Proteolysis of recombinant proteins in bioengineered plant cells

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Abstract

Plants are increasingly used as alternative expression hosts for the production of recombinant proteins offering many advantages including higher biomass and the ability to perform post-translational modifications on complex proteins. Key challenges for optimized accumulation of recombinant proteins in a plant system still remain, including endogenous plant proteolytic activity, which may severely compromise recombinant protein stability. Several strategies have recently been applied to improve protein stability by limiting protease action such as recombinant protein production in various sub-cellular compartments or application of protease inhibitors to limit protease action. A short update on the current strategies applied is provided here, with particular focus on sub-cellular sites previously selected for recombinant protein production and the co-expression of protease inhibitors to limit protease activity.

Introduction

The world-wide demand for recombinant therapeutic and diagnostic proteins requires exploring plant-based protein expression platforms supplementing existing prokaryotic production systems.[1] A number of valuable human recombinant proteins have already been successfully produced in plant-based systems[2-5], ranging from soil-grown plants to plant cells grown in a bioreactor. Cells may be used transiently express the protein over a relatively short time period or be genetically engineered to stably express any recombinant protein. Plants offer the general advantage of a high plant biomass, the ability to perform post-translational modifications on complex proteins when passed through the secretory pathway, correctly folding and assembling complex proteins as well as being relatively safe due to the absence of human pathogens.[6]

Several approaches with various non-food or non-feed plant species, such as tobacco, are currently used for recombinant protein production. In particular, vacuum infiltration as well as infiltration of the tobacco leaf surface with *Agrobacterium* cells using a syringe was recently introduced as more effortless techniques for protein production. This avoids bio-safety concerns of genetically engineered transgenic plants and lengthy plant transformation and selection procedures. These two transient expression techniques are also better suited to satisfy any short-term demand for a recombinant protein with leaf harvest occurring within days.[7-8] With these transient techniques the recombinant protein is expressed in leaves after infection with *Agrobacterium* cells carrying the protein coding sequence for the recombinant protein. An appropriate signal sequence may direct protein accumulation to a particular cellular compartment, which influences protein post-translational modifications and protein yield, depending on the resident proteases. Proteolysis may occur *in planta* or during protein extraction and harvesting, often requiring protease inhibitors to be added to the extraction

buffer to improve protein stability and yield.[9] However, this strategy is expensive and is seldom economically viable with regards to large scale extractions.

The purpose of this short review is to give an overview on the current knowledge of protease action on recombinant proteins produced in plants and to provide an update of some current strategies applied to improve recombinant protein stability in plant-based production system.

Proteases act on recombinant proteins

Protease abundance in plant tissues represents a severe burden to effective recombinant protein production.[10] The degree of proteolysis, either partial or complete, depends on the amino acid sequence of the recombinant protein, susceptibility of sites to proteolytic action and also the number of protease-susceptible sites. Studies on plant proteases have advanced substantially and a more detailed understanding of the role of proteases, particularly in growth, development and pest resistance, is emerging. Hundreds of plant genes encode for proteins involved in proteolysis. In the model plant Arabidopsis, about 1900 genes involved in peptide bond hydrolysis have already been identified, but only a small number of proteases has so far been characterized, with the biological function of only around 40 proteases elucidated.[11-12] Plants with larger genomes are likely to also have a higher number of proteases with highly polymorphic activity profiles in different plant species. Protease functions include assembling and disassembling proteins as well as removing damaged, mis-folded or potentially harmful proteins.[13-14] Based on their active site residues for catalysis, most proteases can be distinguished as serine, cysteine, aspartic, and metallo-types[14] with serine proteases consisting of about 200 members, and the cysteine, aspartic, and metallo-type proteases about 100 members in each class (http://merops.sanger.ac.uk).[15]

In *Nicotiana* species, often used for recombinant protein production, the majority of proteases are of aspartic or cysteine type (papain-like cysteine proteases) and to a lesser extent serine and metallo-type.[16-18] When recombinantly expressing proteins in the leaves of *Nicotiana,* leaves of *N. benthamiana* is considered to contain lower protease activity compared to leaves of *N. tabacum*, consisting mostly of cathepsin L- and legumain-like cysteine proteases.[19] **Table 1** outlines the type of proteases and their localizations so far identified in the different plant species previously used for recombinant protein production.

Selecting the cellular compartment for recombinant production

A cellular localization with limited proteolytic activity may be interesting for recombinant protein stability and ultimately yield. Protease activity is pH dependent and proteases therefore reside in different cellular compartments favourable for their respective activities. These enzymes are found in various cellular compartments including the cytosol, the vacuole, the chloroplast, the mitochondria and the lysosome.[19-22] **Figure 1** provides an overview of the different classes of proteases active in the different compartments of a plant cell. A number of proteases are extracellular, residing in the apoplast to which recombinant proteins can be secreted. For secretion, proteins travel from the endoplasmic reticulum (ER) through the Golgi apparatus to the cell surface.

The cytosol or the vacuoles

Undesired protein modifications changing protein structure folding may occur in the cytosol.[23] The cytosolic ubiquitin-proteasome proteolytic pathway further degrades any improperly folded protein.[24] Recombinant proteins are generally poorly accumulated when

expressed in the cytosol,[25] and this compartment is often regarded as unsuitable for effective recombinant protein production.

Lytic vacuoles are also unsuitable for recombinant protein deposition due to their high protease content. Protein storage vacuoles, abundant in seeds, are more suitable for protein accumulation. Targeting proteins to the storage vacuoles is achieved by a specific amino acids sequence, or sorting signal, within the primary of the protein.[26] An example of accumulation in the vacuole of recombinant proteins achieved through vacuole-targeting is dog gastric lipase production in transgenic tobacco plants.[27]

The ER and the Golgi

The ER has been the production site for several recombinant proteins of industrial and pharmaceutical value. Directing a protein towards the ER results in greater protein yield and lower proteolysis when compared to the cytosol.[10] The value of ER retention has been previously demonstrated where a ER retention signal increased human anti-HIV 2G12 levels in *N. benthamiana* plants.[28] Higher expression of ER-retained proteins has also been demonstrated for a structural poly-protein, P1-2A, as well as for a 3C protease from FMDV serotype O when stably expressed in foliar tomato extract.[29] In contrast, when a signal peptide such as the CTB signal peptide is absent, expression of the viral protein is not detectable possibly due to degradation within the cytoplasm during, or immediately after, synthesis.[30] Proteins that are retained in the ER may also reside in protein bodies enhancing post-translational stability.[31] Greater yield in the ER is very likely due to the action of chaperone proteins supporting proper protein folding.[32] Folding and/or post-translational modification of a recombinant proteins can however differ if post-translational processing occurs in the Golgi apparatus downstream of the ER.[30] Recombinant proteins may be directed to this organelle via KDEL or HDEL signal peptides, however this may also result in undesired, structurally

distinct, proteins due to non-native amino acid additions or non-authentic protein glycosilation patterns.[32-33]

The disadvantage of ER retention is the existance of ER proteolytic pathways acting on mis-folded proteins. Mis-folded proteins are in some cases re-translocated into the cytosol by the ER machinery for proteasomal degradation.[34] Several classes of proteases act in the secretory pathway with pepsin-like (A1), papain-like (C1), trypsin chymotrypsin-like (S1), subtilisin-like (S8) and serine carboxypeptidase-like (S10) the most represented protease families.[19] Unintended processing of recombinant proteins along this pathway by resident proteases has been reported by several research groups.[35-37] Some examples include the systematic processing of mammalian antibodies and the partial trimming of the anti-inflammatory bovine aprotinin protein at the C-and N-termini when retained in the ER.[37-38] Cleavage of the C-terminal region of the human α1-anti-chymotrypsin by intracellular and apoplastic proteases when targeted to the secretory pathway of BY-2 tobacco cells and subsequently detected in the culture medium is another example of unintended processing that may occur.[36]

The apoplast and the chloroplast

A number of recombinant proteins have been successfully expressed in the apoplast with expression of the human interleukin 6 in *N. benthamiana* recently reported as an excellent example.[39] However, abundance and poor specificity of proteolytic enzymes in the apoplast is still a major obstacle.[10, 17, 32, 35] Intact bovine aprotinin was for instance detected in the apoplast of transgenic potato leaves, but final yields *in planta* were much lower when compared to retaining the protein in the ER. In the apoplast proteins are generally exposed to a large number of proteases. The apoplast of *N. tabacum* leaves primarily contains aspartic-,

cysteine- and serine-type proteases [17] while the apoplast in *N. benthamiana* leaves show preferential activity of aspartic- and serine-type proteases.[19]

A fairly new strategy to improve recombinant protein stability and achieve of higher yields is production in the chloroplast compartment of genetically engineered plants.[40] Chloroplast engineering has several advantages including uniform protein expression rates, multiple copies of an integrated transgene and low gene silencing. High plastid number per cell and maternal inheritance of chloroplast DNA leading to minimal transgene escape are also among the advantages.[40] Examples of chloroplast-based production of a recombinant protein include production of a cholera toxin B – pro-insulin fusion in transgenic lettuce and tobacco[41] and production of VP1 structural protein of the foot-and-mouth disease virus in tobacco chloroplasts.[42] It was also recently reported that a chloroplast-derived vaccine candidate was stable at room temperature for 20 months.[43] Despite this success, inability to perform more complex post-translational modifications, such as glycosylation, or to perform protein subunit assembly and proper protein folding, are disadvantages in a chloroplast-based production system. In addition, endogenous proteases are present in the chloroplast that may compromise recombinant protein accumulation. For instance, high protein accumulation of the rotavirus VP6 protein was found in young tobacco leaves whereas in older leaves the amount of VP6 protein decreased possibly due to proteolytic degradation.[44]

Preventing protease action

Different strategies have been proposed to minimize unintended proteolysis *in planta*.[10, 32] Some strategies involve the targeting of recombinant proteins to specific cellular locations using peptide sorting signals[45] or the addition of a stabilizing fusion partner to the protein of interest. Elastin-like peptide (ELP) fusions for instance improved the stability of a

recombinant antibody and affinity-purified antibodies had kinetic binding parameters identical to an ELP-free antibody produced in Chinese hamster ovary cells.[46]

An interesting, relatively new strategy is to minimize proteolysis by co-expression of a recombinant 'companion' protease inhibitor in a transgenic plant or in plants transiently expressing recombinant proteins.[18] Recombinant protease inhibitors have been previously applied as anti-digestive compounds for crop protection against insect herbivory or pathogenic infection.[47] Pleiotropic effects for these proteins have also been reported *in planta* causing altered growth characteristics and protection against abiotic stresses.[47-48] Co-expression of a 'companion' might also be an economically viable option to replace the costly addition of protease inhibitors during recombinant protein recovery and the protein purification process. Knowledge of individual characteristics of each recombinant protein and its sensitivity to proteases is, however, required to decide which inhibitor, or combination of inhibitors, might work best. Since serine protease activity is a major protease activity in plant cells, work has in the past been predominantly focused on serine protease inhibitors active against chymotrypsin and trypsin-like proteases. Co-expression and co-secretion of a soybean Bowman–Birk trypsin inhibitor stabilized recombinant antibodies secreted by transgenic tobacco roots.[49] The introduction of a synthetic serine protease inhibitor II gene further decreased protease activity in transgenic rice callus, indicating its potential as a 'companion' inhibitor for higher accumulation of a recombinant human granulocyte–macrophage colony stimulating factor.[50] Human interleukin 2, a pharmaceutically important cytokine, was also found to be protected against proteolytic action when a trypsin inhibitor I and silk protease inhibitors were co-expressed in transgenic tobacco plants.[51] The potential of a tomato cathepsin D inhibitor as an 'in-built' stabilizing agent for recombinant proteins *in situ* and during the recovery process was further reported for transgenic potato plants.[9, 18] In addition, the *in vivo* expression of a tomato cathepsin D inhibitor (*Sl*CDI) resulted in an increase in leaf protein content with transient expression of human AACT (α_1 – anti-chymotrypsin)

significantly higher in transgenic lines expressing the *Sl*CDI inhibitor.[9] Co-expression of an aspartic/serine 'companion' inhibitor also greatly increased leaf apoplast protein content with more murine diagnostic antibody (C5-1) co-secreted in the apoplast.[19]

There is still very limited knowledge on cysteine protease inhibitors as 'companions' possibly due to observations that less activity has been found in plant systems for cysteine proteases when compared to serine proteases. In a first attempt to demonstrate potential of such strategy, co-expression of the rice cystatin OC-I decreased cysteine protease activity resulting in a stabilizing effect on isolated Rubisco.[9] *Sl*CYS9, an inhibitor of papain- and legumain-like cysteine proteases, had no impact on apoplast-based production, but stabilized the C5-1 antibody *in planta*, presumably upstream in the secretory pathway.[19] In our group, we investigated the use of an 'in-built' protein stabilizing agent in genetically engineered tobacco plants expressing OC-I in the cytosol.[52] Constitutively expressing the rice cystatin in tobacco leaves lowered overall cysteine protease activity and increased the amounts produced of enzymatically active recombinant glutathione reductase, which was used as a model enzyme, when the enzyme was transiently produced in transgenic tobacco leaves after agroinfiltration.

Challenges ahead

Proteolysis caused by plant endogenous proteases is still a key challenge severely compromising recombinant protein yield. However, there is a constant search for new production systems not only to ease purification but also to limit proteolysis. Targeting recombinant proteins either to oil bodies or roots for rhizo-secretion are recent attractive new strategies for easier purification as well as to limit proteolysis.[53-54] Advantage of rhizo-secretion to leaf based-production is that after secretion the hydroponic culture medium has lower and less complex levels of proteolytic enzymes when compared to leaf extracts.

Engineered carnivorous plants have also recently been suggested as production platform system.[55] Carnivorous plants express and transport digestive enzymes into the traps, where any enzymes would be directly accessible for purification in a viscous and sticky liquid without plant destruction allowing continuous harvest. The limitation of this system is the presence of proteases in the juice which might affect recombinant protein stability. However, all these recent new technologies, although interesting, have so far resulted in insufficient protein yields to be considered commercially viable and the search for new innovative production systems for stable recombinant protein production should therefore be an ongoing activity.

Searching for plant species with low proteolytic activity providing better recombinant protein stability should also be relevant in future activities. In comparison to prokaryotic systems, protease-deficient mutant plants do not exist for plant species currently used for recombinant production. More intense screening of plant species useful for recombinant protein production for low protease activity is therefore urgently required. This is in addition to detailed studies on identifying plant endogenous proteases and investigating their expression profiles in various cellular locations. Better knowledge of protease involvement in senescence processes might be particularly helpful to improve protein stability in any transient expression system, as almost all protease families have been associated with some aspects of plant senescence.[56]. There is evidence that leaf infiltration with *Agrobacterium* cells causes leaf senescence resulting in the expression of senescence-related proteases including cysteine proteases.[52] Senescence, the final developmental stage of every plant organ, leads to cell death and senescence-associated proteolysis naturally enables the remobilization of nutrients but might also degrade recombinant proteins

Strategies for protein stabilization might also include application of inducible promoters for induced synthesis of inhibitors or induced down-regulation of proteases. In this regard, antisense or RNA silencing approaches could be of interest to contain proteolysis in

the plant host. Indeed, first evidence that such a strategy might be successful has been recently demonstrated with rice cells where application of the RNA interference technology using a gene to express ihpRNA of alpha-amylase and cysteine protease resulted in a 2.4-fold increase in the production of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) after down-regulation of cysteine protease expression.[57] However, identification of particular proteases involved in recombinant protein degradation is required to avoid any protease involved in vital cellular processes required for growth and development from being targeted.

Future research might also focus on identifying the specific inhibitors of proteases involved in recombinant protein degradation. A better understanding of the exact nature of these inhibitors would allow the design of more active inhibitors. Design by amino acid mutagenesis would optimize their inhibitory activity for application as a 'companion' protease inhibitor in either transient or stable expression of a recombinant protein via genetically engineered plants where proteins and the inhibitor are targeted to cellular compartments high in protein production.[19] Application of transgenic plant material expressing a recombinant protein in addition to co-expressing a 'companion' protease inhibitor is, however, rather complex due pleiotropic effects caused by inhibitor expression affecting plant growth and development.[48] There is so far only limited knowledge about these protease inhibitor actions. Such a strategy might also be problematic when inhibition involves targeting proteases in the secretory pathway.[38] Although such an inhibitor approach might be a major obstacle in a transgenic plant/seed approach, this might possibly be less problematic in short-term transient expression of a recombinant protein, which lasts only a few days.

Recent genomic and proteomic approaches have allowed the large-scale identification of proteases and the elucidation of their particular roles in cellular metabolism. This expanding knowledge will certainly help, in forthcoming years, to develop new techniques for high-throughput analysis of protease activity and identification of target proteins. This will

also advance our knowledge on recombinant protein stability and application of this knowledge in the future will be critical to significantly improving plant-based recombinant protein production.

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Table 1 Cellular locations for recombinant protein production in various plant host, and types of proteases identified at these locations.

Host	Protein	Compartment	Protease	References
Solanum tuberosum cv Desireé	Sea anemone equistatin	Secretory pathway, lytic vacuole, ER	Arginine/lysine-specific, legumain-type Asn-specific cysteine	[1]
Nicotiana tabacum cv Samsun NN	Monoclonal mouse IgG1	Apoplast	Cysteine, aspartic	[2]
Nicotiana tabacum L. Cv. Samsun	Glutathione reductase	Cytosol	Cysteine	[3]
Solanum tuberosum plantlets, cv. Kennebec	human α_1-antichymotrypsin	Cytosol	Aspartic, serine	[4]
Oryza sativa L. cv. Dongin	Human granulocyte–macrophage colony stimulating factor	Secretory pathway	Cysteine	[5]
Oryza sativa L. cv. Dongin	Synthetic serine proteinase inhibitor II gene	Extracellular	Serine	[6]
Oryza sativa L. cv. Dongin	Human granulocyte–macrophage colony stimulating factor	Extracellular	Serine	[7]
Solanum tuberosum L., cv. Kennebec	Bovine aprotinin	Cytosol, ER, apoplast	Serine	[8]
Nicotiana tabacum cv. BY-2	Human α_1-antichymotrypsin	ER, Golgi, apoplast, extracellular	Serine	[9]
Nicotiana tabacum L.	Oryzacystatin-1	ER, chloroplast	Cysteine	[10]
Nicotiana tabacum (var. xanthi)	Human $IgG_1\kappa$ antibody	Apoplast	Cysteine, aspartic, serine	[11-12]
Solanum tuberosum L., cv. Kennebec	Tomato cathepsin D inhibitor (CDI), Bovine aprotinin	Cytosol, ER	Serine	[13]
Nicotiana tabacum cv. '81V9'	Spider dragline silk	ER	Serine	[14]
Solanum tuberosum L., cv. Kennebec	Tomato cathepsin D inhibitor (SlCDI)	Cytosol	Aspartic, serine	[15]
Nicotiana tabacum	Monoclonal antibodies	Secretory pathway	Serine	[16]
Solanum lycopersium var. PED	Human α_1-proteinase inhibitor	ER, apoplast, vacuole, cytosol	Serine	[17]

Figure 1 Protease locations within plant cell subcellular compartments.