Resolving plant transformation recalcitrance by Agrobacterium-mediated protein translocation

Gariboldi, I.

Chapter 4

Tackling plant transformation recalcitrance by *Agrobacterium*-mediated protein translocation

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Abstract

DNA transfer by the soil bacterium *Agrobacterium tumefaciens* (Agrobacterium) is commonly used to generate transgenic plants or for CRISPR-Cas-mediated genome editing. However, Agrobacterium-mediated transformation (AMT) is only efficient in a limited number of plant species or accessions, as many are recalcitrant to this process. This recalcitrance is caused on the one hand by inefficient DNA transfer due to suppression of Agrobacterium virulence by plant cells, and on the other hand by problems with regenerating plants from the transformed cells. It has been shown that Agrobacterium also translocates Virulence (Vir) proteins to plant cells and that this system can be used to introduce heterologous proteins into plant cells. In this chapter, we investigated in *Nicotiana tabacum* (tobacco) leaves whether Agrobacterium-mediated protein translocation (AMPT) can be used to tackle some of the bottle-necks leading to recalcitrance to AMT. Interestingly, AMPT of the *Pseudomonas syringae* avirulence protein AvrPto did not induce severe effector triggered immunity (ETI) leading to leaf necrosis, which is normally observed when *AvrPto* is overexpressed under control of a *35S* promoter. Instead AMPT of AvrPto or the bacterial salicylic acid hydroxylase NahG enhanced the efficiency of both AMT and AMPT, probably by reducing recalcitrance caused by the Agrobacterium induced plant defense responses. In addition, we show that AMPT of the *Arabidopsis thaliana* AT-HOOK MOTIF NUCLEAR LOCALIZED 15 (AHL15) to tobacco leaves reduced the senescence response induced by Agrobacterium. Furthermore, the transfer of AHL15 was able to enhance shoot regeneration on tobacco leaf discs. Based on our result we conclude that AMPT can be used to resolve bottle necks causing recalcitrance to AMT

Introduction

Plants are found all over the world and manage to thrive even in the most difficult natural habitats despite being immobile. Because of this they have evolved elaborate signalling networks for growth, reproduction and defence (Blaacutezquez et al., 2020). In order to respond to internal and external stimuli, plants have to make use of hormone signalling, so-called phytohormones, to communicate with proximal and distal parts (Anfang & Shani, 2021).

Current commercial crops have lost some of this environmental resilience by extensive breeding programs that have focussed on high production capacity. Current breeding programs are aimed at reintroducing resilience traits, but this is a laborious and time-consuming process. With the increasing knowledge on resilience genes and recently developed new techniques, introduction of these traits by directed genome editing would be preferrable. This requires efficient protocols of transformation and regeneration, for which DNA transfer by the soil bacterium *Agrobacterium tumefaciens* (Agrobacterium) is commonly used. Unfortunately, the recalcitrance of many commercial cultivars to Agrobacterium-mediated transformation (AMT) and the subsequent regeneration of genome edited plants still forms a major bottleneck.

The recalcitrance to AMT affects transient expression experiments and makes regeneration of transgenic lines difficult. It is for an important part caused by the fact that plants have developed effective defence systems that enable them to recognise phytopathogens, which in turn have co-evolved together with their host (Anderson et al., 2010). Many phytopathogenic bacteria make use of a delivery system to transfer virulence proteins e.g. to modulate the plant defence or aid in infection. Common delivery systems are the type III (T3SS) and type IV (T4SS) secretion systems, of which the T4SS also transfers DNA (Costa et al., 2021; Deng et al., 2017). Plants on the other hand have the ability to detect these effector or avirulence (Avr) proteins produced by phytopathogens by Resistance (R) proteins, which can either directly recognize the effector proteins or act via 'Guard Model' monitoring, guarding the target of the pathogen effector (Van Der Hoorn & Kamoun, 2008). This recognition induces a rapid defence response, the so-called

hypersensitive response (HR), which prevents spread of the infection by localized cell death (necrosis) on the site of infection (Klessig et al., 2018). Resistance upon infection that radiates throughout the plant is called systemic acquired resistance (SAR). The SAR response is under control of the plant defense hormone salicylic acid (SA) and N-hydroxypipecolic acid (NHP). Exogenous application of SA to Agrobacterium cultures decreased its growth, virulence, and attachment to plant cells (Verberne et al., 2003). *Nicotiana benthamiana* plants treated with SA showed decreased susceptibility to AMT (Anand et al., 2008). Compared to *N. benthamiana* and *Nicotiana tabacum* (tobacco) leaf infiltration, which is abundantly used for transient expression following AMT, Arabidopsis shows recalcitrance to AMT resulting in variable transient expression (Khan, 2017). It was shown that the transient expression efficiency in Arabidopsis leaves can be increased by expressing the *Pseudomonas syringae AvrPto* effector gene under a inducible promoter prior to infiltration (Tsuda et al., 2012b). AvrPto blocks pathogen-associated molecular pattern triggered immunity (PTI) by binding pattern-recognition receptors (PRRs) including FLS2 and EFR (Chinchilla et al., 2006; Zipfel et al., 2006). However, this only works in susceptible hosts, as in non-susceptible hosts AvrPto competes with Pto kinase for binding with PRRs (Xiang et al., 2008) and the interaction of AvrPto and Pto can activate effector-triggered immunity (ETI) (H. Chen et al., 2017). Transient expression of T-DNA is also enhanced by decreasing the endogenous SA levels by expression of *NahG*, encoding an enzyme that can metabolize SA, or by using the SA biosynthesis mutants *sid2* and *ics1* or signaling mutant *npr1* (Rosas-Díaz et al., 2017; Zhu et al., 2017). Expression of *NahG* in Arabidopsis also increased the transformation efficiency (Lawton et al., 1995).

Another bottleneck causing low efficiency in AMT is recalcitrance to regeneration. Plant somatic cells do not normally regenerate new organs or form new embryos, but can be triggered to do so by treatment with phytohormones or by overexpression of specific transcription factors with a key role in zygotic embryogenesis, such as BABY BOOM (BBM), LEAFY COTYLEDON1 (LEC1), WUSCHEL (WUS) or AT-HOOK MOTIF NUCLEAR LOCALIZED15 (AHL15) (Boutilier et al., 2002; Horstman et al., 2017; Karami et al., 2021; Zuo et al., 2002). Generally, stable Agrobacterium-mediated transformation (AMT) was used to obtain lines

overexpressing these transcription factors, leading to increased regeneration efficiencies in various plant species (Heidmann et al., 2011; Horstman et al., 2017; Lowe et al., 2016). Moreover, overexpression of *AHL15* and other *AHL* genes was found to reduce leaf senescence (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013).

Previously, it was shown that the T4SS of Agrobacterium can be used to translocate heterologous proteins to host cells (Sakalis et al., 2014; Vergunst et al., 2000, 2003). In this chapter we investigated the use of Agrobacterium-mediated protein translocation (AMPT) to resolve the two main bottle necks; the recalcitrance to Agrobacterium transformation and the recalcitrance in regeneration. First, the functionality of fusion proteins transferred to or expressed in plant cells via AMPT or after AMT, respectively, on plant physiology was established using AvrPto and AHL15. As expected, transfer or expression of AvrPto induced necrosis whereas AHL15 delayed senescence in *N. benthamiana* leaves. Next we tested AMPT of AvrPto or NahG and observed that this resulted in increased AMPT and transient AMT efficiencies. Interestingly, AMPT of AvrPto did not induce severe leaf necrosis, making it useful to enhance transient expression. Finally, we observed that shoot regeneration from tobacco leaf discs could be increased by AMPT or AMT of AHL15.

Results

AMPT of AvrPto induces necrosis in tobacco leaves

As a first approach to test whether AMPT of an heterologous protein can induce a physiological effect in plants, AvrPto from *Pseudomonas syringae pv. Tomato* (Pto) DC3000 was used, since it induces a strong hypersensitive (HR) response, resulting in programmed cell death at the site of infection in incompatible plants such as *N. benthamiana* and tobacco (Alfano & Collmer, 2004; Choi et al., 2017; Gimenez-Ibanez et al., 2014). Leaves of 4-weeks old tobacco plants were infiltrated with an Agrobacterium strain carrying either a plasmid with *pvirE::GFP$_{11}$:AvrPto:ΔvirF* for AMPT of the AvrPto fusion protein (fp), or a plasmid with *p35S::GFP$_{11}$:AvrPto:ΔvirF::tNOS* (T-DNA fp) or *p35S::AvrPto::tNOS* (T-DNA) for AMT

of a T-DNA expressing the AvrPto fusion protein or AvrPto without tags from the constitutive *35S* promoter (Fig. 1A). At 4 days after infiltration (dpi), transient overexpression of *AvrPto* induced necrosis in almost the entire infiltrated zone (98.1 %) and at 8 dpi this increased to 99.9 % (Fig. 1B). Transient expression of the *GFP$_{11}$:AvrPto:ΔvirF* fusion protein showed a milder necrosis in the leaf tissue at 4 dpi (22.7 %), but at 8 dpi this increased to 84.3 %. AMPT of the AvrPto fusion protein 4 dpi showed necrosis of 2.3 % of the leaf tissue and increased 8 dpi to 13.8 %. These results indicate that AMPT of an AvrPto fusion protein to tobacco leaf cells can induce a physiological effect in the form of necrosis. However, this effect is weaker compared to when the fusion protein or the non-fused AvrPto protein is transiently expressed following AMT. Also, it should be noted that the GFP$_{11}$:AvrPto:ΔVirF fusion protein is significantly less active compared to the AvrPto protein itself in AMT experiments.

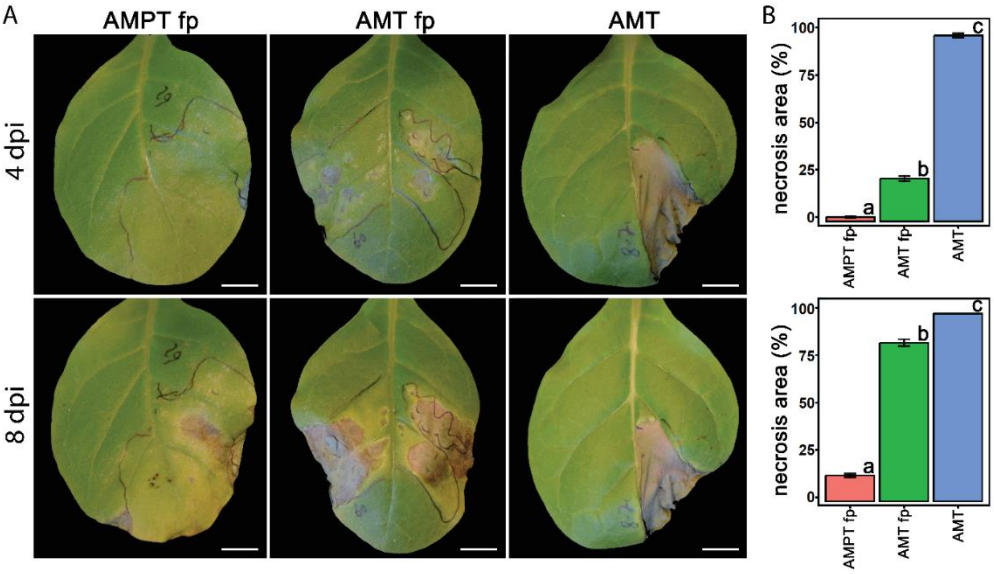

Figure 1. **AMPT and AMT of AvrPto induces necrosis in *N. tabacum* leaves.** (A) Hypersensitive response observed as necrosis in leaves of 4-weeks old tobacco 4 dpi or 8 dpi caused by AMPT of GFP$_{11}$:AvrPto:ΔVirF under control of *pvirE* (AMPT fp), or AMT of a T-DNA construct containing *p35S::GFP$_{11}$:AvrPto:ΔvirF::tNOS* (AMT fp) or *p35S::AvrPto::tNOS* (AMT). Size bars indicate 10 mm. (B) The percentage of the infiltrated leaf surface that showed necrosis at 4 dpi (upper panel for AMPT (2.3 %), AMT fp (22.7 %) and AMT (98.1 %) or at 8 dpi (lower panel) for AMPT fp (13.8 %), AMTfp (84.3 %) and AMT (99.8 %). Letters indicate statistically significant differences ($p < 0.001$) as determined by one-way analysis of variance (ANOVA)

with Tukey's honest significant difference post hoc test. Bars indicate the mean area and error bars indicate the standard error of the mean (n = 12).

AMPT of AHL15 *delays senescence in N. benthamiana leaves*

Next we tested whether AMPT of AHL15 could also induce detectable physiological changes in *N. benthamiana* leaves. Agrobacterium leaf infiltration is known to induce host defense and developmental responses in tobacco and *N. benthamiana* leaves, among which the senescence-related loss of chlorophyll (Ludwig et al., 2005; Pruss et al., 2008). Previous observations on Arabidopsis and tobacco plants overexpressing *AHL15* (Karami et al., 2020) and reports on AHL15 homologs indicated that these AT-Hook motif proteins repress leaf senescence (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013). To test whether AMPT of AHL15 could repress Agrobacterium-induced senescence in leaves of *N. benthamiana*, we infiltrated leaves of 4-weeks old plants with an Agrobacterium strain, either transferring the fusion protein GFP_{11}:AHL15:ΔVirF expressed from *pvirE* (fp), or transferring a T-DNA construct carrying *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (T-DNA fp), *p35S::AHL15::tNOS* (T-DNA) or *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control). Plants expressing a similar fusion with the Cre recombinase were used as control, as previous work has shown that expression of the Cre recombinase does not affect Arabidopsis development (Vergunst et al., 2000). Clear yellowing could be observed in leaves infiltrated with the control strain at 7 dpi, whereas the yellowing was reduced for the other three strains (Fig. 2A). Quantification of the yellowing at 4, 5, 6 and 7 dpi using a handheld device for non-destructive relative chlorophyll content confirmed this observation (Fig 2B, 2C). These results show that AMPT of the GFP_{11}:AHL15:ΔVirF fusion protein is as effective in reducing chlorophyll breakdown as when the AHL15 fusion or the native AHL15 protein is expressed from a T-DNA following AMT.

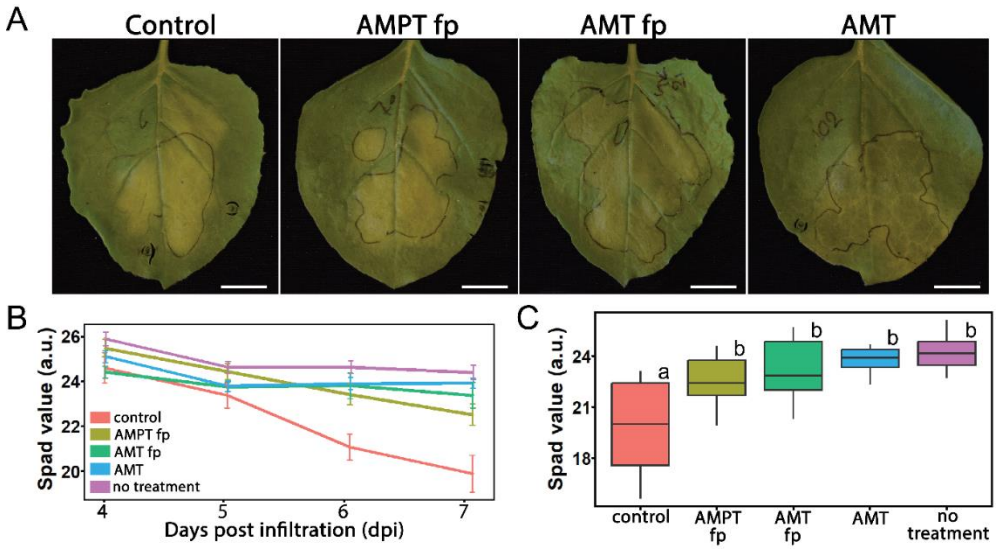

Figure 2. AMPT of AHL15 delays Agrobacterium-induced senescence in *N. benthamiana* leaves. (A) Leaves of 4-weeks old *N. benthamiana* plants at 7 dpi with an Agrobacterium strain carrying *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control), ***pvirE::GFP$_{11}$:AHL15:ΔVirF*** (AMPT fp), *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (AMT fp) or *p35S::AHL15::tNOS* (AMT). Size bars indicate 10 mm. (B) Quantification of the chlorophyll content in the infiltrated area of *N. benthamiana* leaves, as shown in (A) at 4, 5, 6 and 7 dpi. Error bars indicate standard deviation. (C) Quantification of the chlorophyll content of *N. benthamiana* leaves at 7 dpi. Indicated are the median, second and third quartile and whiskers extend the interquartile range by 1.5 (n = 6). Different letters indicate statistically significant differences ($p < 0.05$) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test.

NahG or AvrPto co-translocation increases AMPT and AMT efficiencies in tobacco

After establishing that fusion proteins following AMPT can induce the expected physiological effects in plant cells, we determined the effect of AMPT of AvrPto or NahG on the efficiency of AMPT and AMT by infiltrating 4-weeks old tobacco leaves with the split-GFP^{col} system (Fig. 3A), allowing the simultaneous detection of AMT and AMPT. Agrobacterium strains were used transferring by AMT the T-DNA construct *p35S::GFP$_{1-10}$/p35S::Cherry::tNOS* (where both fluorescent proteins carried a Nuclear Localization Signal (NLS) sequence) and by AMPT the fusion protein GFP$_{11}$:AvrPto:ΔVirF, GFP$_{11}$:NahG:ΔVirF or GFP$_{11}$:BBM:ΔVirF expressed under

control of *pvirE*. The infiltrated leaves were analyzed 4 dpi using confocal microscopy and leaf extracts were used to quantify the GFP and Cherry signals in a plate reader. Confocal analysis showed clear nuclear GFP signal from the split-GFP system, indicative of successful AMPT, co-localizing with the Cherry reporter for AMT (Fig. 3B). The GFP intensity was significantly stronger when the NahG or AvrPto fusion proteins were translocated, compared to translocation of the BBM fusion protein (Fig. 3C). The Cherry signal was enhanced by the co-translocated AvrPto fusion protein, but even stronger with a co-translocated NahG compared to the BBM control fusion protein (Fig. 3C). One has to keep in mind, however, that co-translocation of the AvrPto fusion eventually induces necrosis and can therefore only be used to enhance transient expression and not for stable transformation. The results with the NahG fusion suggested that the transformation efficiency can be increased by lowering the SA concentration in plant cells, implying that SA has a negative effect on Agrobacterium. Indeed, addition of SA to Agrobacterium cultures completely abolished *vir* gene induction (Fig. S1A) and had a severe negative effect on the growth of Agrobacterium (Fig. S1B). Our results indicate that both the AMPT and AMT can be significantly enhanced by co-translocation of NahG or AvrPto, but that concerning the efficiency and for stable transformation co-translocation of the NahG protein seems to be the best choice.

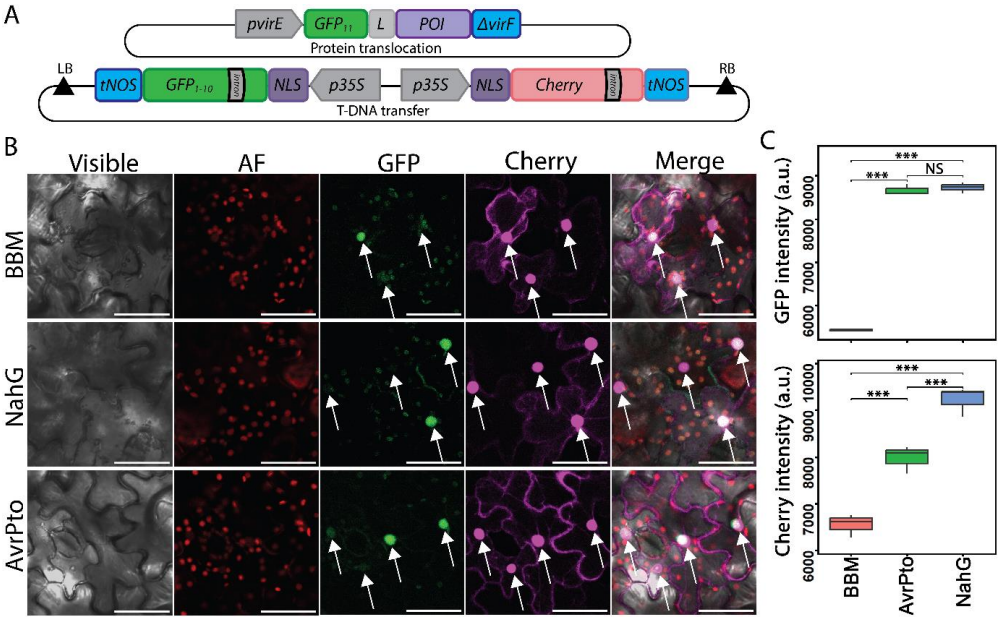

Figure 3. AMPT of AvrPto or NahG enhances protein and DNA transfer by Agrobacterium. (A) Schematic representation of the split-GFP^{col} combined AMPT and AMT detection system. The system comprises of a T-DNA transfer vector containing the *NLS:GFP$_{1-10}$* and *NLS:Cherry* coding regions, both driven by the *35S* promoter. The protein transfer vector encodes a GFP$_{11}$:POI:ΔVirF fusion protein under control of the *virE* promoter. Abbreviation: POI, protein of interest. (B) Confocal images of Cherry and GFP fluorescence observed in 4-weeks old tobacco leaf epidermis cells at 4 dpi with an Agrobacterium strain containing the split-GFP^{col} system: AMT of *p35S::NLS:sfCherry2::tNOS* and AMPT of GFP$_{11}$:BBM:ΔVirF (top), GFP$_{11}$:NahG:ΔVirF (middle) or GFP$_{11}$:AvrPto:ΔVirF (bottom). Scale bars indicate 50 µm. (C) Quantification of GFP (top) and Cherry (bottom) fluorescence in extracts of leaves imaged in (B) using a 96-wells plate reader. Measurements were adjusted to a control treatment (AMT of *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS)*. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 (n = 3). Different letters above the boxplots indicate statistically significant differences ($p < 0.001$) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test.

Functional analysis of AHL15 and BBM protein fusions for AMPT

Next, we tested whether we could use AMPT of AHL15 and BBM to enhance plant regeneration. For many transformation protocols regeneration forms an important rate-limiting step that can be overcome by overexpressing regeneration enhancing proteins, such as WUS and BBM, in the regenerating tissue (Lowe et al., 2016). The problem with this approach is that continuous expression of BBM significantly

alters plant development (Horstman et al., 2017), which is an undesired side effect. Co-translocation of the regeneration enhancing protein via AMPT together with the T-DNA would overcome this problem, as the protein would only be present during the transformation process. However, translocation of proteins to plant cells via AMPT requires the addition of a translocation signal to the C-terminus of the protein of interest and preferably a reporter protein to the N- or C-terminus for detection of translocation. Ideally, these additions should not interfere with the function of a protein. Tagging of proteins at the N- or C-terminus has been reported to interfere with their subcellular location or functionality (Tanz et al., 2013). In order to establish this for AHL15 or BBM, we tested overexpression of the previously generated GFP_{11}:AHL15:ΔVirF and GFP_{11}:BBM:ΔVirF protein fusions for AMPT (Chapter 2) in Arabidopsis using the *35S* promoter. As expected, control plants expressing the Cre recombinase fusion were phenotypical indistinguishable from wild-type Arabidopsis Col-0 plants. In contrast, seedlings overexpressing *GFP_{11}:BBM:ΔvirF* or *GFP_{11}:AHL15:ΔvirF* showed reduced size and abnormal leaf shape (Fig4A). This was observed in 3/60 of the *GFP_{11}:BBM:ΔvirF* and 8/60 *GFP_{11}:AHL15:ΔvirF* overexpressing seedlings. However, many positive transformants could have had too high expression preventing seedling growth and subsequently would have been counterselected. (Fig. S2A).Moreover, whereas wild-type plants showed a termination of flower production, plants overexpressing *GFP_{11}:BBM:ΔvirF* or *GFP_{11}:AHL15:ΔvirF* continued forming new flower buds (Fig 4B, top row). The inflorescence of the plants expressing *GFP_{11}:BBM:ΔvirF* showed disrupted growth and altered morphology, whereas the plants expressing *GFP_{11}:AHL15:ΔvirF* did produce flowers, although angled down slightly and with shorter stamen. Both plants overexpressing the BBM or AHL15 fusion protein did not develop siliques with seeds, not even after hand pollination. The rosette leaves of the 8-weeks old control plant showed complete senescence, however leaves of plants overexpressing the BBM or AHL15 fusion protein were still green at this moment (Fig. 4B, bottom row). The BBM fusion protein caused an abnormal rosette shape and irregular leaf shapes, whereas plants overexpressing the AHL15 fusion protein developed leaves with normal shape, although smaller in size and at a higher number. From T1 transformants with a mild AHL15 overexpression

phenotype T2 seeds could be obtained by hand pollination. The T2 seedlings showed rosette phenotypes according to the Mendelian segregation (Fig. S2B). From this analysis we concluded that the AHL15 and BBM fusions proteins are functional. We cannot exclude, however, that the fusion proteins are less active than the native proteins.

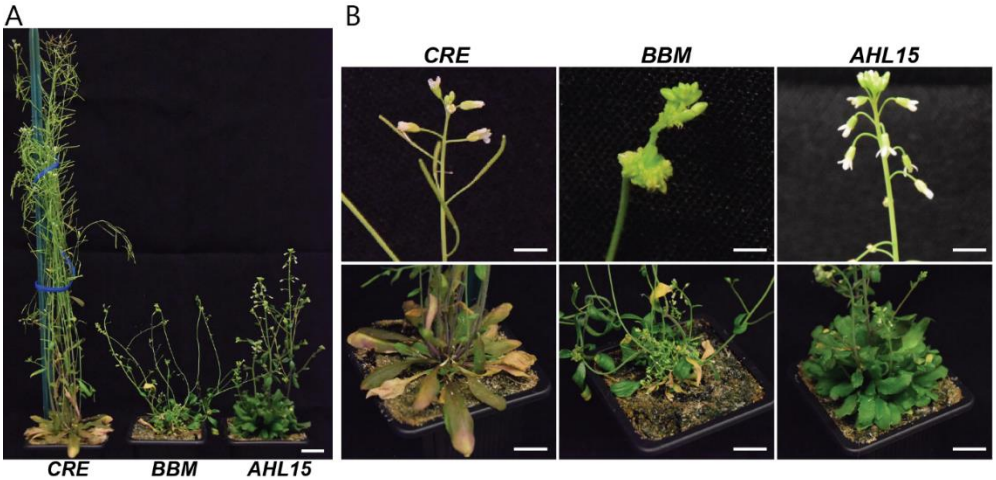

Figure 4. Altered development in Arabidopsis plants overexpressing BBM or AHL15 fusion proteins. (A) The phenotype of 8-weeks old Arabidopsis T1 plants transformed with *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (*CRE*), *p35S::GFP$_{11}$:BBM:ΔvirF::tNOS* (*BBM*) or *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (*AHL15*). (B) Close-up photos of the inflorescence (top) and rosette (bottom) of the plants in (A). Size bars indicate 20 mm.

AMPT of AHL15 increases shoot formation on tobacco leaf discs

Since the BBM and AHL15 fusion proteins for AMPT appeared functional, we selected the AHL15 fusion to see if its AMPT could enhance shoot regeneration. The fourth and fifth leaves of 4-weeks old tobacco plants were infiltrated with Agrobacterium strains transferring by AMT *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control), *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (T-DNA fp) or *p35S::AHL15::tNOS* (T-DNA) or by AMPT GFP$_{11}$:AHL15:ΔVirF (fp). Directly after infiltration, 1.5 cm diameter leaf discs were excised and placed on shoot induction medium for two weeks. The leaf discs were subsequently transferred to medium without hormones and after two weeks this was repeated. Six weeks after infiltration, the leaf discs were photographed (Fig. 5A) and shoot formation was counted (Fig. 5B). Compared to the control

infiltration, the AMPT of the AHL15 fusion protein or its transient expression following AMT significantly enhanced the regeneration of shoots. AMPT or transient expression following AMT of the fusion protein GFP_{11}:AHL15:ΔvirF did not lead to significant differences, suggesting that the amount of protein translocated by Agrobacterium is not rate limiting for enhancing shoot regeneration (Fig. 5B), The strongest effect was observed when AHL15 without N- or C-terminal fusions was expressed from the T-DNA (Fig. 5B), suggesting that the GFP_{11}:AHL15:ΔVirF fusion has reduced activity. In conclusion, our results indicate that AMPT of regeneration enhancing proteins, such as AHL15, may be used to overcome transformation recalcitrance by enhancing plant regeneration.

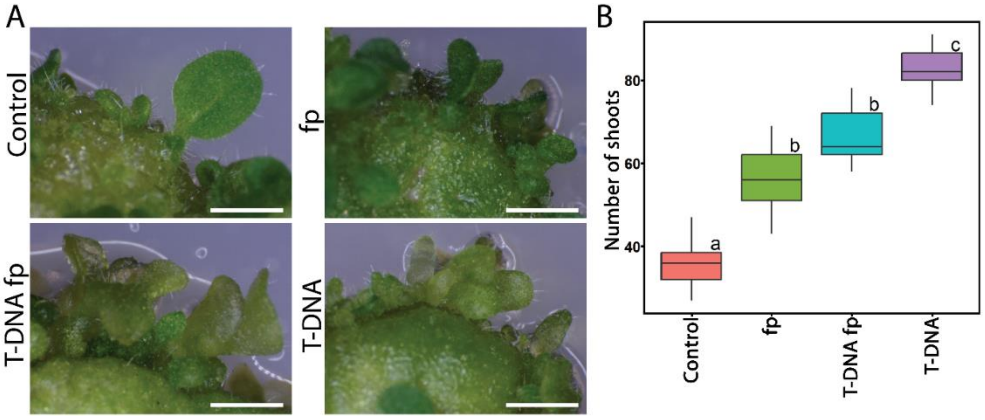

Figure 5. Increased shoot induction by expression or translocation of AHL15 (fusion) proteins in tobacco leaf cells. (A) Shoot formation observed in tobacco leaf discs 6-weeks post infiltration with an Agrobacterium strain transferring a T-DNA with *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control), *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (T-DNA fp) or *p35S::AHL15::tNOS* (T-DNA) or translocating GFP_{11}:AHL15:ΔVirF expressed under control of *pvirE* (fp). Scale bars indicate 2 mm. (B) Quantification of the number of shoots on leaf discs as shown in (A). Boxplot indicates the median, second and third quartile. Whiskers extend the interquartile range by 1.5 (n = 6). Different letters indicate statistically significant differences ($p < 0.05$) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test.

Discussion

The identification of key genes involved in plant resilience and the development of novel methods for directed genome editing in the last decades have provided new opportunities for the rapid introduction of beneficial traits into crop plants. Unfortunately, for many crop species or genotypes, application of this knowledge and methods is limited by their recalcitrance to AMT, which is caused by two main bottle necks; On the one hand Agrobacterium induces the production of SA as a negative feedback mechanism (Wang et al., 2019a), which inhibits Agrobacterium growth, *vir* gene induction and attachment to plant cells (Anand et al., 2008). On the other hand, some crop species or genotypes show recalcitrance to regeneration.

Previously it was shown that the induction of a plant defense response by Agrobacterium could be prevented by either overexpression in the plant cell of NahG or Pseudomonas effectors, leading to increased AMT efficiencies (Anand et al., 2008; Raman et al., 2022; Rosas-Díaz et al., 2017; Tsuda et al., 2012). Here we showed using the split-GFP system that NahG or the Pseudomonas effector AvrPto can be introduced in plants cells via AMPT, and that this enhances the efficiency of both AMT and AMPT. Introducing such proteins via AMPT has two advantages. It obviates the need for generating transgenic lines in which the proteins are continuously expressed. This expression might have a negative effect on the defense response against pathogens. Moreover, high expression of AvrPto causes a strong HR response, whereas AMPT of this protein does not in the first 4 days of infiltration, and thereby allows enhancement of transient expression. To increase the efficiency of the translocation of other proteins of interest, NahG and/or AvrPto can be simultaneously translocated. It remains to be investigated if translocation of NahG by AMPT does lower the SA levels *in planta.*

In order to check whether AMPT can also be used to solve the regeneration bottleneck, we tested translocation of the regeneration enhancing protein AHL15. First, we showed that AMPT of AHL15 reduced the senescence-inducing effect of Agrobacterium on the infiltrated leaf tissue. This is in line with the reported anti-senescence activity of AHL15 homologs (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013), suggesting that the GFP_{11}:AHL15:ΔVirF fusion protein has retained the

activity of AHL15. To confirm this, we generated Arabidopsis lines overexpressing the fusion protein and observed enlarged rosettes with bright green leaves, which was reported previously for Arabidopsis *AHL15* overexpression lines (Karami et al., 2021; Rahimi et al., 2022).

This chapter describes the potential for AMPT to increase the transformation efficiency in tobacco and induce physiological changes in Arabidopsis*, N. benthamiana* and tobacco*.* We expect that our findings will be useful for other plant species or genotypes to lower the recalcitrance to AMT and thereby open up the possibility to do transient expression experiments or even to obtain stable transgenic lines in that species or genotype. In addition, our experiments pave the way to use AMPT as a non-GM system to induce changes in plant development (e.g. flowering) or defense (e.g. SAR) through the translocation of key regulatory proteins (e.g. transcription factors) in those processes. Clear biological effects were shown after AMPT of AHL15, BBM, AvrPto or NahG fusion proteins, however whether they trigger the correct downstream processes still requires further confirmation by reporter and gene expression analysis.

Materials and methods

Agrobacterium strains and growth conditions

The Agrobacterium strain AGL1 (C58, *RecA*, pTiBo542 disarmed, Rif,Cb) (Jin et al., 1987) used in this chapter was grown in modified LC medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH = 7.5) at 28 °C with the appropriate antibiotics at the following concentrations: gentamicin 40 µg/ml; carbenicillin 75 µg/ml; kanamycin 100 µg/ml; rifampicin 20 µg/ml. Plasmids were introduced into Agrobacterium by electroporation, as previously described (den Dulk-Ras & Hooykaas, 1995).

Plasmid construction

The plasmids described in this chapter are listed in Table 1. All cloning steps were performed in *E. coli* strain DH5α (CGSC#: 14231) (Laboratories, 1986). PCR

amplifications were done with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Landsmeer, the Netherlands) and resulting plasmids were verified by sequencing. Primers used to construct the plasmids are listed in Table 2. Sequences were codon optimized using the web base tool OPTIMIZER (Puigbò et al., 2007). The T-DNA transfer vector *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (Khan, 2017) was digested with *Nco*I and *Bst*EII to replace the *Cre* coding region with a PCR amplified *Nco*I *Bst*EII fragment containing the coding regions of *AHL15*, *NahG* or *AvrPto.* The protein translocation vector *pvirE::GFP$_{11}^{opt}$:BBM^{opt}:ΔvirF* constructed in Chapter 2 was digested with *Nde*I and *Bam*HI to replace the coding region with a codon optimized synthetic *Nde*I *Bam*HI fragment containing G*FP$_{11}^{opt}$:AHL15^{opt}:ΔvirF, GFP$_{11}^{opt}$:AvrPto^{opt}:ΔvirF or GFP$_{11}^{opt}$:NahG^{opt}:ΔvirF* (Bio Basic inc., Canada).

Table 1. Plasmids and their function used in this study. In the main text sfCherry2 is referred to as Cherry and the optimized superscript (^{opt}) is omitted.

Plasmid content	**Function**	**Source**
p35S::GFP$_{11}$:Cre:ΔvirF::tNOS/ pNOS::Hyg	T-DNA transfer (Km^r)	Khan, 2017
p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS/ pNOS::Hyg	T-DNA transfer (Km^r)	Khan, 2017
p35S::GFP$_{11}$:BBM:ΔvirF::tNOS/ pNOS::Hyg	T-DNA transfer (Km^r)	Khan, 2017
p35S::GFP$_{11}$:AvrPto:ΔvirF/ pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 4
p35S::AHL15/ pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 4
p35S::AvrPto/ pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 4
p35S::NLS^{opt}:GFP$_{1\text{-}10}^{opt}$::tNOS / pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 2
p35S::NLS^{opt}:GFP$_{1\text{-}10}^{opt}$::tNOS / p35S::NLS^{opt}:sfCherry2^{opt}::tNOS / pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 2
pvirF::GFP$_{11}^{opt}$:BBM^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 3
pVirE::GFP^{opt}	Bacterial expression (Gm^r)	Chapter 3
pVirE::GFP$_{11}^{opt}$:AHL15^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 4
pVirE::GFP$_{11}^{opt}$:AvrPto^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 4
pVirE::GFP$_{11}^{opt}$:NahG^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 4

Agrobacterium induction

For leaf infiltration or co-cultivation of suspension cells, a colony of Agrobacterium strain AGL1 containing the appropriate plasmids (Table 1) from a one-week old plate was resuspended in 10 ml LC medium supplemented with the appropriate antibiotics in a 100 ml Erlenmeyer flask and was incubated at 28 °C under 180 rpm shaking until the culture reached an OD_{600} of 1.0. The bacteria were pelleted by centrifugation in a 50 ml Falcon tube at 4000 rpm for 20 minutes and resuspended in a 20 ml AB minimal medium (Gelvin, 2006) with the appropriate antibiotics and grown overnight at 28 °C under 180 rpm shaking until an OD_{600} of 0.8. The bacteria were pelleted as described above and resuspended in 20 ml induction medium (Gelvin, 2006) containing 200 µM acetosyringone (CAS# 2478-38-8, Sigma-Aldrich, Saint Louis, USA) and cultures were incubated on a rocking shaker at 60 rpm at room temperature.

Plant species and growth conditions

The seed of *Nicotiana tabacum* cv. Petit Havana SR1 (tobacco) and *Nicotiana benthamiana* were stratified for seven days on wet soil and germinated in high humidity under a plastic cover at 24 °C and 16 hours photoperiod. Seedlings were grown in growth chambers at 24 °C, 75 % relative humidity and 16-hours photoperiod for four weeks.

The seeds of *Arabidopsis thaliana* Col-0 were sterilized by a pre-wash with sterile water, followed by one minute in 70% ethanol, 10 minutes in a 10% commercial bleach solution (4.5% active sodium hypochlorite) under constant agitation and five times wash with sterile water. Sterilized seeds were stratified for three days at 4 °C and germinated axenically on 1% sucrose half-strength MS medium (Murashige & Skoog, 1962) solidified with 1% Daishin agar (w/v) (Duchefa Biochemie). Seeds were germinated and seedlings axenically grown at 21 °C and a 16 hour photoperiod.

Floral dip

Arabidopsis was transformed using the floral dip method (Clough & Bent, 1998b), transgenic plants were selected by germinating sterilized seeds on medium with 50 mg/l Hygromycin B and T-DNA integration was verified by PCR analysis (List of PCR primers, table 2). Seedlings were transferred to soil and grown for five days in high humidity under a plastic cover in growth chambers at 21 °C, 50 % relative humidity and a 16 hour photoperiod for four weeks (Rivero et al., 2014).

Table 2. Overview of primers used in this study

Primer name	**Sequence**
AHL15opt Fw	ACTTCACCACCAACAACTCCGG
AHL15opt Rev	GTTGTTGCCGGATTCGTTGTCG
BBMopt Fw	CGTTGACAACCAGGAAAACGGC
BBMopt Rev	TGGTCGTCTTCCTGCTTGAAGC
WUSopt Fw	AACGTCAAGCTGAACCAGGACC
WUSopt Rev	AGTAGTGGTGGTCCATGTTGGC
NahGopt Fw	CCTTAGCACTGGAACTCT
NahGopt Rev	CAACTCGTATAACTCGCC
Cre Fw	CCGCGCGCCTGAAGATATAGAA
Cre Rev	CCATTGCCCCTGTTTCAC
SpeI AvrPto Fw	GG ACTAGT GGAAATATATGTGTCGGCG
SacI AvrPto Rev	C GAGCTC TCA TTGCCAGTTACGGTAC
EcoRI AvrPto Fw	CCG GAATTC GGAAATATATGTGTCGG
HindIII AvrPto Rev	CCCAAGCTTTTGCCAGTTACGGTAC
SpeI NahG Fw	GG ACTAGT AAAAACAATAAACTTGGCTTGCG
SacI NahG Rev	C GAGCTC TCA CCCTTGACGTAGC
EcoRI NahG Fw	CCG GAATTC AAAAACAATAAACTTGGCTTGC
HindIII NahG Rev	CCC AAGCTT CCCTTGACGTAGC

Agrobacterium leaf infiltration and fluorophore measurement

Agrobacterium induction and leaf infiltration was performed as described in Chapter 2. Fluorophore levels were measured using a plate reader as described in Chapter 3.

Senescence measurements

The third and fourth leaf of 4-weeks old *N. benthamiana* plants were infiltrated with Agrobacterium containing the appropriate plasmids (Table 1). The Agrobacterium infiltration was performed as described in Chapter 3. The senescence was measured at 3 dpi using a handheld SPAD-502plus meter (Konica Minolta, Langenhagen, Germany) at three spots of the infiltrated area per leaf using six plants per treatment. The measurements in one leaf were averaged and the statistical analysis was performed per leaf number. The measurements were repeated at 4, 5, 6 and 7 dpi on the same spots on the leaves.

Phytohormone treatment

Agrobacterium virulence induction and growth in response to SA was measured in a 96-wells plate reader. The Agrobacterium cultures were induced as described in Chapter 2 and measurements performed as described in Chapter 3. Each well of the 96-wells plate was loaded with 150 µl induced *Agrobacterium*. The Agrobacterium cultures were treated with SA dissolved in 10% DMSO to a final concentration of 0.425; 2.125; 4.25; 8.50 or 12.75 mM.

Organogenesis quantification

The leaves of soil grown 4-weeks old *N. tabacum* were infiltrated with Agrobacterium containing the appropriate plasmid(s) (Table 2). The position on and the number of the leaf for infiltration and for the subsequent leaf disc was described in Chapter 2. After infiltration, excess Agrobacterium infiltration medium on the leaf disc was removed by a sterile water wash and the leaf was subsequently dried by placing it shortly on sterile filter paper. The round leaf discs (1.5 cm) were

cut using a cork borer, dried on sterile filter paper and immediately placed on solid shoot induction medium containing 1x MS, 3% sucrose, 1% Daishin agar, 200 µM AS, 2 mg/l BAP and 0.2 mg/l NAA. After two weeks, leaf discs were transferred to 3% sucrose MS plates without hormones and AS but containing 100 µg/ml Timentin or 500 mg/l cefotaxime. Six leaf discs were observed per treatment and shoot formation was counted using a Zeiss Axiozoom v16 (Zeiss, Oberkochen, Germany) stereomicroscope.

Laser scanning confocal microscopy

Fluorescence was observed using a Zeiss Imager M1 or a Zeiss Observer (Zeiss, Oberkochen, Germany) microscope equipped with the LSM 5 Exciter confocal laser unit using a 20x and 40x magnifying objective (numerical aperture of 0.8 and 0.65, respectively). GFP signal was detected using a 488 nm argon laser and a 505-530 nm band-pass emission filter. Chloroplast- and other auto-fluorescence was detected using a 488 nm argon laser and a 650 nm long pass emission filter. The Cherry signal was detected using a 561 nm diode laser and a 595 – 500 nm band-pass filter. Visible light was detected using the transmitted light detector. Images were collected using ZEN black edition (Zeiss, Oberkochen, Germany) imaging software and processed in ImageJ (Schneider et al., 2012). The GFP or Cherry fluorescence intensity was measured in ImageJ.

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Author contribution

Ivo Gariboldi, Maarten Stuiver and Remko Offringa conceived and designed the experiments. Ivo Gariboldi, Anton Rotteveel and Koen van Oostrom constructed plasmids and performed the experiments. Ivo Gariboldi performed the microscopic analysis and statistical analysis. Ivo Gariboldi and Koen van Oostrom performed the plate reader experiments. Ivo Gariboldi and Remko Offringa analyzed the results and wrote the manuscript.

Supplemental figures

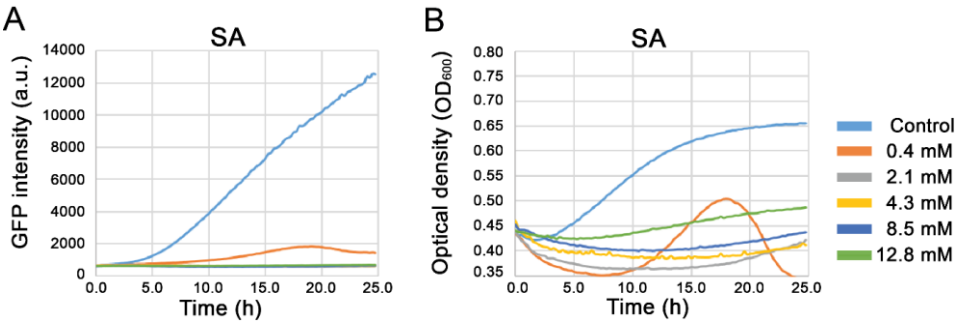

Figure S1. Agrobacterium *vir* gene induction and growth is severely inhibited by SA. (A) Time lapse measurements of GFP fluorescence from and Agrobacterium strain expressing *GFP* under control *pvirE* treated with 200 uM acetosyringone in the absence or presence of SA. (B) Timelapse measurement of the optical density (OD_{600}) of the Agrobacterium cultures in (A). Data represent the mean of three replicates.

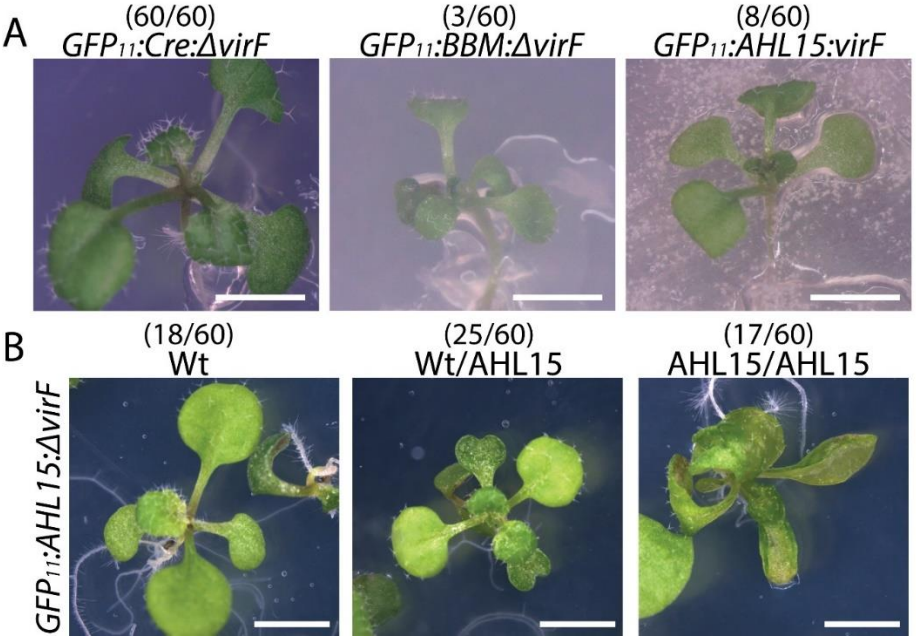

Figure S2. Phenotypes of seedlings overexpressing BBM or AHL15 fusion proteins (A) Phenotype of T1 seedlings overexpressing the indicated fusion protein under the *35S* promoter. The numbers above the pictures indicate in how many of 60 seedlings the phenotype was observed. (B) The phenotypes of T2 seedlings of an Arabidopsis line follow a typical Mendelian ratio and show either wild-type phenotype (Wt) or are heterozygous (Wt/AHL15) or homozygous (AHL15/AHL15) AHL15 phenotypes by overexpressing *GFP11:AHL15:ΔvirF* under control of the *35S* promoter. The number of observations is indicated above the figure. Size bars indicate 10 mm.

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