (pharmaceutical) protein was separated from the membrane and deposited into the PSVs in transgenic tobacco seed [9,19]. Such protein transport to the PSVs for accumulation, using ER-derived vesicles and bypassing the Golgi, would prevent unwanted complex glycan modifications to pharmaceutical proteins in plant bioreactors.

vacuoles an attractive and intrinsic compartment for storing large amounts of pharmaceutical proteins in plant bioreactors [7].

In plant cells, proteins reach PSVs either by the Golgi or the ER; in the latter case, they bypass the Golgi directly (Figure 1). In pea cotyledons, Golgi-derived dense vesicles (DVs) mediate the transport of storage proteins to PSVs [8]. However, several studies in other plants show that proteins reach PSVs directly from the ER, mediated by various morphologically distinct ER-derived transport vesicles or organelles (Figure 1). For example, ER-derived DIP (dark intrinsic protein) organelles might serve as transport vesicles or prevacuolar compartments for proteins targeting to PSVs in tobacco seeds [9,10]. Similarly, ER-derived PAC (precursor accumulating) vesicles are responsible for transporting the 2S albumin storage proteins to PSVs directly from the ER in developing pumpkin seeds [11]. In developing rice and wheat, the storage protein prolamine aggregates in the ER lumen, and is then packaged into the protein body directly [12,13]; however, the transport of the other storage protein, globulin, to PSVs in developing rice endosperm was mediated by PAC-like vesicles derived, directly, from the ER [14]. Therefore, pharmaceutical proteins can be delivered by either ER-derived transport vesicles or organelles to PSVs for storage (Figure 1). Such direct ER-to-PSV delivery would avoid the unwanted Golgi-specific complex glycan modifications to pharmaceutical proteins, enable their stable accumulation in plant bioreactor PSVs and provide an attractive and simple alternative for producing plant-derived and non-immunogenic pharmaceutical proteins. The challenge is how to deliver pharmaceutical proteins to PSVs by the Golgi-independent pathway, which uses ER-derived vesicles and/or organelles? In general, proteins reach PSVs because they contain vacuolar-sorting determinants that are recognized by vacuolar-sorting receptor (VSR) proteins [4], where a RMR (a receptor-like protein) and several VSRs might function as sorting receptors for the PSV pathway [9,15,16]. In addition, unique transmembrane and cytoplasmic sequences of integral membrane proteins might serve as anchors for delivering pharmaceutical proteins to vacuoles directly from the ER [17,18]. For example, the sequences of BP-80

transmembrane domain (TMD) and the cytoplasmic tail (CT) of α-TIP (tonoplast intrinsic protein) function as anchors to deliver a reporter to PSVs using ER-derived DIP organelles in transgenic tobacco seeds (Figure 1) [9]. More recently, when a YFP (yellow fluorescent protein) was fused to the same TMD and CT sequences, and the resulting fusion was expressed in transgenic tobacco plants, the membrane-anchored YFP fusion protein was successfully delivered to the PSVs, directly from the ER. Upon reaching seed PSVs, the YFP portion was proteolytically separated from the membrane anchor by the enzymatic system of the cell and, thus, resulted in the accumulation of soluble YFP within this organelle, enabling easy downstream purification (Figure 1) [19].

In conclusion, unwanted complex N-linked glycan modifications to pharmaceutical proteins can be avoided by manipulation of protein transport pathways; currently, three approaches are on the table for making non-immunogenic plant-derived pharmaceutical proteins. The strategy of ER accumulation is successful but over-accumulation of proteins within the ER lumen might induce protein degradation by the ER-associated degradation (ERAD) pathway or cause ER-associated unfolded protein response (UPR) [20]. The approach of knockout or knockdown of Golgi-specific enzymes, such as glycosyltransferases, requires the generation of knockout transgenic plants as a starting platform for further transformation, which might encounter the problem of gene silencing. In addition, these Golgi-specific enzymes might be essential for the function of a plant. The third approach discussed here would use unique targeting sequences to deliver biopharmaceutical proteins to the PSVs using ER-derived transport vesicles or organelles. Seed PSVs provide an ideal and attractive compartment for protein accumulation and subsequent easy purification.

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