Expression of Multiple Novel Proteins in Plants from Single Polyprotein Constructs

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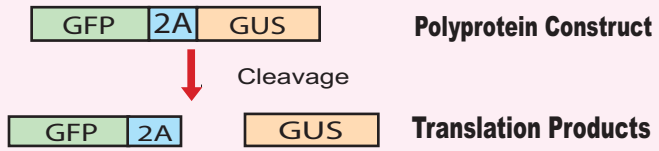
2A

2A is a 20 amino acid peptide from Foot and Mouth Disease virus. The 2A peptide is described as self-'cleaving', as dissociation of the viral polyprotein occurs at the 2A carboxy-terminus. 2A can be used to separate different coding sequences within a chimeric polyprotein, resulting in the expression of multiple peptides from a single open reading frame. However, 2A does not really separate from the coding sequences; a ribosomal 'skip' occurs, preventing the formation of a peptide bond. Originally it was hypothesised that 2A worked via proteolysis; that is the polyprotein was formed and subsequently cleaved in two, by lysis of a peptide bond. However it has since been proposed that breakage of a peptide bond does not occur; rather a peptide bond is not formed in the first place. It is now thought that 2A works by modifying ribosome activity, so that the first protein is prematurely released from the translational complex, preventing a peptide bond being formed. Translation then proceeds to form the second protein. 2A has been successfully used to co-express multiple proteins for biotechnological applications in plant, animal and yeast cells.



Development of a GUS Assay

Aim
To develop a transient assay system to allow 2A constructs to be tested quickly.

A transient assay was developed for testing the efficiency of the 2A constructs. This allows constructs to be tested quickly, without the time and effort required to make transgenic plants. The constructs are transformed into Agrobacterium and subsequently introduced into plants by agroinfiltration, and then 48h post-injection the leaves can be harvested, protein extracted and expression measured. To demonstrate that cleavage of the polyprotein is occurring, western blots are used. However, there needs to be enough protein present in the samples to detect by western blotting, therefore a GUS assay is used to determine if sufficient protein is present in the sample to be visualised by western blotting. The relative fluorescence of GUS had to be above 20 000 units to allow visualization of the protein on a western blot.

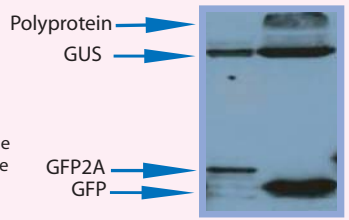


Figure 1. Lane 1 shows cleavage in plant cells of construct GFP-2A-GUS, lane 2 shows cleavage of GUS-2A-GFP.

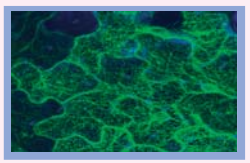


Figure 2. GFP expression from construct GUs-2A-GFP. GFP is ER targeted.

Alternative Sequences

Aim
To find alternative sequences with similar cleavage abilities to 2A. Ideally these sequences would be from a non-pathogenic animal virus.

It is unlikely that the use of 2A in GM crops, or in the development of biotechnological products would be accepted by the public, as the peptide is from a pathogenic animal virus, although the 2A sequence itself is not pathogenic. Similar sequences to 2A are found in the insect *Thosea asigna* Virus (TaV) and Porcine Teschovirus (PTV). These sequences, particularly TaV may be deemed more acceptable by the public. I have examined the cleavage activity of these alternative sequences in plant cells, and found them to be equally efficient to the FMDV 2A. In particular the TaV sequence appears to leave little uncleaved polyprotein.

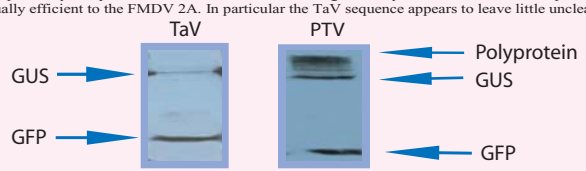


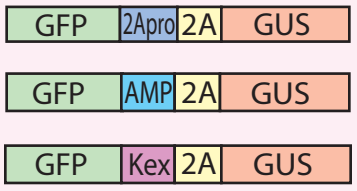
Figure 3. Comparison of cleavage between constructs GFP-TaV-GUS and GFP-PTV-GUS.

A Fully Cleavable 2A

Aim
To develop a 'fully-cleavable' 2A, so that the 2A peptide is completely excised from the protein products.

Upon 'dissociation' of the polyprotein, the protein anterior to 2A gains 19 amino acids at its C-terminus, while the posterior protein gains only one amino acid. The addition of 19 amino acids has not affected the proteins used in the constructs, but it is possible that in more complex molecules the additional amino acids could interfere with protein folding and targeting. This creates the need for a 'fully cleavable' 2A, where the excess residues would be completely excised. Constructs containing linker sequences were designed to attempt to produce a 'fully-cleavable' 2A. The linker sequences used were Kex from yeast and AMP from *Balsamina impatiens*; these sequences contain cleavage sites which are recognised by cellular proteases. Another linker used was the 2A protease (2Apro) from Human Rhinovirus (HRV), which elicits cleavage at its N-terminus.

Results from the western blot show that the 2Apro and Kex constructs produce GFP of the correct size, but GUS can not be detected on the blot. The GUS assay for these constructs showed little GUS activity, so this suggests translation is terminated after GFP production. The construct containing the AMP linker sequence yields both GFP and GUS, indicating that 2A has functioned as expected, but the AMP linker sequence does not appear to have cleaved from GFP by the cellular protease



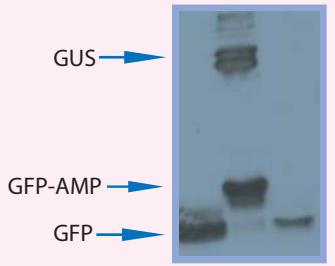


Figure 4. Western blot of constructs containing linker sequences.
Lane 1: GFP-2Apro-2A-GUS
Lane 2: GFP-AMP-2A-GUS
Lane 3: GFP-Kex-2A-GUS

Lpro

It is known that there are alternative viral sequences with similar cleavage abilities to 2A. It was thought that the cleavage ability of the Leader protease (Lpro) from FMDV might be suitable for producing a 'fully-cleavable' 2A. This protease cleaves at its C-terminus and then cleaves (at an 18 amino acid recognition sequence) within the polyprotein. Lpro is toxic when expressed in animal cells and unfortunately, is also toxic to Agrobacterium, preventing the use of agroinfiltration into plants. Therefore direct transformation using biolistics has to be used to introduce the constructs to plants. Tobacco seedlings were bombarded with the Lpro construct and then stained for GUS expression. From the picture of the seedlings blue GUS spots can clearly be seen, showing GUS expression has occurred. However, the quantity of protein produced is not enough to be detected on a western blot, to show conclusively that cleavage is occurring, and the linker sequence is being removed. The experiment, however, indicates that although toxic to Agrobacterium and mammalian cells, Lpro is not toxic to plant cells.

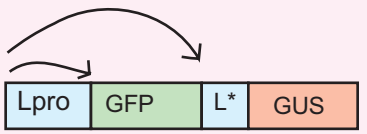


Figure 5. Diagram showing points of cleavage in Lpro construct.

Figure 6. GUS staining of a *Nicotiana tabacum* seedling, bombarded with the Lpro construct.

Future Research

Future research will concentrate on designing a fully cleavable 2A, and trying to get levels of expression from the Lpro construct high enough to detect on a western blot. Viral vectors are being examined as a possible method to boost expression of the Lpro construct. Constructs are also being designed to demonstrate the utility of 2A to metabolic engineering in plants, by expressing multiple genes (phytoene synthase and phytoene desaturase) to manipulate the synthesis of carotenoids in potato plants.