Post-translational modifications of recombinant proteins produced in plants: Review

T.H. Mamedov[1,2]*, N. Gun[1]

[1] *Department of Agricultural Biotechnology, Faculty of Agriculture, Akdeniz University, Antalya, Turkey*
[2] *International Laboratory of Molecular Bioengineering, Institute of Molecular Biology & Biotechnologies, Azerbaijan National Academy of Sciences, 11 Izzat Nabiyev, Baku AZ1122, Azerbaijan*
*** *For correspondence:*** *tmammedov@gmail.com*

Plant transient expression system has become one of the promising expression platforms for the production of a variety of vaccines, therapeutic proteins, enzymes and antibodies. Plant-based expression systems have a number of potential advantages, such as high production capacity, scalability, safety and low cost of production of biologically active products. In addition, plant expression systems possess eukaryotic posttranslational modifications (PTMs). However, the plant expression system lacks some important PTMs, present in mammalian cells, which are critical for the functional activity of produced proteins. PTMs significantly affect the protein properties such as stability, solubility, interactions with other molecules and localization. Thus, for the expression of various proteins with different PTM statuses, more flexible approaches are needed to preserve their native sequence and biological function. In this mini-review, we discuss the PTMs of recombinant proteins produced in plants and their importance for the production of functionally active recombinant proteins for medical, agricultural, and industrial applications.

Keywords: *Post-translational modifications, N-glycosylation, in vivo deglycosylation, sialylation, furin processing, gamma-carboxylation, sulfation, plant transient expression system*

INTRODUCTION

The selection of a suitable expression system is important for the production of properly folded, stable, safe and functional active recombinant proteins. Plant transient expression system became the promising platform for expression of important recombinant proteins such as vaccines, therapeutic proteins, monoclonal antibodies, human, industrial enzymes, bacterial toxins (colicins), etc. (Gils et al., 2005; Bendandi et al., 2010; Jul-Larsen et al., 2012; Pua et al., 2016; Stoger et al., 2014; Fischer and Buyel, 2020; Tusé et al., 2015; Wang et al., 2019; Rybicki, 2014; 2018; 2020; Lomonossoff et al., 2020; Mamedov et al., 2012; 2016; 2017; 2019a, 2019b, 2020, 2021). Recently, taking advantage of plant molecular and protein engineering, strategies have been developed that allow the successful production of complex recombinant proteins in their native-like form. (Parsons et al., 2012; Loos et al., 2015; Kallolimath et al., 2016; Mamedov et al., 2012; 2017; 2019a; Margolin et al., 2020). Notably, the majority of proteins undergo PTMs, which is most important for functional activity, immunogenicity, transport, stability, solubility of proteins (Gomord and Faye, 2004). PTMs are covalent processes, generally enzymatic modification of proteins that make alterations on the primary structure of proteins (Webster and Thomas, 2012). PTM can occur at the protein's C- or N- termini or on the side chains or amino acids (Voet and Pratt, 2006). These modifications can affect the protein structure, stability, subcellular localization and interactions. Notably, many PTMs were shown evolutionarily conserved, however, there are also some important kingdom-specific modifications that should be taken into account when expressing recombinant proteins (Kwon, 2005). Plant-based expression systems obtain valuable acceptance as a platform for the expression of heterologous recombinant proteins with eukaryotic PTMs. In addition, the production of low-cost, scalable, fast, and safe product features, makes plant expression systems an excellent alternative for mammalian and other conventional expression systems (Nandi et al., 2016). In this mini-review, we evaluate the importance of PTMs on the production of recombinant proteins in plants.

N-Glycosylation PTM. Glycosylation is the most common PTM in eukaryotic cells and defines the attachment of glycans to the peptide backbone of the proteins. The attached carbohydrates to proteins are classified in two major categories: i) N-linked glycans, where glycans link the amide group

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of asparagine (Asn) and ii) O-linked glycans, where glycans link to the hydroxyl group of Serine (Ser), threonine (Thr), hydroxylysine (Hyp) residues of proteins. N-linked glycosylation or Asparagine (N) -linked glycosylation is one of the most important and common types of PTMs, which can affect the proper folding, stability, transport of proteins (Helenius and Aebi 2001, 2004). N-glycosylation also significantly contributes to the volume and charge of glycoproteins, which is important for ligand binding and conformational stability (Hu et al., 1994, Li and d'Anjou, 2009, Srikrishna et al., 2002). A number of studies showed that there is a relationship between the N-glycosylation status of cellular proteins and drug sensitivities (Faye et al., 2005). N-linked glycosylation of proteins occurs in the endoplasmic reticulum (ER) and Golgi apparatus for the proteins entering the secretory pathway (Strasser, 2016). Over the protein maturation, only properly folded proteins are passed to the cell surface whereas misfolded proteins are retained in the ER (Lannoo & Van Damme, 2015). Retention of the proteins in ER lumen is based on the addition of H /KDEL sequences to the C-terminal end of the protein (Gomord et al., 1997). ER-resident proteins contain high 6-9 mannose type N-glycans (Man6-Man9) that are structurally the same in plants and mammals (Saint-Jore-Dupas et al., 2007; Strasser et al., 2009). For successful production of recombinant proteins, one important approach is to engineer the sequence of target proteins of interest by fusing the C-terminus with the ER retention signal (KDEL) to produce proteins in the endoplasmic reticulum (ER) with high mannose N-glycan structure, which is common in humans, yeast, and plants (Saint-Jore-Dupas et al., 2007; Strasser et al., 2009). In addition, ER is equipped well with molecular chaperones and folding assistants (Margolin et al., 2020; Stronge et al., 2001), thus enabling protein folding as well as PTMs of target proteins. This approach has been successfully used for rapid and cost-effective production of a variety of recombinant proteins such as vaccine candidates such as Ffs48/45 of *Plasmodium falciparum*, PA83 of *Bacillus anthracis,* an heptameric form of PA63 of *Bacillus anthracis* (Mamedov et al., 2012; 2016; 2017; 2019a, 2019b, 2021), plague vaccine candidate (Mett et al., 2007), recombinant hemagglutinin (HA) vaccine candidates targeting influenza H1N1 and H5N1 (Shoji et al., 2011), antibodies against HIV (Yusibov et al., 2011) and complex mammalian proteins, enzymes such as furin and Factor IX (Mamedov et al., 2019a). This approach provided the production of recombinant glycoproteins with a high mannose structure that is common in humans, yeast, and plants.

In other approaches, plant-specific α1,3-fucose and β1,2-xylose attachment to N-glycan core (GnGn) in N-glycan formations in plants (Wilson et al., 2001) were eliminated, using gene silencing (sense and antisense RNAi), genome editing, and knockout strategies. These strategies were successfully applied to species *N. benthamiana* (Li et al., 2016), *Nicotiana tabacum* BY2 cells (Hanania et al., 2017), *Physcomitrella patens* (Koprivova et al., 2004), *Lemna minor* (Coxet et al., 2006) which are often used for the production of recombinant proteins. In addition, recently, these α1,3-fucose and β1,2-xylose residues were successfully removed with CRISPR-Cas9 strategy in *N. benthamiana* plants (Jansing et al., 2020).

O-linked Glycosylation. O-linked glycosylation is the second major glycosylation type. This PTM plays a critical role in the growth and development of plant-microbe interactions in plant secretory pathways (Seifert and Roberts, 2007, Stulemeijer and Joosten, 2008). O-glycosylation type is most related with Ser or Hyp residues in plant glycoproteins. The addition of O-glycans to the hydroxyl group of Hyp is unique to plants (Gomord et al., 2010; Saint-Jore-Dupas et al., 2007). There is little attention have paid to plant O-glycosylation. Recent genetic engineering studies have made it possible to exclude O-glycans from the plant secretory pathway (Parsons, 2016; Schiavinato, 2019).

Mannose-6-phosphate (M6P) modifications. Mannose-6-phosphate (M6P) modifications, which are very important for the storage of lysosomal proteins are absent in plants. In this line, the M6P elaboration pathway has been recently engineered in plants (Zeng et al., 2019) that enabled the production of safe and affordable recombinant therapeutics for enzyme replacement therapy (Shaaltiel et al., 2007; Van Patten et al., 2007). Another limitation of plant-based expression systems for the production of recombinant proteins is the formation of truncated N-glycans (paucimannosidic), produced by removing terminal GlcNAc (MMXF) residues from the core N-glycan (Liebminger et al., 2011). Using gene silencing and knockout strategies, hexosaminidase enzyme expressions can be eliminated, preventing GlcNAc hydrolysis from GnGn structures and causing an increase in complex GlcNAc-terminating N-glycans at the expense of paucimannosidic structures (Shin et al., 2017).

Specific proteases of the plant secretory pathway can significantly degrade functionally active recombinant proteins (Castilho et al., 2010)

during production in plants. Strategies such as targeting recombinant proteins to organelles, engineering the protein sequence to remove proteases sensitive regions, and co-expression of various protease inhibitors (PIs) can protect recombinant proteins from degradation in a plant expression system (Grosse-Holz et al., 2018).

Sialylation. Sialylation is an important PTM and plays a role in the immunological response, cell-cell interaction, and mediating signaling and in normal cells. Sialylated glycans, which are present on proteins and lipids, are catalyzed by enzymes namely sialyltransferases (STs), localized in Golgi (Bhide and Colley, 2017). It has been demonstrated that sialylation affects the biological activity of many therapeutically important proteins (Matsumoto et al., 1995; Mamedov et al., 2011; Bhide and Colley, 2017). For example, asialylated hEPO recombinant human erythropoietin (hEPO) protein, was shown to possess very low erythropoietic activity in vivo compared to sialylated hEPO (Fukuda et al., 1989; Spivak et al., 1989). Sialylated N-glycans have been also demonstrated to play essential roles in pathogen recognition and cancer (Bhide and Colley, 2017). It was also demonstrated that the addition of sialic acid on the N-glycans could increase the clearance rate, affect the half-life and functional activity of glycosylated PMPs (Sato et al., 2013). Numbers studies were demonstrated that in plants, protists, archaea, eubacteria, and fungi sialic acids are absent. Thus, it was shown that plants are unable to add terminal sialic acid and transfer galactose residues to the produced therapeutic proteins, in contrast to their mammalian counterparts. Plants lack N-glycans with β1,4 galactose, which is linked with sialic acid. The addition of β1,4 galactose on N-glycans is a mammalian type modification, which has not been reported to occur in plants (Bakker et al., 2001, Jacobs and Callewaert, 2009). The absence of β1,4-galactose residues is the main obstacle to the production of recombinant antibodies in plants with sialylated glycoproteins. It was demonstrated that the addition of β1,4 galactose on N-glycans facilitates complement-dependent cytotoxicity and binding (Raju, 2008). The lack of sialic acid modifications in plants limits the use of this platform for the production of important complex therapeutic mammalian proteins, antibodies, and enzymes. To address this need, the production of proteins with human-type N-glycans with sialic acid was achieved by stable or transient expression of the corresponding enzymes and pathways in *N. benthamiana* plant (Montero-Morales et al., 2018; 2019; Goritzer et al., 2019).

Notably, the presence of endogenous sialylated N-glycans was discovered in green-algae *Chomydomonas reinhardtii* (Mamedov et al., 2011), which makes this system attractive for the production of functionally active mammalian complex proteins in green algae.

***In-vivo* enzymatic deglycosylation strategy.** Plant expression systems may not be a suitable platform for proteins that do not carry N-linked glycans in their native glycan form. The in-vivo deglycosylation strategy of proteins with deglycosylation enzymes is considered an important strategy for the production of recombinant proteins in non-glycan forms in plants (Mamedov et al., 2012, 2013, 2017). Recently, Mamedov et al. (2012; 2017) developed an *in vivo* deglycosylation strategy for the production of recombinant proteins in non-N-glycosylated form in *N. benthamiana* plants, by co-expressing with bacterial deglycosylation enzymes PNGaseF (Mamedov et al., 2012) and EndoH (Mamedov et al., 2017). It was demonstrated that PNGaseF in-vivo deglycosylated proteins are highly immunogenic and appear to be more stable than their glycosylated counterparts (Mamedov et al., 2012, 2013, 2016; 2017). Notably, PNGaseF removes N-glycans from glycoproteins, causing amino acid changes in the expressed proteins due to the deamidation reaction (Mamedov et al., 2012). On the other hand, EndoH cleaves the bond between the two acetylglucosamine residues of the N-linked oligosaccharides without amino acid changes in the produced proteins while preserving the native protein structure (Mamedov et al., 2017). Moreover, EndoH in-vivo deglycosylated proteins have superior properties compared to PNGaseF counterparts and the glycosylated form of the same protein (Mamedov et al., 2019a, 2019b). For example, EndoH in-vivo deglycosylated form of Protective antigen (PA) of *Bacillus anthracis* was a more stable, active, and highly immunogenic compared to its PNGaseF deglycosylated counterpart (Mamedov et al., 2017), which greatly increases the life and duration of vaccine storage and thereby greatly reducing the cost of the vaccine considerably (Mamedov et al., 2017). The purification yields of Endo H deglycosylated Pfs48/45 or Pfs48/45-10C proteins were about two times higher than that of plant-produced PNGase F counterparts (Mamedov et al., 2019b). Moreover, sera from mice immunized with Endo H deglycosylated full-length Pfs48/45 showed the strongest inhibition (100%) in SMFA compared to the PNGase F deglycosylated counterpart (which was less than 80%) (Mamedov et al., 2019b). Although there have been many decades of effort,

malaria remains the leading cause of morbidity and mortality among the human population globally and no vaccine is currently available that provides a satisfactory level of protection. Using in vivo enzymatic deglycosylation strategy, the most advanced, a full-length Pfs48/45 based malaria antigen has been produced in plants, which may have the potential to save millions life (Mamedov et al., 2019b). All these findings demonstrate that Endo H *in vivo* enzymatic deglycosylated Pfs48/45 is a promising candidate for the development of an affordable TB vaccine. Using Endo H in vivo deglycosylating strategy, the deglycosylated form of Receptor Binding Doman (RBD) of Spike protein of SARS-CoV-2 was recently successfully produced in N. benthamiana plant (Mamedov et al., 2021) as a vaccine candidate against COVID-19. It was demonstrated that in vivo deglycosylated RBD variant of SARS-CoV-2 induced stronger neutralizing responses in mice compared to the glycosylated form of RBD (Mamedov et al., 2021) and deglycosylated RBD antigen is promising vaccine candidates for the prevention of COVID-19. Thus, all of the above data demonstrate a more native folding of the deglycosylated forms of Endo H in vivo compared to the glycosylated form.

***In vivo* post-translational processing of target proteins by Furin.** Proteolytic cleavage is one of the important PTMs required for the activation of various proteins. In this sense, human furin is a very important protein that is involved in the activation of a wide range of proteins such as, blood clotting factors, hormones, growth factors, and their receptors (Nakayama K 1997). Thus, the development of furin processing in vivo in plants can ensure the production of functionally active mammalian proteins especially coagulation proteins and also other proteins in plants, which require furin processing for their activation. Recently, the human truncated form of Furin was successfully produced in *N. benthamiana* plants, and Furin processing has been successfully established in plants, which enabled the processing of recombinant target proteins, Factor IX and PA83 (Mamedov et al., 2019a). It was demonstrated that plant-produced human furin is highly active both *in vivo* and *in vitro* and specifically cleaved the tested target proteins, Factor IX (FIX) and Protective Antigen (PA83), Mamedov et al., 2019a. Thus, using this important strategy, various target proteins of interest can be modified and produced in plants by co-expressing target proteins with furin *in vivo*.

Gamma-carboxylation and sulfation. The activity of coagulation factors is modified by the γ-carboxylation PTM, which is catalyzed by vitamin-K-dependent γ-carboxylase enzyme (Gomord 2004). It should be noted that the PTM of γ-carboxylation in plants has not yet been achieved. Thus, the development of PTM γ-carboxylation in plants is the most challenging task due to the complexity of the vitamin K-dependent γ-carboxylase enzyme.

Tyrosine sulfation is one of the mammalian cell PTMs where a sulfate group is added to a tyrosine moiety by tyrosylprotein sulfotransferase (TPST) (Yang et al., 2014). This PTM is critically important for neutralizing antibodies that target HIV envelope glycoproteins (Lee et al 2017). Although plants were shown to contain TPSTs, however, these proteins exhibit different enzymatic properties (Moore, 2009; Stone et al., 2009). Tyrosine sulfation has been achieved in plants by co-expression of human TPST1 (hsTPST1) with neutralizing anti–HIV-1 monoclonal antibodies (PG9) (Loos et al., 2015). This is the only report so far about the achievement of sulfations in plants and it was shown that PG9 is efficiently sulfated in plants by co-expression of an engineered human tyrosylprotein sulfotransferase (Loos et al., 2015).

Hydroxylation. The hydroxylation of proline (Pro) residues is a precondition for O-glycosylation in plants (Merle C 2002) and could affect the conformational stability, enzymatic activity, and protein-protein interactions (Gorres and Raines, 2010). The hydroxylation of Pro residues is catalyzed by the prolyl 4-hydroxylases (P4H) enzymes. The plant prolyl 4-hydroxylases (P4H) differ from their mammalian counterparts. Hydroxylation of Pro residues using a plant-based expression system has been reported (Eskelin et al., 2009; Karnoup et al., 2005; Weise et al., 2007; Xu et al., 2010).

CONCLUSIONS

Plant-based expression systems offer potentially safe, low-cost, scalable, high yield, and fast production of recombinant proteins. However, for the expression of various proteins with different post-translational modification statuses, more flexible approaches are needed to preserve their native sequence and biological function. Using flexible approaches a number of difficult-to-express proteins, vaccines, therapeutic proteins, antibodies, and human enzymes have been successfully produced in plants. In this mini-review, we discussed the development and importance of PTMs for the successful production of valuable recombinant proteins in plant-based expression systems.

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Bitkilərdə istehsal olunan rekombinant zülalların post-translasiya modifikasiyası: İcmal

T.H. Məmmədov [1,2] , N. Gün[1]

[1]Ağdəniz Universitetinin Kənd təsərrüfatı fakültəsinin Kənd təsərrüfatı biotexnologiyası Departmenti, Antalya, Türkiyə
[2]AMEA Molekulyar Biologiya və Biotexnologiyalar İnstitutunun Molekulyar biomühəndislik beynəlxalq laboratoriyası, Bakı, Azərbaycan

Bitkinin müvəqqəti ekspressiya sistemi müxtəlif vaksinlər, terapevtik zülallar, fermentlər və anticisimlər istehsalı üçün perspektivli platformadır. Bitki əsaslı ekspressiya sistemləri yüksək məhsuldarlıq potensialı, miqyaslılıq, təhlükəsizlik və bioloji aktiv məhsulların istehsalının aşağı dəyəri kimi bir sıra potensial üstünlüklərə malikdirlər. Bundan başqa, bitki ekspressiya sistemləri eukaryotik posttranslasion modifikasiyalara (PTM) malikdirlər. Bununla belə, bitki ekspressiya sistemində istehsal olunan zülalların funksional fəaliyyəti üçün vacib olan və məməli hüceyrələrində mövcud olan bəzi mühüm PTM-lər yoxdur. PTM-lər sabitlik, həll olma qabiliyyəti, digər molekullarla qarşılıqlı təsir və lokalizasiya kimi zülal xüsusiyyətlərinə əhəmiyyətli dərəcədə təsir göstərir. Beləliklə, müxtəlif PTM statusları olan müxtəlif zülalların ekspressiyasına nail omaq, onların nativ ardıcıllığını və bioloji funksiyasını qorumaq üçün daha çevik yanaşma üsulları tətbiq edilməlidir. Bu mini icmalda biz bitkilərdə istehsal olunan rekombinant zülalların PTM-lərini və onların tibbdə, kənd təsərrüfatı və sənayedə istifadə etmək məqsədilə funksional aktiv rekombinant zülalların istehsalı üçün əhəmiyyətini müzakirə edirik.

Açar sözlər: *Posttranslyasion modifikasiyalar, N-qlikozilləşmə, in vivo deqlikozilləşmə, sialilləşmə, furinlə prosessinq, qamma-karboksilləşmə, sulfatlaşma, bitki ekspressiya sistemləri*