Brief Communication

Plant-based production can result in covalent cross-linking of proteins

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Antibodies, antigens and enzymes for replacement therapies and virus-like particles (VLPs) have all been produced successfully in plants as part of the concept of ‘Molecular Farming’ (Lomonossoff and D’Aoust, 2016). There have been several differences noted between plant-expressed proteins, and their equivalents produced in other systems, such as CHO cells, particularly regarding their glycosylation. However, several publications have also indicated that preparations of plant-expressed proteins, including antibodies, VLPs and soluble molecules such as HIV gp120, have a higher proportion of multimers or aggregates than their CHO- or yeast-expressed equivalents (e.g. Mechtcheriakova et al., 2006; Ramesar et al., 2008; Rosenberg et al., 2013). The cause of this has not been investigated in detail because the molecules expressed in the different systems are often not identical and may have been purified to different extents.

As part of our studies on virus maturation, we have expressed the coat protein of the insect virus, Nudaurelia capensis omega virus (Nov) in both Nicotiana benthamiana (a heterologous expression system) and insect cells (the homologous system). Nov belongs to the Tetraviridae, a family of viruses with non-enveloped T = 4 capsids and single-stranded positive-sense RNA genomes that infect Lepidoptera. Maturation of the virus particles from procapsid to mature capsid involves a compaction of the genomes that infect Lepidoptera. Maturation of the virus particles enveloped T = 4 particles from 48 to 41 nm diameter with a concomitant reducing the pH of a suspension of procapsids from 7.6 to 5.0 to the mature particles extracted from insect cells and to contain the cleaved α-peptide (Figure 1a). This is consistent with the previous identification of dimers by mass spectrometry and Western blotting in samples of plant-expressed Nov VLPs (Castells-Graells, 2019). Since identical conditions were used to extract and purify the procapsids and the denaturing conditions used for the SDS-PAGE analysis were the same in each case, the formation of the oligomers must be a specific consequence of using plants for expression. Given the fact that the oligomers resisted denaturation, it is probable that they result from covalent cross-linking of subunits.

To investigate whether cross-linking occurred within the plant cells or during extraction, we incubated insect cell-produced Nov procapsids with plant extracts prepared in the same buffer used to extract procapsids (50 mM Tris-HCl pH 7.6, 250 mM NaCl) and in which cleavage of the α-peptide does not occur. The conditions of the incubation (4 h at 4 °C) were approximately the same as those used to prepare procapsids up to the sucrose gradient step. Western blot analysis (Figure 1b) showed that incubation of insect cell-produced procapsids in plant extracts results in the appearance of dimers and additional high molecular bands (lanes 2 to 4), not seen when the procapsids were incubated in buffer alone (lane 1). This pattern of higher bands is similar to that found in plant tissue expressing the α-peptide (lane 8), though in this sample maturation products are also seen since the procapsids were not purified. The formation of dimers occurred irrespective of whether the insect cell procapsids were incubated with extracts prepared from uninfiltreated leaves (lane 2), leaves infiltrated with the empty pEAQ-HT vector (pEAQ-HT-EV; lane 3) or leaves incubated with pEAQ-HT-GFP (lane 4), indicating that the act of infiltration or the presence of Agrobacterium did not influence the result. The negative controls (lanes 5 to 7) containing the equivalent plant extracts without the added...
procapsids did not show any N\textsubscript{x}V-specific bands demonstrating the specificity of the antiserum. These results strongly imply that some component present in the plant extracts, such as peroxidases, induces the cross-linking of the N\textsubscript{x}V coat proteins and that this cross-linking occurs during extraction and purification. Varying the conditions of extraction using, for example, buffers at different pHs (5.0, 7.6, 10.0) or adding DTT to 1mM did not make a detectable difference to the result.

The observation of cross-linking has implications for the production of proteins in plants. In the case of N\textsubscript{x}V, we propose that such cross-linking is detrimental to the protein rearrangements necessary for the efficient \textit{in vitro} maturation of the procapsids, thus affecting the final assembly. We conclude that it is the time taken for the isolation and purification of the procapsids (approximately 4 h) that allows the cross-linking to occur, thereby interfering with the maturation process \textit{in vitro}; however, cross-linking within cells prior to extraction might also occur. Cross-linking can still occur post-maturation, but this does not affect the structure of the particles.

Though cross-linking is clearly deleterious for the maturation of N\textsubscript{x}V capsids, in certain cases it may not be harmful or even beneficial. For example, in the case of plant-produced antibodies, the presence of aggregates increases the IC\textsubscript{50} of preparations several fold compared to CHO-produced material (Ramessar \textit{et al.}, 2008; Rosenberg \textit{et al.}, 2013). Likewise, partial cross-linking of subunits can also aid the stability and structural integrity of VLPs (Peyret \textit{et al.}, 2015); indeed, VLPs and virus particles intended for vaccine purposes are often deliberately cross-linked during formulation. Nonetheless, it is important to be aware of cross-linking in plant-produced material so that methods for its control or elimination can be developed. If the enzymes involved can be identified, cross-linking could be addressed by removing them, using genetic engineering techniques like CRISPR-Cas9 (Belhaj \textit{et al.}, 2015). Controlling cross-linking should increase interest using plants for the production of pharmaceuticals and other relevant biological products (Lomonossoff and D'Aoust, 2016).

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**Conflict of interest**

G.P.L. declares that he is a named inventor on granted patent WO 29087391 A1 which describes the HyperTrans expression system and associated pEAQ vectors used in this manuscript.
Author contributions
R.C-G and G.P.L conceived the study. R.C-G. designed the constructs and conducted expression tests in plants and insect cells. G.P.L. supervised the work and provided scientific advice. Both authors contributed to writing and editing the manuscript.

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