COMMUNICATION

N-glycosylation at noncanonical Asn-X-Cys sequences in plant cells

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The vesicular transport pathway in plant cells is often used for higher accumulation of recombinant proteins. In the endoplasmic reticulum, which acts as a gateway to the vesicular transport pathway, N-glycosylation occurs on specific Asn residues. This N-glycosylation in recombinant proteins must be carefully regulated as it can impact their enzymatic activity, half lives in serum when injected, structural stability, etc. In eukaryotic cells, including plant cells, N-glycans were found to be attached to Asn residues in Asn-X-Ser/Thr (X ≠ Pro) sequences. However, recently, N-glycosylations at noncanonical Asn-X-Cys sequences have been found in mammals and yeast. Our laboratory has discovered that N-glycans are attached to Asn residues at Asn-Thr-Cys sequences of double-repeated B subunit of Shiga toxin 2e produced in plant cells, the first reported case of N-glycosylation at a noncanonical Asn-X-Cys sequence in plant cells.

Keywords: Asn-X-Cys sequence / B subunit of Shiga toxin 2e / N-glycosylation, plant cells

Introduction

Recombinant pharmaceutical proteins are expressed in a wide range of host cells including Escherichia coli, yeast, mammal and plant cells. The plant-based expression system is considered to have some advantages over the other systems, such as lower production costs and lower risk of contamination by animal viruses (Daniel et al. 2001; Yoshida et al. 2004). For the high-level accumulation of a target protein in plant cells, regulation of transcriptional, translational and also post-translational processes are important. For example, stronger transcription is possible when the expression cassette contains a transcriptional terminator derived from the Arabidopsis thaliana heat shock 18.2 gene (Nagaya et al. 2010), and translational enhancement is achieved by use of the 5’-untranslated regions of alcohol dehydrogenase genes (Satoh et al. 2004; Sugio et al. 2008). At the post-translational level, sending the target protein into the vesicular transport pathway often leads to its successful accumulation (Matsui et al. 2003; Yoshida et al. 2004).

In the vesicular transport pathway of eukaryotic cells, post-translational modifications including N-linked glycosylation can occur. It is important to regulate the N-glycosylation of recombinant proteins for several reasons. For many proteins, such as erythropoietin and antibodies, the attachment of N-glycans is important for their activity and/or function (Jelkmann 2007; Jefferis 2009). On the other hand, allergies can be caused by plant-specific N-glycan structures (Altmann 2007), and methods of producing human-compatible N-glycan structures in plant cells are under development (Ko et al. 2008). In other proteins, unintended N-glycosylation can lead to the deterioration of the protein. For example, when E. coli β-glucuronidase (GUS) was sent into the vesicular transport pathway of plant cells, active GUS protein was not obtained as a result of its being N-glycosylated (Iturriaga et al. 1989). However, when a modified GUS protein, in which Asn358 was substituted with a Ser residue in order to avoid N-glycosylation, was sent through the same pathway, active GUS protein accumulated in the transgenic plants as intended (Firek et al. 1994).

In eukaryotic cells, including plant cells, N-glycans are thought to be co-translationally and/or post-translationally attached to Asn residues in the sequences Asn-X-Ser/Thr (X ≠ Pro) of acceptor proteins in the endoplasmic reticulum (ER) (Pattison and Amtmann 2009). The Ser and Thr residues in the third position are considered to be important for the determination of the N-glycosylation site. However, recently, N-glycosylation at noncanonical sequences has also been reported. For some recombinant proteins produced in mammalian cells, N-glycans are attached to Asn residues in Asn-X-Cys sequences (Sato et al. 2000; Wang et al. 2008; Borisov et al. 2009). This type of N-glycosylation signal was also found in yeast (Chi et al. 2010). Another example of a noncanonical N-glycosylation sequence is the Asn-Ser-Gly-Ala-Leu sequence of antibody fragment (Vulliere-Douglass et al. 2009).

In this study, we characterized post-translational modifications on the double-repeated B subunit of Shiga toxin 2e...
(2× Stx2eB) designed to accumulate in the ER of plant cells. We found that N-glycans were attached to Asn residues in Asn-Thr-Cys sequences of 2× Stx2eB in several plant species. This is the first report of N-glycosylation at noncanonical Asn-X-Cys sequence in plant cells.

Results and discussion

We previously reported that the ER is a suitable compartment for the accumulation of the B subunit of Shiga toxin 2e (Stx2eB) in lettuce plants (Matsui et al. 2009) and that the double-repeated Stx2eB (2× Stx2eB (NTC/NRS) in Figure 1) accumulated to higher levels than the single Stx2eB (Matsui et al. in press). In a western blot analysis of stable transformants of lettuce plants expressing 2× Stx2eB (NTC/NRS), three bands for Stx2eB were detected (bands 1, 2 and 3 in Figure 2, and Matsui et al. in press). Bands 1, 2 and 3 have molecular weights of about 17, 20 and 23 kDa, respectively. Three bands were also detected when A. thaliana culture cells were used as a host (Figure 2). When a gene for 2× Stx2eB designed to accumulate in the cytosol was expressed, only a single band having a molecular weight equal to band 1 was detected (data not shown) and, therefore, we supposed that band 1 corresponded to nonmodified 2× Stx2eB and bands 2 and 3 were 2× Stx2eB with modifications presumably specific to the ER. We checked whether the appearance of bands 2 and 3 were due to N-glycosylation by treating cellular extracts with glycopeptidase F (GPF), which removes N-glycans from Asn residues, before western blotting. The signal intensities of bands 2 and 3 decreased after GPF treatment in both lettuce and A. thaliana (Figure 2), indicating that in both 2× Stx2eB had been N-glycosylated. Considering the signal intensities and the molecular weights of the bands, it can be inferred that bands 2 and 3 correspond to 2× Stx2eB having one and two N-glycans, respectively.

Previously, N-glycans were found to be attached to Asn residues in the sequences Asn-X-Ser/Thr (X ≠ Pro) of acceptor proteins in the ER (Pattison and Amtmann 2009). Stx2eB does not contain an Asn-X-Ser/Thr sequence within the primary amino acid sequences itself, but this sequence exists in the 2× Stx2eB sequence used, 2× Stx2eB (NTC/NRS), where the genes join the C-terminal linker peptides (-Lys-Phe-Asn-Arg-Ser-Pro-), the underlined Asn residue corresponds to the last amino acid of Stx2eB followed by the linker peptide in italics (Sites 2 in Figure 1). In order to check whether these Asn residues are N-glycosylated, a gene for 2× Stx2eB linked by another linker peptide was constructed (2× Stx2eB (NTC/NRA) in Figure 1). To evaluate these constructs by convenient means, transient expression analysis was performed using protoplasts derived from A. thaliana cultured cells. In the case of the expression of 2× Stx2eB (NTC/NRS), bands 1 and 2 were detected, while band 3 was below the detection limit in this experimental system (Figure 3A). By GPF treatment, band 2 disappeared as had been observed for the stable transformants. In the case of 2× Stx2eB (NTC/NRA) expression, band 2 was still detected indicating that Sites 2 were not major N-glycosylation sites. A proline residue at the C-terminal of the Asn-X-Ser sequon abolished N-glycosylation in a cell-free system using reticulocytes, in oocytes from Xenopus laevis, and also in yeast cells (Roitsch and Lehle 1989; Melloquist et al. 1998). Similarly, plant N-glycosylation machinery may not prefer Asn-X-Ser/Thr-Pro sequences as acceptor sequences.

In mammalian and yeast cells, N-glycans were found to be attached to Asn residues in noncanonical Asn-X-Cys sequences in certain proteins (Sato et al. 2000; Wang et al. 1999). This is the first report of N-glycosylation at noncanonical Asn-X-Cys sequence in plant cells.

Fig. 1. Schematic representations of constructs used in this study. Amino acid sequences of putative N-glycosylation sites (Sites 1 and 2) and those of modified versions are shown below. Amino acid sequences of linker peptides are indicated in italics. Substituted amino acid residues are underlined. mStx2eB5, codon-modified gene for Stx2eB; p35S, Cauliflower mosaic virus 35S RNA promoter; pNOS, A. tumefaciens nopaline synthase (NOS) gene promoter; NOS-T, transcription terminator from A. tumefaciens NOS gene; HSP-T, transcription terminator from A. thaliana HSP18.2 gene; NtADHmod, 5’-untranslated region of tobacco (N. tabacum) alcohol dehydrogenase gene with a modification (nucleotides immediately upstream of the initiating AUG were changed for efficient translation initiation); SP, signal peptide for ER translocation derived from tobacco β-n-glucan exohydrolase; HA, HA peptide tag to detect the Stx2eB protein (Tyr-Pro-Tyr-Asp-Tyr-Pro-Asp-Tyr-Ala); HDEL, ER retention signal (Ser-Glu-His-Asp-Glu-Leu). An arrow indicates the translation start site, and a triangle indicates the putative cleavage site after translation.
Stx2eB contains a 73Asn-Thr-Cys sequence (Sites 1 in Figure 1); mutations were introduced into these sites to check for N-glycosylation. The 73Asn residue was substituted with a Ser, chosen because one is located in that position in Stx2B, a closely related protein in the Shiga toxin family, and a gene for 2× Stx2eB (STC/NRA) was constructed (Figure 1). Only band 1 was detected when 2× Stx2eB (STC/NRA) was expressed in a transient expression assay (Figure 3A), which means that 2× Stx2eB was N-glycosylated at the noncanonical Asn-Thr-Cys sequence. We also generated stable transformants of A. thaliana and lettuce using the 2× Stx2eB (STC/NRA) construct and found that only nonglycosylated 2× Stx2eB was detected in these transformants (Figure 3C and D). As future works, it is important to analyze impacts of amino acid substitution and N-glycosylation on immunogenicity of 2× Stx2eB proteins using these plant materials. At the same time, mass spectrometric analysis will be significant in order to confirm that N-glycans are attached to 73Asn residues in 2× Stx2eB (NTC/NRS) and also to clarify glycan structures.

To evaluate the importance of the Cys residues in the sequence, we constructed a gene for 2× Stx2eB (NTA/NRA) in which 75Cys was substituted with Ala (Figure 1). When the 2× Stx2eB (NTA/NRA) was transiently expressed in A. thaliana protoplasts, only one band was detected (Figure 3B), which means that the third Cys residue plays an important role in the determination of the N-glycosylation site. We also checked by transient expression assay whether other plant species had the ability to add an N-glycan moiety to the Asn residue in the Asn-Thr-Cys sequence. Cultured cells of rice (Oryza sativa), leaves of cutleaf evening-primrose (Oenothera laciniata) and cultured cells of tobacco (Nicotiana tabacum) were used to prepare protoplasts. O. sativa is a monocot, and the latter two species are dicots, as are lettuce and A. thaliana. In protoplasts of all species tested, both bands 1 and 2 were detected when 2× Stx2eB (NTC/NRA) was expressed, while only band 1 was detected when 2× Stx2eB (STC/NRA) was expressed (Figure 4).

In mammals and yeasts, Asn residues in Asn-X-Cys sequences are less efficiently N-glycosylated than those in Asn-X-Ser/Thr sequences (Sato et al. 2000; Gil et al. 2009). This appears to hold true for plant cells as well since, estimated from the signal intensity of the bands in the western blots in Figures 2 and 3, occurrence of the N-glycosylation at the Asn-X-Cys sequence was as low as 10%. Considering the data shown in this paper and previous reports, N-glycosylation of Asn residues at Asn-X-Cys sequences appears to be a well-conserved ability of glycosylation machineries in eukaryotic cells.

**Materials and methods**

*Construction of plasmids for the expression of 2× Stx2eB*

Binary vector pRI909 (TaKaRa Bio Inc., Shiga, Japan) was used as a backbone plasmid for construction of 2× Stx2eB.
cassettes, because this plasmid can be used for both genetic transformation of plant cells and transient expression analysis owing to its high copy numbers in E. coli cells. 2× Stx2eB (NTC/NRS) is the same as 2BH in our previous report (Matsui et al. in press). 2× Stx2eB (NTC/NRA) was constructed as follows: a DNA fragment was PCR amplified using 2× Stx2eB (NTC/NRS) as a template and ADH KpnI-F (5′-aaatcagaggtactatttaactcagattcagaaac-3′) and PG12ver2-R (5′-aagatcagaggtactatttaactcagattcagaaac-3′) as primers. The resulting DNA fragment was digested with KpnI and BglII, and inserted into the KpnI–BglII sites of 2× Stx2eB (NTC/NRA) to generate 2× Stx2eB (NTC/NRA).

2× Stx2eB (STC/NRA) and 2× Stx2eB (NTA/NRA) were constructed as follows: a DNA fragment encoding Stx2eB, in which 73Asn was substituted with Ser, was amplified using 2× Stx2eB (NTC/NRS) as a template and ADH KpnI-F (5′-aaatcagaggtactatttaactcagattcagaaac-3′) and PG12ver2-R (5′-aagatcagaggtactatttaactcagattcagaaac-3′) as primers. The resulting DNA fragments were end-blunted and self-ligated (plasmid 30). A DNA fragment encoding Stx2eB, in which 73Cys was substituted with Ala, was amplified by inverse PCR using mStx2eB5 (Matsui et al. in press) as a template and Stx2eBN73S-F (5′-gacctgcttacctgtcctgttgg-3′) and Stx2eBN73S-R (5′-gcagagatgctagctgctagc-3′) as primers. The resulting DNA fragments were end-blunted and self-ligated (plasmid 31). In order to add PG12 spacers to DNA fragments for Stx2eBN73S and Stx2eBC75A, oligonucleotides PG12-F (5′-gatcctgccggtctgtgctgctgc-3′) and PG12-R (5′-gacctgcttacctgtcctgttgg-3′) were annealed and phosphorylated with T4 polynucleotide kinase. The resulting phosphorylated PG12 fragments were inserted into the BglII sites of plasmids 30 and 31 to generate PG12-added Stx2eBN73S (plasmid 32) and PG12-added Stx2eBC75A (plasmid 33), respectively. DNA fragments were PCR amplified using plasmid 32 or 33 as a template and ADH KpnI-F and PG12ver2-R as primers. The resulting DNA fragments were blunt-ended and inserted into the HinflII site of pUC118 to generate PG12-ver2-added Stx2eBN73S (plasmid 34) and PG12ver2-added Stx2eBC75A (plasmid 35), respectively. The DNA fragment for mStx2eB5N73S–PG12ver2 was digested from plasmid 34 with BamHI and BglII, and inserted into the BamHI site of plasmid 34 to generate 2× mStx2eB5N73S–PG12ver2 (plasmid 36). The DNA fragment for mStx2eB5C75A–PG12ver2 was digested from plasmid 35 with BamHI and BglII, and inserted into the BamHI site of plasmid 35 to generate 2× mStx2eB5C75A–PG12ver2 (plasmid 37). The DNA fragments for 2× mStx2eB5N73S–PG12ver2 and 2× mStx2eB5C75A–PG12ver2 were digested from plasmids 36 and 37 with BamHI and BglII, and each inserted into the BamHI–BglII sites of 2× Stx2eB (NTC/NRS) to generate 2× Stx2eB (STC/NRA) and 2× Stx2eB (NTA/NRA), respectively.

**Fig. 4.** N-glycosylation at Asn-Thr-Cys sequence in another three-plant species. Protoplasts derived from rice (*O. sativa*), cutleaf evening-primrose (*O. laciniiata*) and tobacco (*N. tabacum*) cultured cells were transiently transfected with 2× Stx2eB (NTC/NRA) and 2× Stx2eB (STC/NRA). Mock plasmids that did not contain Stx2eB gene were transfected as a negative control. Bands for N-glycosylated and nonglycosylated 2× Stx2eB are indicated by solid and open triangles, respectively.

### Genetic transformation of plant cells

Transformation of lettuce (*Lactuca sativa* L cv. green wave) was carried out as described previously (Matsui et al. 2009).

A. thaliana T87 cells were cultured in a modified MS medium, containing MS medium (Murashige and Skoog 1962) supplemented with 200 mg/L KH2PO4, 100 mg/L myo-inositol, 1 mg/L thiamine–HCl, 0.2 mg/L 2,4-dichlorophenoxyacetic acid, 3% sucrose, pH 5.7. A 300 mL flask containing 100 mL of cell suspension was incubated at 22°C with shaking under continuous light, and 2 mL of culture containing fully-grown cells was transferred to a new medium every 7 days for maintenance. For transformation, *Agrobacterium tumefaciens* EHA105 cells (Hood et al. 1993) harboring the binary vector were cultured in 2× YT liquid medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 mg/L kanamycin at 28°C for 2 days. On the third day of subculture, the *A. thaliana* suspension culture was combined with 500 μg/mL acetosyringone, inoculated into the medium, and cultured in 2× YT liquid medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 mg/L kanamycin at 28°C for 2 days. This suspension was then transferred to a sterile centrifugation tube, and *A. thaliana* cells were collected by centrifugation (60× g for 5 min at room temperature). The upper phase, containing the cell suspension, was transferred to a new medium every 7 days for maintenance. For transformation, *Agrobacterium* was removed and the precipitated *A. thaliana* cells were mixed with 20 mL of modified MS medium containing 250 mg/L carbencillin. This wash step was repeated five times. Collected *A. thaliana* cells were transferred to a new flask containing 100 mL of modified MS medium supplemented with 250 mg/L carbenicillin, and cells were cultured for another 2 days at 22°C with shaking under continuous light. *A. thaliana* cells collected by centrifugation (60× g for 5 min at room temperature) were re-suspended in modified MS medium.
medium supplemented with 250 mg/L carbenicillin so that the cell volume accounted for about half the total volume. A 500 μL sample of this suspension was spread onto modified MS medium, supplemented with both 250 mg/L carbenicillin and 40 mg/L kanamycin, and solidified with 3 g/L gerungum in a Petri dish (9 cm in diameter). The Petri dishes were sealed with parafilm, and placed under continuous light at 22°C. After a few weeks, kanamycin-resistant calluses were picked up for analyses.

**Transient expression analysis**

Transient expression analysis was performed according to the procedure previously reported (Matsui et al. 2009). For preparation of protoplasts of lettuce and cutleaf evening-primrose, leaves were cut into pieces with a diameter of about 5 mm and used as a starting material. For cultured cells (O. sativa, A. thaliana and N. tabacum), fourth day cultures were centrifuged (40×g for 5 min at room temperature), and collected cells were further treated with protoplastization enzyme solution. A 5 μg sample of plasmid was used in each 2×Stx2eB transformation.

**GPF treatment**

Leaves of transgenic lettuce and transgenic cultured cells of A. thaliana were ground in liquid nitrogen and mixed with 10 mM Tris–HCl, pH 8.0, for protein extraction. For protoplasts transiently expressing Stx2eB, precipitated protoplasts were mixed with 10 mM Tris–HCl, pH 8.0, followed by sonication on ice. After centrifugation (20,000× g, 20 min at 4°C), supernatant was collected, and denatured proteins were treated with GPF (TaKaRa Bio Inc.) according to the instruction manual.

**Western blot analysis**

Protein samples were mixed with SDS sample buffer (4% [w/v] SDS, 20% [v/v] glycerol, 0.05% [v/v] bromophenol blue, 300 mM β-mercaptoethanol, 125 mM Tris–HCl, pH 6.8), followed by thermal denaturation at 95°C for 5 min. Proteins were separated in a 15% acrylamide gel and blotted onto a polyvinylidene fluoride membrane (Hybond-P; Amersham Biosciences, Piscataway, NJ) using an electrotransfer system. Anti-HA antibodies (clone 3F10, Roche, Mannheim, Germany) were used to detect Stx2eB.

**Abbreviations**

ER, endoplasmic reticulum; GPF, glycopeptidase F; GUS, β-glucuronidase.

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**References**


