Use of Fluorescent Chimeras to Characterise ALY Domains Involved in Nucleolar Targeting and Interaction with the TBSV P19

Tomas Canto and Stuart MacFarlane

Scottish Crop Research Institute
Scottish Crop Research Institute,
Invergowrie, Dundee DD2 5DA, Scotland, UK
Tel: 00-44-(0)1382-562731 Fax: 00-44-(0)1382-562426

Introduction

We previously found that the suppressor of gene silencing P19 from *Tomato bushy stunt virus* interacts with the central RNA Recognition Motif (RRM) of plant ALY proteins, in yeast-two-hybrid assays (Uhrig *et al.*, Plant Physiol.135, 2411-2423). Using fluorescent tags and confocal microscopy, the subcellular distribution of P19 and those of the four *Arabidopsis thaliana* (At) and four *Nicothiana benthamiana* (Nb) ALYs was determined, in the absence or presence of P19. In the absence of P19, all AtALYs showed nuclear and, with one exception, nucleolar targeting. In the presence of P19, two AtALYs and two NbALYs relocalised from the nucleus to the cytoplasm, whereas the other four remained nuclear. Here we characterise the ALY domains involved in nucleolar targeting and the relocation induced by the P19 protein using chimeras. The effect that transiently expressed ALY proteins had on the subcellular distribution of P19 and on its activity as a suppressor of gene silencing is also presented.

Methods

Green fluorescent protein (GFP) or monomeric red fluorescent protein (mRFP) tags were added to the C-terminus of ALY and P19 proteins. Wild type and fluorescently tagged proteins were transiently expressed, either alone or in mixtures, in epidermal cells of *Nicothiana benthamiana* leaves by agroinfiltration techniques. The subcellular distribution patterns were then analysed by confocal microscopy. In order to characterise the ALY domains involved in nucleolar targeting and the relocation induced by P19, chimeras were made between *Arabidopsis thaliana* (At) ALY 2, which does not accumulate in the nucleolus and does relocate to the cytoplasm in the presence of P19, and AtALY 3, which accumulates in the nucleolus and does not relocate to the cytoplasm in the presence of P19. Nucleic acid extracts from infiltrated patches were analysed for GUS mRNA and siRNAs by Northern blotting.

Results

We have determined the subcellular distribution of four ALY homologs from *Arabidopsis thaliana* and of another four from *N. benthamiana*. When expressed alone or together with the suppressor P1-HCPro, all ALY-GFPs accumulated almost exclusively in the nucleus, including, save one case, the nucleolus (Figure 1, left field and nuclei panels). By contrast, co-expression with the suppressor P19 resulted in the redistribution of some of the ALY-GFPs to the cytoplasm (Figure 1, right field and nuclei panels).

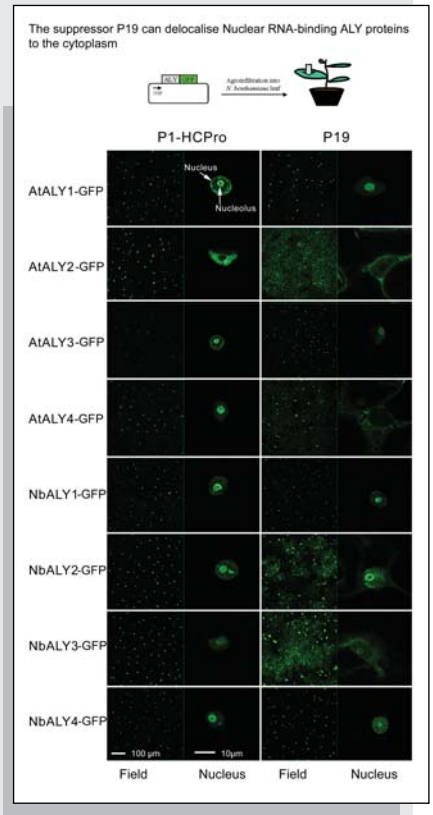

Figure 1. The viral suppressor P19 induces relocalisation of a subset of plant ALYs from Nucleus to Cytoplasm. All four GFP-tagged *A. thaliana* ALYs (AtALYs) and all four *N. benthamiana* ALYs (NbALYs) localised to the cell nucleus, including (with the sole exception of AtALY2) the nucleolus, when expressed either alone or together with the suppressor P1-HCPro of Potato Virus Y (left field and nuclei panels). By contrast, fluorescence from AtALYs 2 and 4, and NbALYs 2 and 3 shifted from nucleus to cytoplasm when co-expressed with the suppressor P19 (rigth field and nuclei panels).

Using chimeras between AtALYs 2 and 3, we found that nucleolar targeting by AtALY 3 is determined by the C-terminal and the Central RRM domain of the protein (Figure 2A). The domain responsible for the nucleus-to-cytoplasm delocalisation of AtALY2 by P19 localised to the C-terminal half of the central RRM domain (Figure 2B).

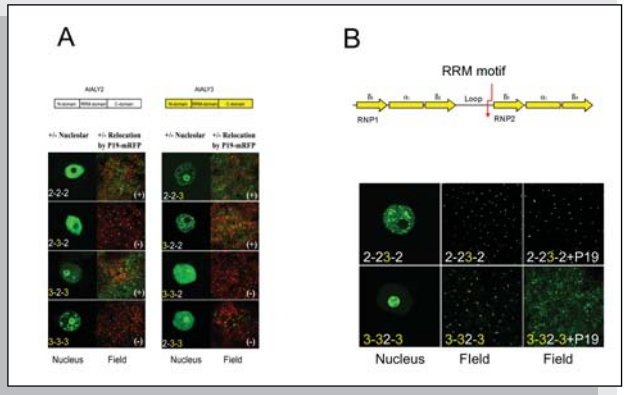

Figure 2. Characterisation of ALY domains involved in nucleolar targeting and the delocalisation induced by P19. Chimeras were made between *Arabidopsis thaliana* (At) AtALY 2, which does not accumulate in the nucleolus and does relocate to the cytoplasm in the presence of P19, and AtALY 3, which accumulates in the nucleolus and does not relocate to the cytoplasm in the presence of P19. **A.** For any chimera to target the nucleolus, either the Central RRM or the C-terminal domain must originate from AtALY 3 (nucleus panels). For any chimera to be delocalised by P19, the central RRM must originate from AtALY2 [field panels, delocalisation indicated by a (+)]. **B.** Further mapping of the delocalisation domain within the RRM motif using intra-RRM chimeras showed that only the C-terminal half of the RRM domain of AtALY2 is required to allow the delocalisation of the chimera by P19 (lower panels).

Co-expression of mRFP- or GFP-tagged P19 with ALY also affected the subcellular distribution of the former protein: expressed alone, fluorescence was confined mostly to the cytoplasm, with very little nuclear fluorescence, and no nucleolar fluorescence at all. In presence of ALY, P19-associated fluorescence shifted to the nucleus/nucleolus (Figure 3). The presence of overexpressed ALY also affected the activity of P19 as suppressor (Figure 4), in a way that seems to bear relation with the changes in subcellular localisation patterns.

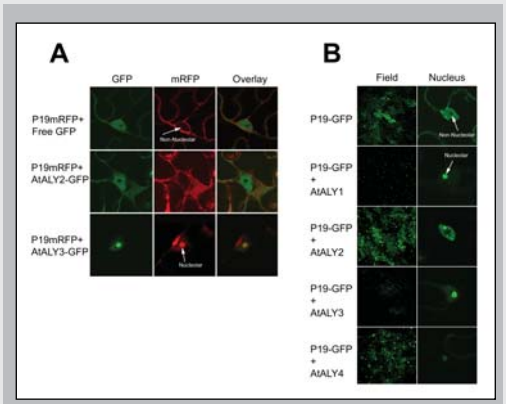

Figure 3. Co-expression with ALY alters the subcellular distribution of tagged P19. **A**. Red fluorescence from mRFP-tagged P19 was found mostly cytoplasmic, a little nuclear and not nucleolar, when co-expressed with free GFP (upper panels), but when co-expressed with AtALY3-GFP, P19-mRFP accumulated strongly in the nucleolus (lower panels). **B**. Likewise, distribution of P19-GFP on its own was mostly cytoplasmic (top field and nucleus panels), but when co-expressed with AtALYs, it accumulated in the nucleus/nucleolus (top vs. lower panels).

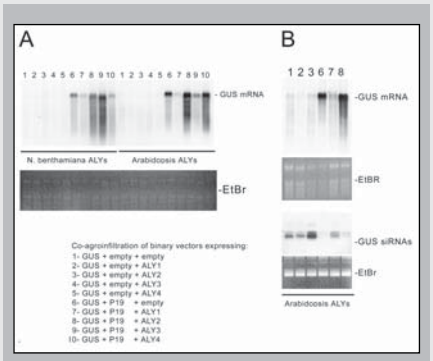

Figure 4. Overexpression of ALY affects the suppressor activity of P19. An *Agrobacterium* culture containing a binary vector expressing GUS was mixed with cultures containing empty vectors, vectors expressing P19, or expressing ALY proteins (samples 1 to 10) and infiltrated into *Nicothiana benthamiana* leaves. **A**, Northern blot analysis of nucleic acids from the infiltrated patches showed an increase in the steady state level of GUS mRNA in presence of P19 (lane 6). However, co-expression of P19 with ALYs that did not delocalise decreased P19 suppression activity (ie. lane 7). **B**, The effect was mirrored in the levels of siRNAs to GUS (compare lanes 6 and 7).

Conclusions

Using fluorescent tags and chimeras, we have mapped domains involved in the interaction between nuclear plant ALYs and the suppressor P19, and in their subcellular localisation. We have shown that this interaction between ALY and P19 proteins results in the transfer to the cytoplasm of some ALYs, but also in the transfer of P19 from the cytoplasm to the nucleus/nucleolus. These data suggest the existence of a turnover of both proteins in and out of the nucleus. An effect of these interaction phenotypes on the biological activity of the P19 as suppressor was also found.