

Article Addendum

The CaMV 35S promoter has a weak expression activity in dark grown tissues of moss *Physcomitrella patens*

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The constitutive Cauliflower Mosaic Virus 35S promoter (CaMV 35S) is widely used as a tool to express recombinant proteins in plants, but with different success. We previously showed that the expression of an F-actin marker, GFP-talin, in *Physcomitrella patens* using the CaMV 35S promoter failed to homogeneously label moss tissues. Here, we show a significant diminution of the GFP fluorescence in dark grown old moss cells and complete lack of labelling in newly differentiated cells. Furthermore, we demonstrate that stable moss lines harbouring a resistance cassette driven by the CaMV 35S are unable to grow in darkness in the presence of the antibiotic. In contrast to the CaMV 35S, the heat inducible promoter, *hsp17.3B* showed uniform expression pattern in all cells and tissues following a mild heat shock.

The CaMV 35S promoter is regularly used to drive the expression of recombinant proteins in plants. In angiosperms, several studies showed that reporter genes driven by this promoter display unequal tissue and developmental expression patterns.¹⁻⁶ Several versions of the CaMV 35S promoter are being used in different plant species and an 800 bp *HindIII-BamHI* CaMV 35S fragment⁷ is the most commonly used in the moss *Physcomitrella patens*. CaMV 35S was intensively used to drive the expression of resistance genes, cDNAs as well as genomic sequences.⁸⁻¹⁰ Comparison between several true constitutive promoters in *P. patens* revealed that CaMV 35S has a much weaker activity as compared to the rice actin-1 and the maize ubiquitin-1 promoters.^{11,12} Although the expression of resistance genes using CaMV 35S is sufficient

for the selection of transformed moss plants, the attempts to overproduce proteins of interests by CaMV 35S remain rather inefficient in moss. For instance, the complementation of *abi3* mutant phenotype was not successful when 35S::ABI3 expression cassette was introduced in *P. patens* genome.¹³ Moreover, under standard conditions (16 h light/8 h dark), the expression of actin microfilament marker (GFP-talin) using CaMV 35S promoter, in 35S-GT line, resulted in detectable GFP fluorescence only in old cells while GFP levels were weak and undetectable in actively differentiating protonemal and meristematic cells.¹⁴ Our attempts to overcome this problem by using stronger promoters such as the rice actin-1,¹⁵ or the maize ubiquitin-1,¹⁶ promoters failed probably due to the high toxic amounts of produced GFP-talin (data not shown). These obstacles were overcome by employing the inducible soybean *hsp17.3B* promoter¹² to drive GFP-talin expression in HGT line.¹⁴

Caulonemal cells are the only protonemal cell type that can grow and divide in darkness in presence of a carbon source.¹⁷ To verify whether the expression of GFP-talin could be achieved in these newly differentiated cells, we monitored fluorescent labeling in dark grown caulonemal cells in 35S-GT and HGT lines. Whereas HGT cells remained unlabeled, a steady attenuation of GFP fluorescent signal was observed in the previously labeled tissue of 35S-GT line (Fig. 1A). Moreover, no GFP signal was detected in newly differentiated caulonemal cells (Fig. 1B). Whereas a short mild heat shock (1 h at 36°C) had no influence on labeling in 35S-GT line, a uniform GFP labeling was detected in all HGT newly developed cells (Fig. 1C).

To further study the phenotypic consequences of the above-mentioned CaMV 35S-driven expression pattern, we compared the resistance to hygromycin of two moss lines both carrying the hygromycin phosphotransferase (*hph*) resistance gene driven either by CaMV 35S promoter (35S-Hygro^R) or the rice actin-1 promoter (Act-Hygro^R). We monitored caulonemal differentiation in 35S-Hygro^R and Act-Hygro^R lines grown under continuous darkness (Fig. 2A and B). In the presence of 25 mg/l hygromycin, differentiation and growth of 35S-Hygro^R caulonemal cells was drastically reduced compared to the one observed in Act-Hygro^R line. Under standard light conditions, colonies of both

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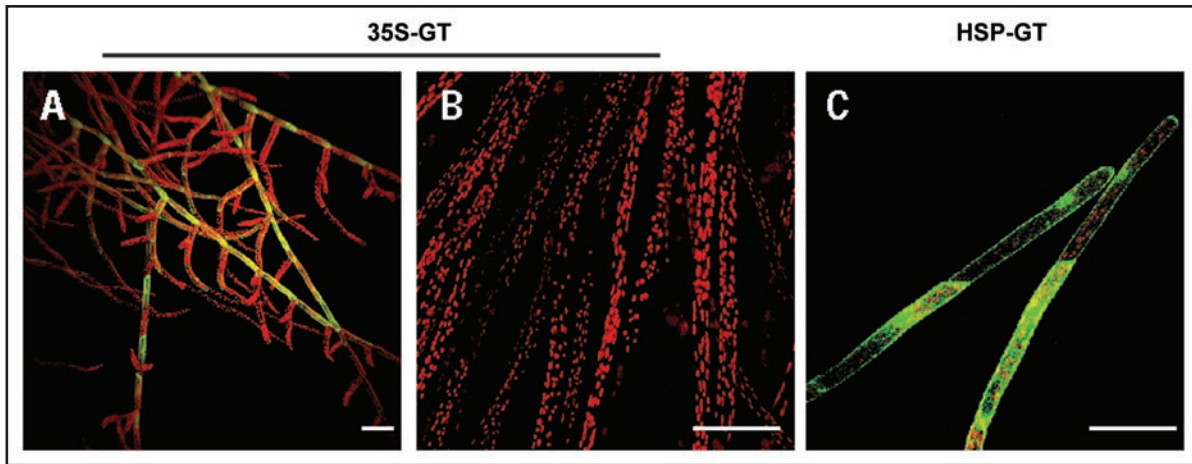


Figure 1. Confocal images showing GFP-talin expression in 35S-GT and HGT lines. (A–C) Merged confocal images displaying auto fluorescence of chloroplasts (in red) and fluorescence signal of GFP (in green). (A) 35S-GT grown under standard conditions. Ten days old, dark grown caulonemal cells of (B) 35S-GT and (C) HGT lines. The 35S-GT cells were imaged immediately after light exposure, whereas HGT cells were heat treated in darkness for 1 h at 36°C and imaged 16 hours after treatment. Bars: 100 μm.

lines proliferated optimally and at similar rate regardless of the presence of hygromycin (Fig. 2C and D). Similarly, dark grown strains with integrated 35S-*nptII* (neomycin phosphotransferase II) expressing cassette could not produce etiolated caulonemal cells on medium containing G418 antibiotic (data not shown). These data indicate that resistance to antibiotic is seriously diminished during etiolating processes in lines harboring the resistance gene driven by CaMV 35S promoter.

We conclude that moss etiolation make expression of transgenes from CaMV 35S promoter unattainable to the point of loss of transgene phenotypical characteristics. We may only speculate that the weak activity of CaMV 35S promoter is intrinsically more repressed in darkness or it is attributable to the lack of certain non-identified transcription factor regulated by light. In moss cells, when expression of transgenes needs to be maintained in darkness, we therefore advocate the use of endogenous promoters (i.e., housekeeping genes) by gene conversion. Yet, if overexpression or ectopic expression is required the rice actin promoter is better choice than CaMV 35S promoter. Alternatively, when the permanent overexpression of proteins of interest causes adverse effect to *P. patens*, the use of the *hsp17.3B* inducible promoter is thus the best approach to date.

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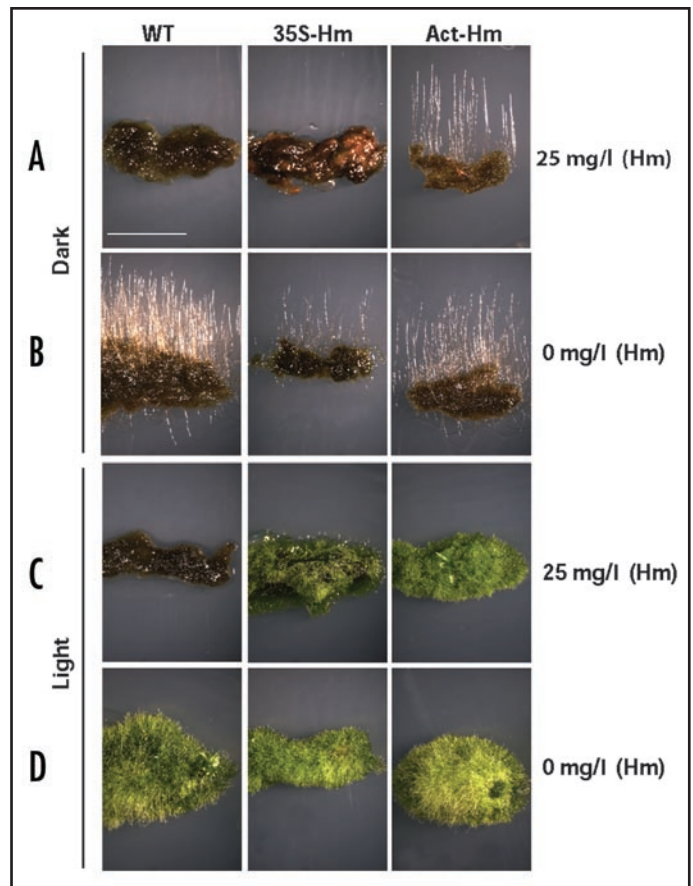


Figure 2. 35S-Hm line is unable to grow in the dark when hygromycin is supplemented to the medium. Growth of WT (left), 35S-Hm (middle) and Act-Hm (right) lines using mediums supplemented with (A and C) or without (B and D) hygromycin (Hm, 25 mg/l). Six days old *P. patens* colonies were transferred to dark (A and B) or continued to grow in standard growing conditions (C and D) during ten days. Pictures were taken immediately after. Bars: 10 mm.

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