Molecular engineering of plant development using Agrobacterium-mediated protein translocation

Khan, M.

ARABIDOPSIS AHL15-INDUCED REJUVENATION PROMOTES LONGEVITY AND POLYCARPY IN NICOTIANA TABACUM

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ABSTRACT

Plant rejuvenation and senescence are interrelated and interdependent developmental processes. Early maturation and poor rejuvenation potential restricts flowering plants to a monocarpic life history strategy and thus to a seasonal life span, which is generally shortened by environmental factors such as biotic and abiotic stresses. In contrast, delay in senescence of adult shoots and rejuvenation of (axillary) shoot meristems may enhance plant longevity and allow plants to flower multiple times (polycarpy). Recently, we identified the AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 15/REJUVENATOR (AHL15/RJV) protein as a key switch between monocarpic and polycarpic life history strategy in *Arabidopsis thaliana*. Here we analyzed the effect of heterologous expression of an inducible version of AHL15 (AHL15-GR) on *Nicotiana tabacum* development. Early activation of AHL15-GR delayed seed germination and arrested seedling development, resulting in callus formation rather than the somatic embryogenesis observed in *Arabidopsis*. Late AHL15-GR activation enhanced plant longevity by reducing leaf senescence, delaying flowering, and by shoot meristem rejuvenation, leading to an increased number of branches, leaves and seeds produced per plant. But the quality of the produced seeds in polycarpic *35S::AHL15-GR* tobacco plants was affected at the cost of seed quantity. Our data indicates that the overall function of *AHL15* seems conserved in different families of flowering plants, but also points to specific differences that require further study before AHL15-induced polycarpy can be used as a generic tool to enhance biomass and seed production in monocarpic crop species.

Keywords: AHL15-GR. Rejuvenation. Senescence. Polycarpy. *Nicotiana tabacum*.

INTRODUCTION

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The life span of a flowering plant depends on its genetic potential for longevity and on environmental conditions (Woo et al., 2013). The plants dying after one flowering and seed production period are called monocarpic. A monocarpic perennial plant lives for two or more years, then flowers once, sets seed and dies, while a polycarpic perennial lives for a number of years, often many years, flowering and setting seed annually throughout its life time. The removal of flowers is regarded to extend plant life up to 50% in monocarpic plants (Sadras et al., 2000; Pic et al., 2002). For annual herbaceous plant species, such as *Arabidopsis thaliana*, germination, vegetative growth, reproduction and senescence are the four main phases that can be distinguished during the life of these plants but their life is mainly limited by leaf senescence (Sharabi-Schwager et al., 2010). In contrast, woody perennials, such as deciduous trees rejuvenate themselves after passing through winter stress conditions by reactivation of shoot meristems and development of new leaves and branches (Andersson et al., 2004; Xu et al., 2016). Similarly, in herbaceous perennial plants some meristems remain vegetative and initiate new shoots after a short dormant stage, producing new leaves and branches throughout the year, even at advanced age (Munne-Bosch, 2008; Xu et al., 2016).

Developmental processes such as organogenesis and developmental phase changes in plants are orchestrated by complex regulatory networks comprising hormone- or peptide signaling and downstream transcription factors that change gene transcription through direct (in)activation, or by inducing epigenetic changes involving chromatin remodeling (Sparks et al., 2013). For example, the SQUAMOSA promoter binding protein-like (SBP or SPL) family of transcription factors regulated by microRNAs miR156 and miR157 are responsible for various plant developmental processes like heteroblasty (juvenile to adult vegetative phase change), apical dominance, inflorescence branching, flowering time and fruit ripening in various flowering plants such as *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum* (Preston and Hileman, 2013). Similarly, phytohormones working as upstream regulators in plant developmental pathways have multiple effects in interconnecting different signaling pathways (Jibran et al., 2013). The plant hormone cytokinin can extend the plant life by delaying senescence and enhancing production of multiple new shoots (Wang and Irving, 2011). The delay in maturation and formation of juvenile shoots is of great importance for longevity in plants, especially in clonal forestry where different physical methods such as serial propagation, micropropagation and serial grafting are used to maintain juvenility (Wendling et al., 2014).

Senescence occurs at the final stage of plant development, and is defined as the age-dependent programmed degradation and degeneration process of the cells, organs or the entire organism, leading to death (Lim et al., 2007a). Sometimes this normal developmental process is induced by various biotic and abiotic stresses, which by increasing the level of reactive oxygen species negatively affect the developmental processes and leads to early maturation and death of the plants resulting in decrease of productivity (Petrov et al., 2015). Leaf senescence is a highly complex genetic and epigenetic program that is controlled by interconnected regulatory pathways at the level of chromatin and transcription, as well as by post-transcriptional, translational and post-translational regulation (Woo et al., 2013; Ay et al., 2014). Dark-induced senescence of *Arabidopsis* leaves, for example, involves 137 miRNAs that control many genes

(Huo et al., 2015), and the expression of *SENESCENCE-ASSOCIATED GENES* (*SAGs*) is regulated at the chromatin level by HISTONE DEACETYLASE 3 (HDA6), the AT-Hook motif nuclear Localized (AHL) protein ORE7 and other members of AHL family (Woo et al., 2013). Lim et al. (2007) have also shown that increased expression of the AHL protein encoding gene *ORE7* markedly extends the leaf longevity.

AT-hook is a small protein motif that binds the minor groove of the DNA at AT-rich regions and is associated with High Mobility Group (HMG) proteins in animal cells (Aravind and Landsman, 1998). The HMG proteins influence gene transcriptional regulation by participating in the formation of multi-protein complexes on the promoter regions of the genes they regulate (Bustin et al., 1990; Reeves and Nissen, 1990; Tjian and Maniatis, 1994). It is shown that HMGI/Y proteins mostly play a role as a cis-acting enhancers in the enhancement of gene activation by regulating both specific protein-DNA and protein-protein interactions at the promoter region (Reeves and Beckerbauer, 2001). Aravind and Landsman, (1998) extracted these AT-hook motifs from a non-redundant protein sequence database at NCBI and classified these motifs into three types according to their sequence similarity and found that they are prevalent in many eukaryotic nuclear proteins in single or multiple copies.

AHL proteins have been shown to have key roles in growth and development and act by modifying the chromosomal architecture to co-regulate transcription of a set of genes. In *Arabidopsis thaliana*, AHL proteins are encoded by a family of 29 genes, and they contain two conserved structural units, the AT-hook motif and the plant- and prokaryote-specific (PPC) domain. *Arabidopsis* AHL protein evolved in two clades: Clade A proteins contain a type 1 AT-hook motif, while clade B AHL proteins contain a type 2 and some also a type 1 AT-hook motif (Zhao et al., 2013). Previous analysis has shown that AHL proteins function in a large variety of processes, modulating plant size and biomass, yield and size of seeds, senescence and life cycle, ploidy and branching, immunity and stress resistance, production of secondary metabolites, tissue patterning, somatic embryogenesis, rejuvenation, regeneration and floral induction (Cai-Zhong, 2004; Zhao et al., 2013; Zhou et al., 2013; Karami, 2015).

The *Arabidopsis* AT-Hook nuclear Localized protein AHL15/REJUVENATOR (RJV) was previously identified as a DNA binding factor in a yeast one-hybrid screen (Hooykaas and Jacobus, 2004). Our recent functional analysis of this gene has revealed that *AHL15/RJV* overexpression maintains juvenile traits in the adult reproductive phase, thereby inducing polycarpic behavior in *Arabidopsis* (Karami et al., 2017). Here, we analyzed the effect of heterologous expression of a Dexamethasone (DEX)-activatable version of *Arabidopsis* AHL15 (AHL15-GR) in *Nicotiana tabacum* SR1 (tobacco), and showed that DEX activation of this fusion protein significantly increased shoot branching, delayed flowering and leaf senescence, and maintained axillary meristems in the vegetative state with juvenile features, thereby allowing the monocarpic tobacco plants to become polycarpic.

RESULTS

Arabidopsis AHL15 delays seed germination and arrest seedling development in tobacco

Previous research has shown that overexpression of *AHL15/RJV* can slow down various developmental processes such as germination, vegetative phase change, flowering and senescence in *Arabidopsis thaliana* (Karami et al., 2017). To demonstrate that this effect of AHL15/RJV overexpression was not specific for *Arabidopsis*, we generated *AHL15/RJV* overexpression lines for another annual plant species from a different family, being *Nicotiana tabacum* SR1. Initial attempts to generate lines with the *35S::AHL15/RJV* construct did not lead to transformants. We therefore transformed tobacco cells with the *35S::AHL15-GR* construct, expressing a translational fusion between the AHL15 protein and the glucocorticoid receptor (GR), allowing conditional activation of the fusion protein through its dexamethasone (DEX)-dependent nuclear import. In total 11 independent transgenic lines were obtained, of which we selected four single locus T-DNA insert lines based on their segregation for the phosphinothricin (ppt) resistance marker present on the T-DNA construct.

Germination of segregating *35S::AHL15-GR* T1 seeds on medium with or without 10 μM DEX and/or ppt showed that the development of the transgenic seedlings on DEX containing medium was significantly delayed compared to wild type seedlings. Whereas two weeks old SR1 wild-type and non DEX treated *35S::AHL15-GR* seedlings already developed one or two leaves next to normal looking cotyledons (Fig. 1b and c), the DEX treated *35S::AHL15-GR* transgenic seedlings showed comparatively short cotyledons and no leaves after two weeks of germination (Fig. 1d and e), suggesting that heterologous expression of *AHL15-GR* significantly delayed germination, and also reduced cotyledon growth. When we tested T3 seeds homozygous for the *35S::AHL15-GR* construct, germination on DEX-containing medium was again delayed, but now root and shoot development completely stopped in early seedling stage. Detailed studies showed that *35S::AHL15-GR* seedlings had lost the root and shoot meristem function and were converted to callus like structures (Fig. 1f and g). Also the cotyledons were thickened and produced callus on the upper and lower surfaces (Fig. 1h). Transfer of the root and shoot parts with callus to DEX-free medium neither recovered development nor resulted in shoot or root regeneration, suggesting that stem cell activity was permanently lost. These results also explain why no transgenic lines could be obtained with the non-inducible *35S::AHL15* construct.

Arabidopsis AHL15 delays flowering and leaf senescence in tobacco

Non DEX-treated *35S::AHL15-GR* plants slightly lagged behind in development and had greener leaves compared to wild-type plants (Fig. 2a), probably because of leaky nuclear import of the AHL15-GR fusion protein in the absence of DEX. To study the effect of *35S::AHL15-GR* expression on flowering, we DEX-sprayed (see schedule in Table. 1) 6 weeks old wild-type and *35S::AHL15-GR* tobacco plants just before flowering (Fig. 2a). One week after DEX treatment, wild-type plants developed elongated inflorescences with open flowers, whereas the *35S::AHL15-GR* plants showed a short inflorescence with closed flower buds (Fig. 2b). Two weeks after DEX treatment, the elongated *35S::AHL15-GR* inflorescences carried opened flowers, whereas the wild-type plants were already in the fruit ripening stage (Fig. 2c). These results suggested

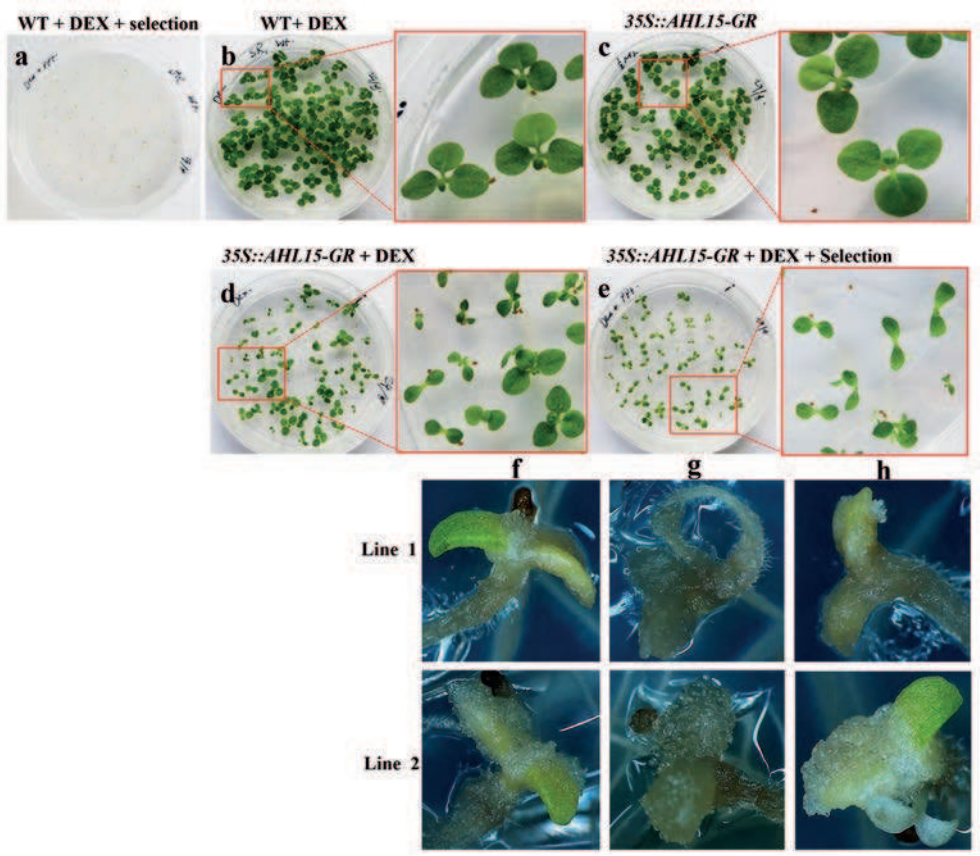

Figure 1. DEX activation of AHL15-GR delays seed germination and arrests seedling development in *Nicotiana tabacum*. (a-e) Germination of *Nicotiana tabacum* wild-type (a,b) and heterozygous *35S::AHL15-GR* (c-e) seeds on medium with DEX and phosphinothricin (selection) (a,e), with DEX but without selection (b,d) and without DEX and selection (c). Red boxed right panel in (b-e) shows enlarged part of the left panel. (f-h) Two weeks old seedlings from two homozygous *35S::AHL15-GR* lines (1 and 2) after germination on DEX containing MS medium, showing loss of meristem function and formation of callus-like structures.

that *AHL15* overexpression increased the flowering time in tobacco, just like overexpression of this gene or the *AHL22* gene did in *Arabidopsis* (Yun et al., 2012).

We noticed that leaves of DEX-sprayed *35S::AHL15-GR* plants generally stayed greener compared to the DEX-sprayed wild-type leaves (Fig. 2c), suggesting that *AHL15* overexpression also reduced leaf senescence. When we left these plants growing for 3 more weeks, we observed an even more significant difference in leaf senescence. On wild-type plants, leaves had already senesced or showed strong signs of senescence, whereas most leaves on DEX-treated *35S::AHL15-GR* plants remained green (Fig. 2d). After the seed pods of these plants were harvested, plants were cut back so that only a 6 inch main stem with attached leaves was left behind (Fig. S1a). Repotting of these remaining stems and root systems to fresh soil led to renewed shoot growth, which was more vigorous and branched for the DEX sprayed *35S::AHL15-GR* stems than for the wild-type stems (Fig. S1b).

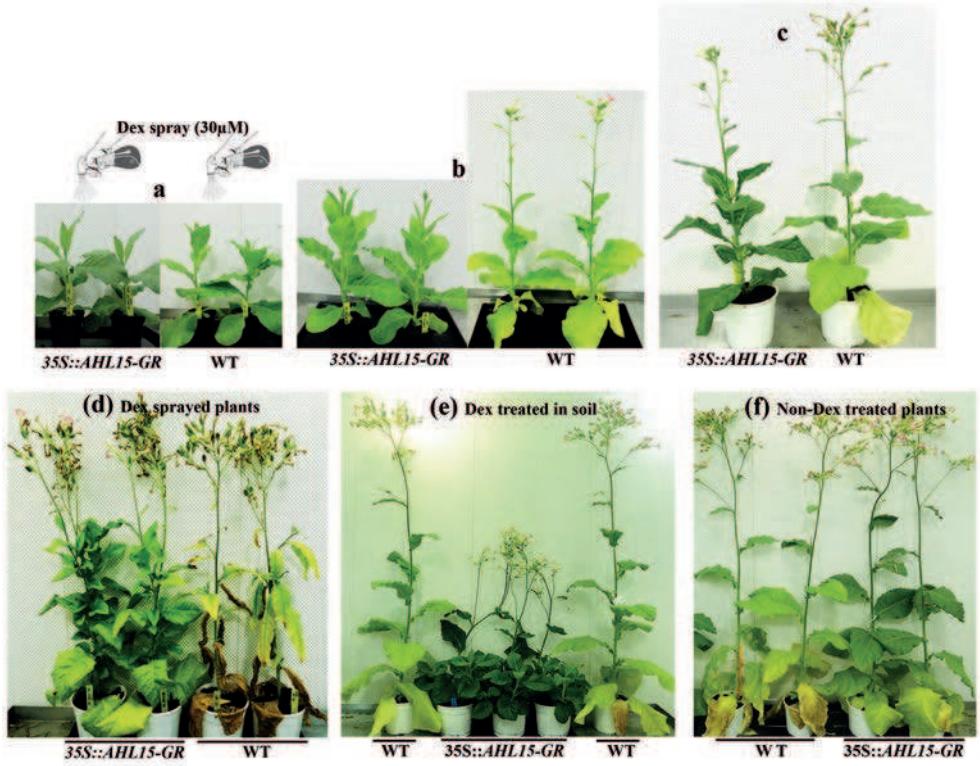

Figure 2. AHL15-GR activation delays flowering and senescence and enhances branching in *Nicotiana tabacum*. (a-c) 6 weeks old *Nicotiana tabacum* wild-type (WT) and *35S::AHL15-GR* plants were sprayed with 30 µM DEX before flowering (a), and subsequently photographed one week (b), two weeks (c), or 5 weeks (d) after spraying. (e, f) 2 weeks old *Nicotiana tabacum* wild-type (WT) and *35S::AHL15-GR* plants treated with DEX in the soil and spray (e), or non-DEX treated (f) and photographed after 6 weeks.

In another experiment, 2 weeks old wild-type or *35S::AHL15-GR* plants were DEX-treated by watering and spraying according to the schedule in table. 2. After 6 weeks, the *35S::AHL15-GR* plants showed a strong delay in development with short and branched stems and smaller dark green leaves, whereas wild-type plants were fully developed and started to show senescence of the bottom leaves (Fig. 2e), similar to the non-DEX treated control plants (Fig. 2f). The leaves of non-treated *35S::AHL15-GR* plants remained darker green compared to wild type, in line with the earlier observed slight leakiness of the AHL15-GR system. Also *35S::AHL15-GR* plants cultured *in-vitro* on DEX medium showed delayed development compared to DEX-treated wild-type or non-treated *35S::AHL15-GR* plants. Moreover, plants were very bushy and showed a strong delay in senescence compared to the controls (Fig. S2). These phenotypic effects of induced *Arabidopsis* AHL15 activity in tobacco corroborate our observations in *Arabidopsis* and indicate that this protein has a general effect in delaying plant development including germination, flowering time and leaf senescence.

Arabidopsis AHL15 induces rejuvenation in young and adult tobacco plants

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Like *Arabidopsis*, tobacco plants are heteroblastic. In the juvenile vegetative phase the leaves are smaller and round with a relatively long petiole (Fig. S3a), while after the vegetative phase change the adult leaves are much larger with a shorter petiole and a clear central midrib (Fig. S3b).

To see the effect of heterologous AHL15 expression on the timing of the vegetative phase change in tobacco, both wild-type and *35S::AHL15-GR* seeds were germinated on MS medium (Murashige and Skoog, 1962) with or without DEX. Wild-type plants on DEX containing medium or *35S::AHL15-GR* plants on medium without DEX developed normally, showing the same timing of the vegetative phase change (Fig. 3a and 3b), while the DEX grown *35S::AHL15-GR* plants extended their juvenile phase with 5 to 6 leaves (Fig. 3c), or even stayed in the juvenile phase (Fig. S2a). When adult *35S::AHL15-GR* plants grown on normal medium were transferred to DEX medium, the newly formed leaves showed juvenile traits (Fig. 3d and S4c) while after 4 months, it was seen on the same plants that all the adult leaves stopped further development and new shoots with juvenile leaves appeared from the lateral buds (Fig. S5a). In reverse, transfer of DEX grown plants to medium without DEX resulted in an immediate shift of the newly formed leaves from juvenile to adult morphology (Fig. S4d,f). While the plants developed on non-DEX medium and after 40 days of development DEX-induced for only 15days and then transferred back to non-DEX medium, gave rise a thick branched bushy appearance (Fig. S5b). Whereas the plants continuously staying on DEX-containing medium produced branches with minute juvenile leaves and then completely stopped further development (Fig. S5a). These results indicated that, like in *Arabidopsis*, AHL15 is not only able to slow down but also to reverse development in tobacco.

To test whether activation of AHL15 would also lead to rejuvenation of senesced tobacco plants, we transferred 3 months old wild type and *35S::AHL15-GR* plants from which seeds had been harvested (approximately 60 cm long stem with root system) to bigger pots with fresh soil (Fig. 4a). One set of plants (wild type and *35S::AHL15-GR*) were DEX-treated in the soil, whereas a control set of plants (*35S::AHL15-GR*) was just treated with water. Only the DEX-treated *35S::AHL15-GR* plants developed lateral shoots form the axillary meristems producing adult leaves, and a few shoots from the transition area between roots and shoots producing juvenile leaves (Fig. 4b middle plant). The DEX-treated wild-type and mock-treated *35S::AHL15-GR* plants showed no shoot production (Fig. 4b right and left plants). These results indicate that apart from the ability to rejuvenate active meristems, AHL15 is also capable to reactivate dormant axillary meristems after the plant has senesced.

Arabidopsis AHL15 overexpression significantly increases branching and leaf number in tobacco

The biomass and yield of crop plants mostly depends on the plant architecture, which is determined for an important part by the level of apical dominance (Reinhardt and Kuhlemeier, 2002). Wild-type tobacco plants generally show a strong apical dominance, producing a main stem with a single inflorescence. For tobacco *35S::AHL15-GR* plants, however, we observed that induction of AHL15 activity significantly induced branching, by breaking the apical dominance

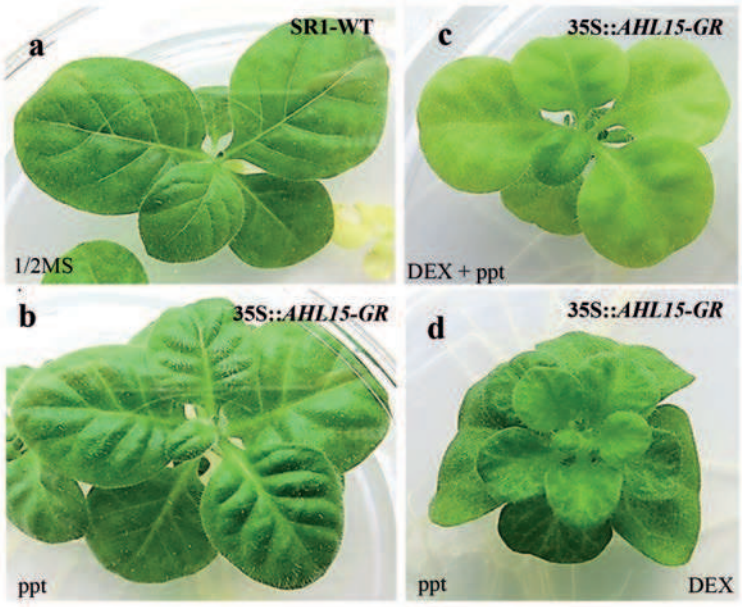

Figure 3. DEX induction of *Nicotiana tabacum 35S::AHL15-GR* plants extend juvenile phase and rejuvenate the adult plants. (a-d) 3 weeks old *Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants development on non-inducible (a,b) DEX inducible (c,d) MS medium. WT (a) and *35S::AHL15-GR* (b) plants show 4-5 adult leaves in the same developmental stage. *35S::AHL15-GR* plant (c) shows 4-5 juvenile leaves while *35S::AHL15-GR* plant (d) after transferring to MS medium with DEX shows switch from adult to juvenile leaf morphology.

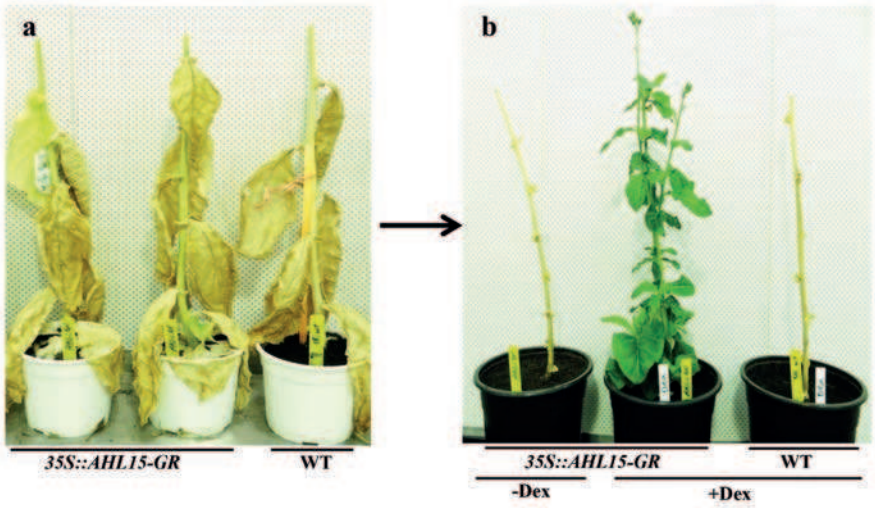

Figure 4. AHL15 triggers activation/rejuvenation of axillary meristems in *N. tabacum 35S::AHL15-GR* plants upon DEX induction. 3 months old *Nicotiana tabacum* (a) non DEX-treated wild type (one on the right side) and *35S::AHL15-GR* (two on the left side) plants with two feet stem along with roots transferred to fresh soil with DEX-treatment (only white tagged plants). (b) After 3 weeks only DEX-treated *35S::AHL15-GR* (middle) plant shows development of all lateral shoots along with juvenile shoots from transition area between stem and roots.

and by induction of lateral shoots, and that this completely changed the morphology of the plant as compared to wild type (Fig. 5a and 6a). The number of branches and leaves were significantly enhanced in *35S::AHL15-GR* plants grown *in vitro* on DEX medium as compared to wild-type or non-DEX-treated *35S::AHL15-GR* plants (Fig. 5a-c). Also for soil grown DEX-treated *35S::AHL15-GR* plants the number of leaves and branches was significantly enhanced (Fig. 6b and 6c) compared to DEX-treated wild-type or non-treated *35S::AHL15-GR* control plants (Fig. 6a). These results indicate that, like in *Arabidopsis*, enhanced expression of *AHL15* results in loss-of-apical dominance, and enhanced biomass production by increased shoot initiation.

Heterologous expression of *Arabidopsis AHL15* induces polycarpy in tobacco in the presence of sufficient nutrients.

Tobacco is an herbaceous annual plant that is very sensitive to temperature, light and humidity. Tobacco plants therefore complete their life cycle by flowering and producing offspring once in a single growing season (around 6 months). In contrast, our transgenic tobacco *35S::AHL15-GR* plants after DEX-treatment not only extended their life by delaying flowering time and leaf senescence, but also converted from seasonal monocarpic to polycarpic plants.

To further show that AHL15 induces perenniality and polycarpy in tobacco, we DEX-treated *35S::AHL15-GR* plants (either grown in tissue culture or on soil) and followed them

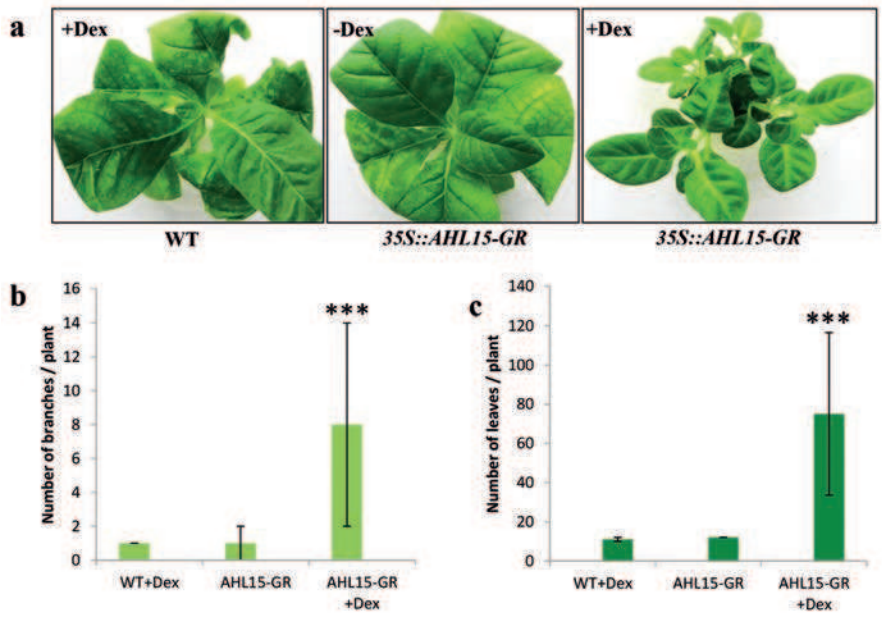

Figure 5. DEX induced *in vivo Nicotiana tabacum 35S::AHL15-GR* plants significantly enhances branching and leaf number. (a) 2 months old *Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants developed on DEX-inducible and non-inducible MS medium. WT (right) and *35S::AHL15-GR* (middle) plants with normal morphology and *35S::AHL15-GR* (left) plant with branched morphology on DEX-inducible medium. The graphs show significant enhancement in (c) branching and (d) leaves number per plant in *35S::AHL15-GR* plants on DEX medium. Significant increase is indicated by asterisks (*) ($p < 0.05$).

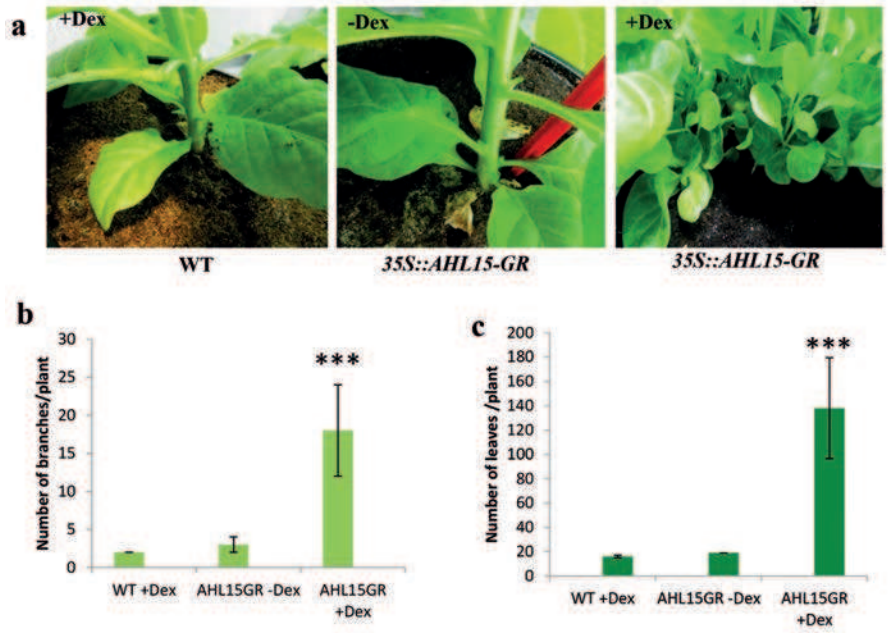

Figure 6. AHL15 induces enhanced branching and leaves number in *Nicotiana tabacum 35S::AHL15-GR in vitro* plants. (a) 2 months old *Nicotiana tabacum* wild type and *35S::AHL15-GR* plants developed in the green house. WT (right plant) with DEX treatment and *35S::AHL15-GR* (middle plant) without DEX shows normal morphology while *35S::AHL15-GR* plant on soil DEX-treatment shows branched morphology. The graphs show significant enhancement in (c) branching and (d) leaves number per plant in DEX-treated *35S::AHL15-GR* plants. Significant increase is indicated by asterisks (*) ($p < 0.05$).

for one year next to non-treated *35S::AHL15-GR* and DEX-treated wild-type control plants. After one year of *in vitro* culture (without media refreshment) the DEX-treated *35S::AHL15-GR* plants were still alive, having some green leaves near the shoot apex, whereas the control plants were completely dried out and dead (Fig. 7a). Also the soil-grown DEX-treated *35S::AHL15-GR* plants continuously produced multiple lateral and new juvenile shoots (Fig. 7b), and even after one year and three rounds of seeds harvesting just by soil refreshment and DEX-treatment the plants remained green and healthy with multiple shoots producing flowers and fruits (Fig. 7c). In contrast, the control plants completed their life cycle in less than six months and died after a single fruit set even when the soil was refreshed (Fig. 4). But DEX-treated *35S::AHL15-GR* plants even without soil refreshment remained green and produced flowers and seeds along with developing lateral and juvenile shoots, only some of the leaves turned yellow (Fig. 8a). By transferring the plants (seedpods, flowers and extra branches removed) to fresh soil, they completely rejuvenated producing fresh leaves and new shoots (Fig. 8b), suggesting that the yellowing of leaves was because of nutrient deficiency. To confirm this, stems of first generation DEX-treated *35S::AHL15-GR* plants were allowed to grow and regenerate new shoots without soil refreshment. We observed that all the newly developed shoots produced whitish yellow leaves (Fig. 8c); however, when 500 ml MS medium was added to the pots, we found that after two weeks the plants developed multiple new green shoots (Fig. 8d) in

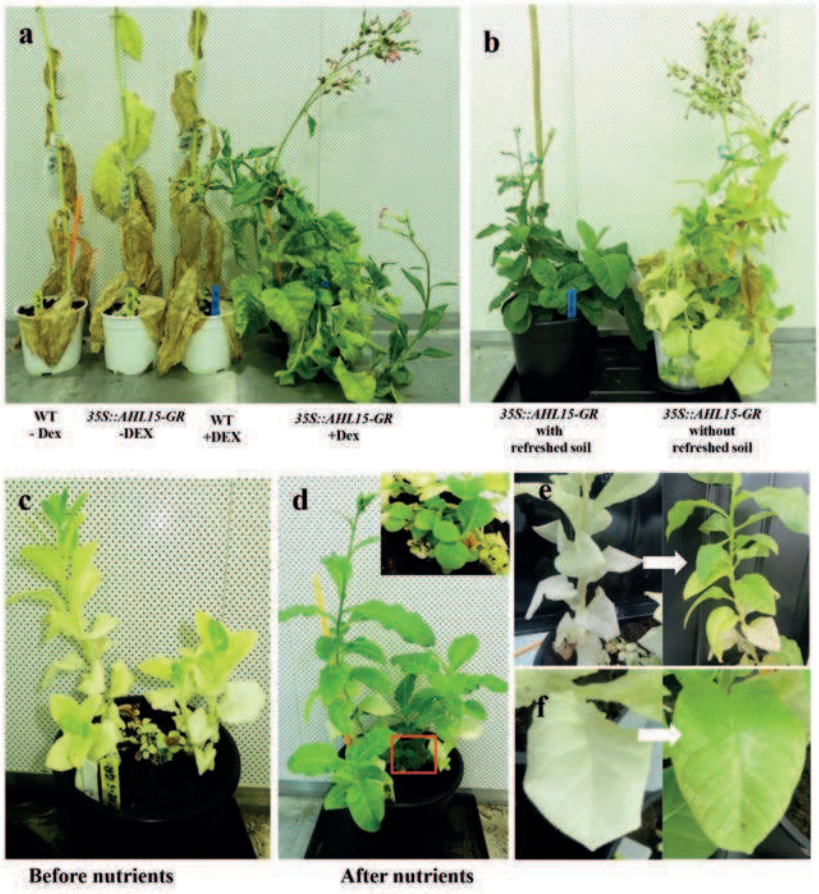

Figure 7. AHL15 induces perenniality and polycarpy in *35S::AHL15-GR* plants upon DEX treatment. (a) *in vivo Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants developed on 100 ml DEX-inducible and non-inducible MS medium. The picture taken after one year shows WT (right) on inducible medium and *35S::AHL15-GR* (middle) plant on non-inducible medium completely died while *35S::AHL15-GR* (left) plant on inducible medium alive with some green leaves. While (b) *35S::AHL15-GR* plant maintained *in-vitro* by periodically soil refreshment and DEX-treatment shows juvenile (highlighted part) and (c) multiple lateral shoots with flowers and fruits after three rounds of seed harvesting.

a similar way as when the DEX-treated *35S::AHL15-GR* plants were transferred to fresh soil (Fig. 4 and 8b) and the whitish-yellow leaves became green (Fig. 8e and f) indicating nutrients restoration. Like the previous observations, these plants were continuously producing new shoots, flowers and fruits but only because of deficiency of nutrients the leaves were turning whitish-yellow. This data shows that heterologous expression of *Arabidopsis AHL15* in tobacco (*35S::AHL15-GR* plants) in the presence of sufficient nutrients maintains the plants in a polycarpic and perennial-like state via delay of senescence, rejuvenation, and enhanced regeneration of lateral shoots throughout the year.

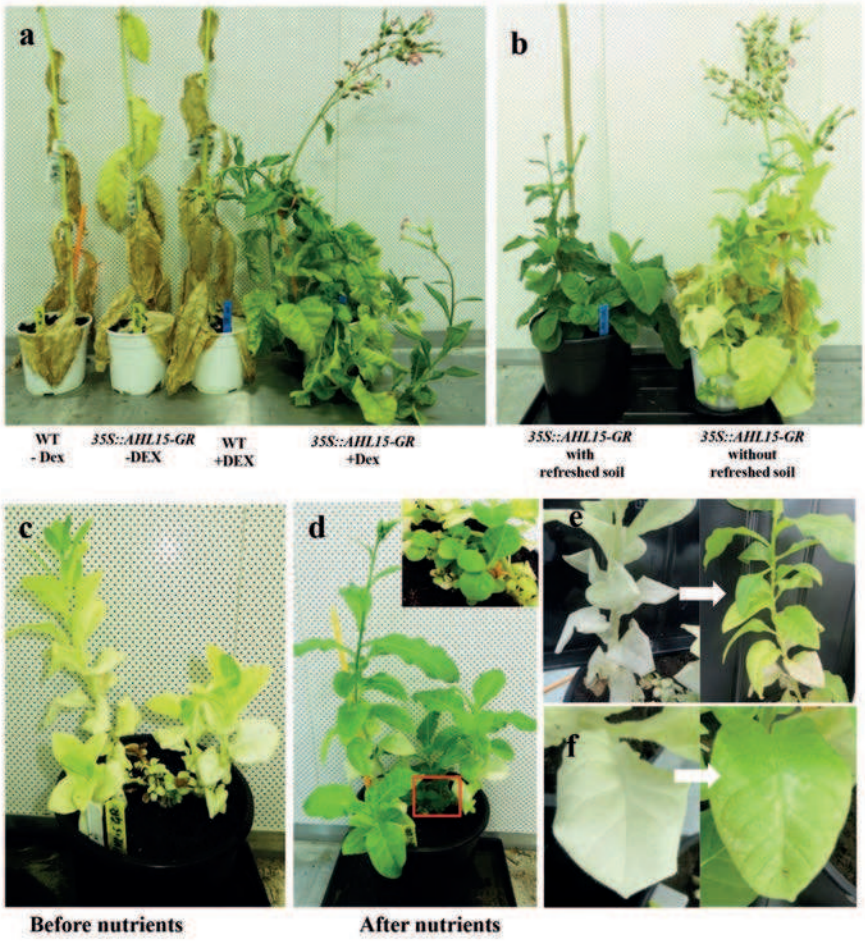

Figure 8. DEX-treatment with sufficient nutrients availability leads to perenniality longevity and rejuvenation in *35S::AHL15-GR* tobacco plants. (a) 3 months old *Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants with and without DEX-treatment. Non-induced WT and *35S::AHL15-GR* plants (with yellow tag) and induced WT plant (with blue tag) show normal senescence at the same time. Whereas DEX-induced *35S::AHL15-GR* plant (with blue tag) shows delay in senescence with development of lateral shoots. (b) *35S::AHL15-GR* DEX-induced plants without soil refreshment shows whitish-yellow leaves (right plant) and slow aging process while with refreshed soil (left plant) shows green leaves with lateral and juvenile shoots. (c) *35S::AHL15-GR* second generation plants (DEX-induction during 1st generation only) developed new shoots with whitish-yellow leaves from the 5 inches stem. (d) After two weeks of adding 100 ml MS medium into the soil the whole plant turned green with the development of new shoots (highlighted part) and already present (e) whitish-yellow and wrinkled shoots turned green and fresh. (f) Shows the changing of white leaf to green upon nutrients availability.

Heterologous *AHL15* expression increases seed quantity at the cost of seed quality in tobacco

Survival at the cost of reproduction is common both in plants and animals (Obeso, 2002; Tabatabaie et al., 2011). Aragon et al. (2009) have shown that the survival rate of perennial plants significantly increases when flowers are removed. This suggests that the high reproduction

burden often observed in annual plants restricts them to a monocarpic mode of life (Suzuki et al., 2012). Like *35S::AHL15 Arabidopsis* plants, the DEX-treated *35S::AHL15-GR* tobacco plants continuously produced many lateral branches that flowered and produced seeds, causing the plants to carry new shoots, flower buds, opened flowers, green fruits and ripened fruits at the same time (Fig. S6a). To quantify the effect of *AHL15* expression on seed production we harvested seeds from all the ripened fruits from DEX-treated and non-treated wild-type and *35S::AHL15-GR* plants. The average weight of individual seed from all four samples was determined by weighing 300 seeds. The total number of seeds produced by a plant was calculated by dividing the total weight of all the seeds of a plant by its average single seed weight. After statistical analysis we found that the total number of seeds produced per DEX-treated *35S::AHL15-GR* plant was two times more than the control plants (Fig. 9a). But when we compared the total weight of seeds of all four types of plants, surprisingly there was no significant difference (Fig. 9b). We also compared the volume of four types of seeds by putting them in 50ml falcon tube, interestingly the volume of DEX-induced *35S::AHL15-GR* seeds was more than the volume of other three types of seed (Fig. S6b). This discrepancy between the calculated seed number, and the quantified total seed weight and volume per plant led us to look into the seed morphology and viability. Electron micrographs of the four types of seeds showed clear shape and size abnormalities in the seeds harvested from DEX-treated *35S::AHL15-GR* plants (Fig. 10 d1), whereas seeds harvested from the other plants (non-treated wild type and *35S::AHL15-GR* and DEX-treated wild type) were normal round and oval shaped (Fig. 10 a1, b1 and c1). Observation of the seeds with a stereomicroscope using dark field lighting showed that most seeds from DEX-treated *35S::AHL15-GR* plants were empty (Fig. 10 d2). Germination of 500 seeds per seed batch showed that the seeds from the control plants germinated normally (Fig. 10 a3, b3, and c3), but that the germination efficiency of the seeds collected from the DEX-treated *35S::AHL15-GR* plants was reduced to 2-3 percent (Fig. 10 d3). These results suggest that the abundant and repeated flowering induced by DEX treatment of the *35S::AHL15-GR* tobacco plants affected their reproduction efficiency. At this moment we cannot exclude,

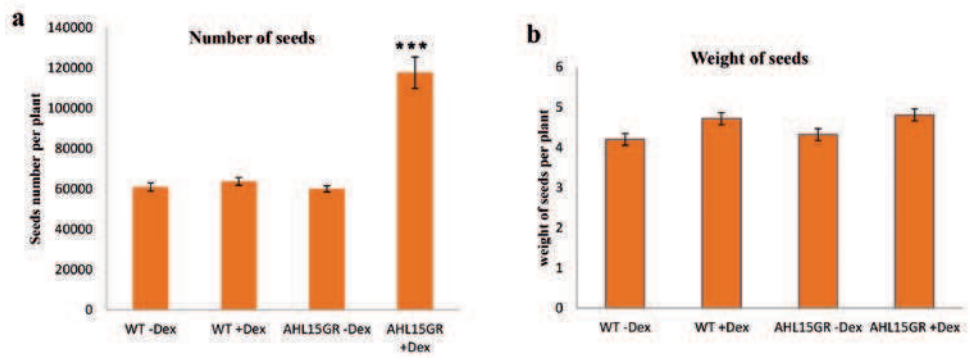

Figure 9. AHL15 induction in *Nicotiana tabacum 35S::AHL15-GR* plants significantly enhances total seed number without affecting total seeds weight. The total number of seeds per plant is calculated by dividing the total weight of seeds of a plant by the average weight of a single seed of that plant. The comparison of (a) total seed number per plant shows that AHL15 induction in *35S::AHL15-GR* plants significantly enhances number of seeds per plant as compared to induced wild type plants and non-induced wild type and *35S::AHL15-GR* plants. The comparison of seed weight (b) shows no significant difference in total seed weight per plant. Significant increase in indicated by asterisks (*) ($p < 0.05$) and (***) indicate 2x difference.

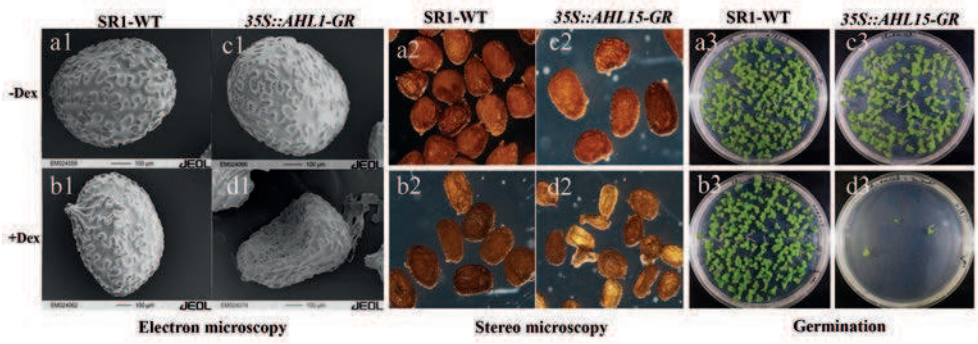

Figure 10. AHL15-induction in *Nicotiana tabacum 35S::AHL15-GR* plants affects seed quality. Electron microscopy of the *Nicotiana tabacum* seeds harvested from non DEX-treated wild type (a1) and *35S::AHL15-GR* (c1) plants and DEX-treated wild type (b1) plants show normal oval/round morphology and that of *35S::AHL15-GR* DEX-treated (d1) plants with irregular shapes. Stereomicroscopy shows all the seeds with normal and oval/round morphology (a2, b2, c2 and some in d2) compact while all the abnormal seeds (d2) with irregular morphology are empty from inside. The germination of the seeds from non-induced wild type (a3) and *35S::AHL15-GR* (c3) and induced wild type (b3) plants on MS medium without DEX and selection shows 100% efficiency while only 2-3% of the seeds from induced *35S::AHL15-GR* plants (d3) show germination.

however, that the poor seed quality was induced by nutrient deficiency, which was observed previously for DEX-treated *35S::AHL15-GR* tobacco plants that were allowed to rejuvenate without refreshing the soil (Fig. 8).

DISCUSSION

The annual and perennial life cycles are two different life history strategies of flowering plants that allow them to battle against harsh environmental conditions. Common in both types of life history strategies is the developmental transition from vegetative to reproductive phase (Friedman and Rubin, 2015). In annual plants, the reproductive phase is followed by senescence and death of the plant while survival of the species is guaranteed by the seed. In contrast, in perennials the hardy part of the vegetative plant body is maintained, which allows the plant itself to survive the harsh winter conditions and re-enter the reproductive phase again and again (Albani and Coupland, 2010). So polycarpy and longevity in flowering plants depend on maintaining the vegetative plant body and development of lateral or new shoots with juvenile characteristics having the potential to pass through the reproductive phase. In this study we showed that heterologous expression of the *Arabidopsis AHL15* gene changes the monocarpic tobacco into a polycarpic plant, inducing branching by activating axillary meristems and by keeping these meristems in the vegetative state. Other phenotypes observed in DEX-treated *35S::AHL15-GR* plants were delayed germination and seedling development even leading to callus formation, and delayed flowering and leaf senescence. All these observations indicated that *AHL15* overexpression has the same effects on tobacco as it has on *Arabidopsis* (Karami et al., 2017), and that it generally is able to revert developmental transitions, thereby keeping plant tissues in a juvenile state.

However, there are also clear differences in the phenotypes induced by *AHL15* overexpression in *Arabidopsis* or tobacco. For example, in *Arabidopsis*, *AHL15* overexpression, similar to *BABY BOOM* (*BBM*) overexpression, induces somatic embryos on cotyledons and also callus-like structures that later convert to somatic embryos (Boutilier, 2002; Karami et al., 2017). In tobacco, however, *AHL15* overexpression only led to the formation of callus, whereas *BBM* overexpression leads to shoot and root induction. *BBM* overexpression requires cytokinin to be added to the medium to induce somatic embryogenesis (Srinivasan et al., 2007), and possibly the same hormonal treatment is required before *AHL15* overexpression can induce somatic embryos on tobacco seedlings, but this needs further testing. In any case, it is clear that activation of AHL15-GR by DEX-treatment in homozygous *35S::AHL15-GR* seedlings completely arrests development, probably by converting organized stem cell zones into undifferentiated callus tissues. This suggests that in tobacco *AHL15* expression completely inhibits cell differentiation and organogenesis, which might explain why *35S::AHL15* plants that constitutively overexpress *AHL15* could not be obtained. This difference in phenotypes for *AHL15* or *BBM* overexpression in tobacco and *Arabidopsis* suggests that there is a significant difference in the set of target genes that are up- or downregulated by AHL15 or BBM in these plant species. The fact that for BBM this can be restored by adding cytokinin to the medium, hints that a major difference might lie in plant hormone-related genes.

35S::AHL15-GR tobacco plants first grown in the absence of DEX developed normally like wild-type plants in the presence or absence of DEX. DEX-treatment in *35S::AHL15-GR* plants resulted in the activation of axillary meristems, not only delaying senescence and enhancing branching, but also leading to the production of leaves with juvenile features. Similar to *Arabidopsis*, AHL15 can rejuvenate development in tobacco, as *in-vitro* induction experiments showed that AHL15 activation brings the adult plant morphology back to juvenile state (Fig. 3d & S4), and shoots with juvenile features could be induced on soil-grown plants (Fig. 7b and 8d). Similarly, we observed that the DEX activation of AHL15 in *35S::AHL15-GR* plants, delayed leaf senescence and caused a delay in flowering time. This suggests that, like in *Arabidopsis*, the vegetative phase change is important for flower initiation and also preludes the leaf senescence (maturation) process.

DEX-treated *35S::AHL15-GR* tobacco plants continued to grow for at least 2 years. Continuous refreshment of the soil was essential for their proper development. If not, leaves turned yellow and eventually white. But this could be restored by transferring them to fresh soil again. Like in *Arabidopsis*, *AHL15* overexpression resulted in polycarpy in tobacco, giving rise to more seeds. Interestingly, in tobacco this came at the cost of reduced seed quality, which could be because the *35S* promoter driving the *AHL15-GR* expression is more active in the seeds during embryogenesis in tobacco. Alternatively, the more branched growth of th DEX treated *35S::AHL15-GR* tobacco plants might limit nutrient availability. Tobacco is considered as heavy feeder, and the refreshment of soil alone might not provide sufficient nutrients, as additional nutrient supply resulted in a rapid but transient conversion of the leaves from yellow to dark green (Fig. 8b,d). Moreover, in contrast to *Arabidopsis*, *AHL15* overexpression in tobacco might not result in the production of photosynthetic leaves that produce sufficient energy to support the renewed seed set. This might explain why polycarpy in this plant species comes at the cost

of reduced seed quality, which is typically a seed size versus seed number trade-off in larger-seeded perennial species (Leishman and Westoby, 2000). For example, the average number of seeds produced per plant in *Arabidopsis thaliana* is much more than its perennial relative *Arabis alpina* that produces significantly larger seeds (Boyes et al., 2001; Andrello et al., 2016). Like with our *35S::AHL15-GR* tobacco plants, it is generally observed that in evolution plants adopt the polycarpic life history at the cost of reproduction (Friedman and Rubin, 2015). That this is not the case in *Arabidopsis* plants overexpressing *AHL15* is quite surprising (Karami et al., 2017). Our observations on the *35S::AHL15-GR* tobacco lines suggest that the outcome of a switch from monocarpic to polycarpic growth probably depends on how efficient a plant uses its resources. At the same time *AHL15* overexpression in *Arabidopsis* and tobacco due to enhanced number of branches resulted in more seeds production.

Like the effect of ipt gene on axillary buds in tobacco (Hewelt et al., 1994), *35S::AHL15-GR* induction strongly reduces apical dominance which results in branched morphology and smaller leaf size (Fig. 6a and S7). In case of soil-DEX treatment of *35S::AHL15-GR* plants, leaves kept the juvenile characteristics and new juvenile shoots appeared from the transitional area between shoots and roots (Fig. 6a, 7b & 8d) while the non-DEX treated *35S::AHL15-GR* and wild type plants passed normally through the developmental processes and died after one flowering period with a single main stem. A comparison of flowers, inflorescence and fruits morphology of wild type and induced *35S::AHL15-GR* plants did not show any significant differences in their size and shape (Fig. S8). Only the inflorescence of *35S::AHL15-GR* was more scattered as compared to wild type which could be because of the branched morphology induced by *35S::AHL15-GR* induction.

Our data add to the general picture that *AHL* genes are key regulators of plant developmental processes. Our results show that ectopic expression of *Arabidopsis AHL15* leads to similar phenotypic changes in *Arabidopsis* (Karami et al., 2017) and tobacco, suggesting that *AHL15* could be a generic switch between monocarpic and polycarpic life history strategy in flowering plants. However, our results also indicate that the strategy to use *AHL15* overexpression to enhance seed production will not work in all plant species. It is important to understand what is at the basis of this difference, and also to determine the molecular mechanism underlying action of *AHL15* so that we can successfully apply this knowledge to enhance the yield of important crop plants.

MATERIAL AND METHODS

Plant material and growth conditions

Nicotiana tabacum SR1 (tobacco) plants were grown axenically on MS medium (Murashige and Skoog, 1962) in 1l glass jars. To establish this axenic plant culture, seeds were surface sterilized by a first wash with sterile Milli-Q water (MQ), followed by one minute incubation in 70% ethanol, a wash with sterile MQ, 10 minutes incubation in 50% Glorix (commercial solution containing 4.5% active chlorine, and <5% sodium hypochlorite) with periodically shaking, and finally 4 to 5 washes with sterile MQ.

For *in-vitro* seed germination, half strength MS medium with 0.8% agar (w/v) (Diachin agar) and 1.5% sucrose was used, while for transformation and regeneration normal MS medium

with 0.7% agar, 3% sucrose 2 mg/l BAP and 0.2 mg/l NAA was used. *In-vitro* germination, plant growth and regeneration were carried out at 24°C and 16 hours photoperiod, while plants were grown on soil in a growth room at 25°C, 75% relative humidity and 16 hours photoperiod.

Generation of *35::AHL15-GR* tobacco lines

The *35S::AHL15-GR* construct was obtained by replacing *BBM-GR* fragment in a binary vector pSRS031 with a synthetic *Pst*I-*Xho*I fragment containing the *AHL15-GR* fusion (Passarinho et al., 2008). Fresh single *A. tumefaciens* colonies were obtained from a -80°C stored glycerol stock by making a pure streak on LC plates (10 g/l tryptone, 5 g/l yeast extract, 8 g/l NaCl, 0.8 % agar) containing 20 µg/ml rifampicin and 75 µg/ml carbenicillin and 250 µg/ml spectinomycin to select the *A. tumefaciens* AGL1 strain (Jin et al., 1987) containing the *35::AHL15-GR* construct. Plates were incubated at 29°C for two days. For liquid culture, a single colony was inoculated in 25 ml LC medium (without agar) in a 100 ml flask that was incubated at 30°C with 180 rpm rotation for two days. The liquid cultures were transferred to 50 ml falcon tubes (SATSTED) and centrifuged at 4000 rpm for 20 minutes. The supernatant was discarded and after washing with MQ, the pellet was re-suspended in induction medium (Gelvin, 2006) with 100 µM acetosyringone and induced for overnight in the dark on 50 rpm rotator at room temperature.

For *Agrobacterium* leaf disc infiltration, round leaf discs were cut from the 3rd and 4th leaf of non-sterile 4 to 5 weeks old greenhouse grown wild type tobacco plants with the help of a blue cap tube of 5 cm diameter. The non-sterile round leaf discs were surface sterilized with a first wash in sterile MQ followed by 15 minutes incubation in 10% Glorix (commercial solution containing 4.5% active chlorine, and <5% sodium hypochlorite) with gentile rotation and finally 4-5 washes with sterile MQ (Baltes et al., 2014). The surface sterilized leaf discs were infiltrated with overnight induced *A. tumefaciens* culture of OD_{600} 0.6-0.8. The infiltrated leaf discs were blotted for 2 to 3 minutes and then transferred to co-cultivation plates containing 25 ml MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA and 40 mg/l acetosyringone. The cocultivation was carried out for three days in the dark at 24°C. Selection and regeneration was carried out on MS medium without acetosyringone using 15 mg/l phosphinothrycine (ppt) for selection and 500 mg/l cefotaxime for killing *Agrobacterium*. Ppt resistant shoots were transferred to 1l jars with hormone free MS medium containing 15mg/l ppt and 500mg/l cefotaxime for rooting. The rooted transformed plants were transferred to soil and grown in growth rooms at 25C° with 75% relative humidity and 16 h photoperiod.

T2 seeds were germinated on selection medium to identify single locus T-DNA insertion lines based on their 3:1 segregation ratio. Four lines were selected and used in the subsequent experiments. To check for the presence of the *35S::AHL15-GR* construct, genomic DNA was isolated from leaf tissue of T2 plants of the transgenic lines via CTAB method (Doyle and Doyle, 1990) and PCR was performed using the primers A-GR-fw CATTTGGAGAGGACTCGAGCTCAT and A-GR-rev CGCTGTACCATGCATGATCTGGAT. Homozygous T3 plants were selected by segregation analysis.

Phenotypic analysis and morphometry on wild-type and *35S::AHL15-GR* plants

To compare phenotypes, generally 3 plants per line were grown and images of only representative plants are shown. To quantify the effect of induced AHL15 activity on number of leaves and branches, 3 representative plants were grown each *in vitro* on MS medium with or without 10μM DEX and on soil of same quality and quantity with 30μM DEX-treatment via spray or watering. To quantify the seed number and weight per plant, one plant of each lines was grown and the DEX treatment was done via spray and watering in soil (see Table. 2). All the seeds were harvested from completely senesced tobacco control plants while in positive tobacco plants the seeds were harvested 2-3 weeks later only from ripe fruiting bodies only.

Photography, Stereo- and scanning electron microscopy

Plants photography was done with Canon camera (model: pc1742) with 12.1 mega pixels and 20X zooming power. Stereomicroscopy of seeds was done with Leica MZ16FA stereo fluorescent microscope with Leica DFC 420 Camera and cable -5 megapixel,6 pin firewire and seedlings and callus pictures were taken with Leica MZ12 with LEICA DC 500 microscope CCD Camera Head 12447108 12-33 vdc Firewire. For scanning electron microscopy dry clean seeds were fixed to specimen stubs with adhesive and placed on the revolving discs of a sputter coater E5100 (Polaron Equipment ltd) where each seed was uniformly coated with gold. These specimen tubes were then fixed to the specimen holder of scanning electron microscope (Joel JSM6400) maintained at an accelerating potential of 10KV. The images were taken at different angles and magnifications. Images were modified assembled in PowerPoint (Microsoft office 2010).

DEX treatment

A stock solution of 30 mM DEXamethasone (Sigma-Aldrich®) was prepared in 70% alcohol. For *in-vitro* treatments, DEX was added at a final concentration of 10 μM to sterile MS medium. For *in-vivo* treatments, DEX was added to water at a final concentration of 30 μM and directly sprayed on plants or 100ml DEX-water was added to the pots per soil treatment, according to the schedules in Tables 1 and 2.

Table 1. Three days DEX spray schedule for 50 days old plants

Spray	Day	Time
1st	1st day	16:00 pm
2nd	2nd day	10:00 am
3rd	3rd day	10:00 am

Table 2. Combined soil DEX-treatment and DEX-spray schedule for two weeks old plants

DEX treatment	Soil DEX treatment	DEX spray
In-vitro seedlings transferred to soil	100 mL (Friday)	No spray
1st Week	100 mL (Tuesday)	1st Tuesday 2nd Friday
2nd Week	100 mL (Tuesday)	3rd Tuesday 4th Friday
3rd Week	-------	5th Tuesday 6th Friday

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SUPPLEMENTARY FIGURES

2

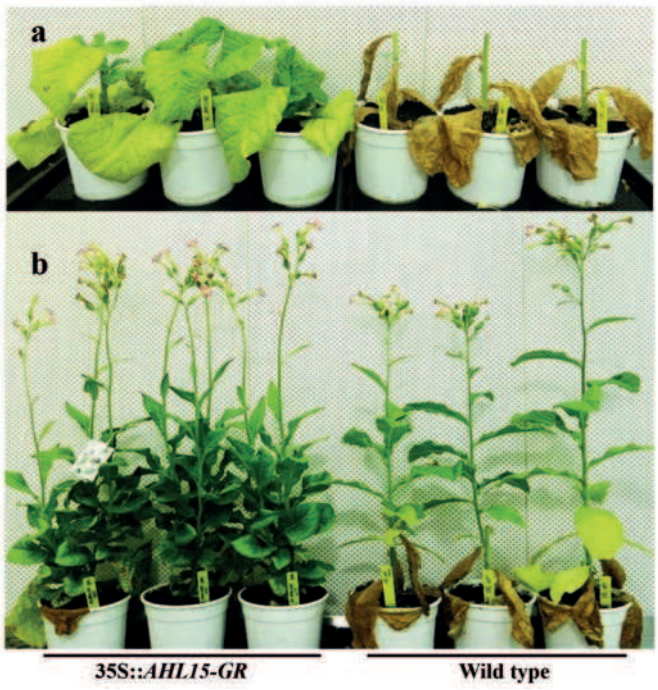

Figure S1. AHL15-induction maintains the delay in senescence in *35S::AHL15-GR* tobacco plants during the second generation. (a) 4 months old *Nicotiana tabacum* first generation DEX-treated wild type (senesced) and *35S::AHL15-GR* (delayed senescence) plants 6 inches stems DEX sprayed. (b) After 5 weeks wild type stems show development of single shoot with early senescence and *35S::AHL15-GR* plants show development of multiple lateral shoots with delayed senescence.

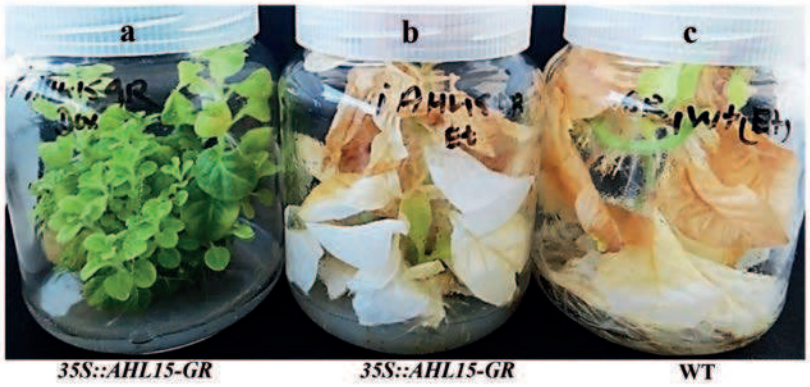

Figure S2. 4 months old *Nicotiana tabacum*. (a) *35S::AHL15-GR* plant developed on DEX-induced medium shows delayed senescence with multiple juvenile and some adult shoots (b) *35S::AHL15-GR* plant on non-DEX medium and (c) wild type on DEX medium shows completely senesced adult leaves and having no juvenile shoots or leaves.

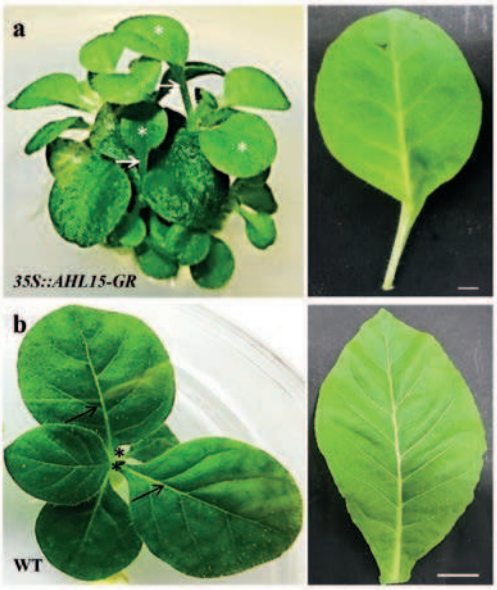

Figure S3. Adult and juvenile leaf morphology in *35S::AHL15-GR* and wild type *Nicotiana tabacum*. *Nicotiana tabacum* (a) DEX-treated *35S::AHL15-GR* plant shows small round leaves (white asterisks) with long petiole (white arrows) and no clear veins as juvenile characteristics and (b) wild type plant shows adult morphology having large elongated leaves with short petiole (black asterisks) and a clear central midrib (black arrows). The right panel shows the juvenile and adult leaf morphology difference in *in-vivo* plants. Scale bar is 10 mm.

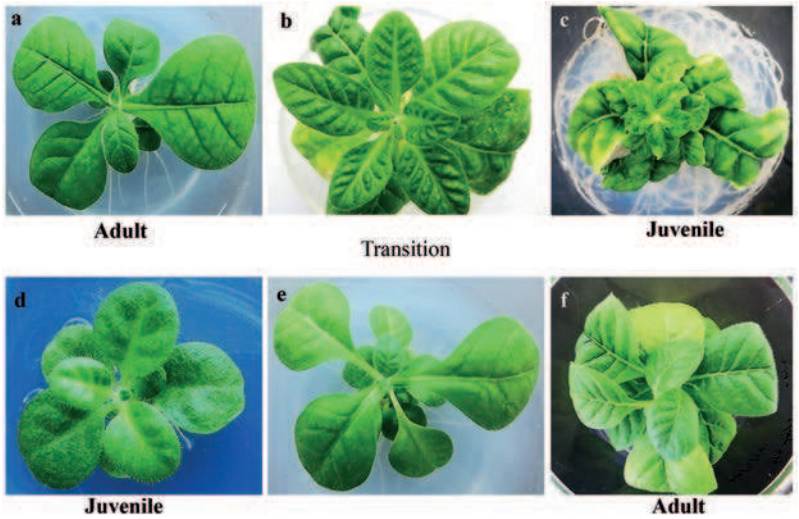

Figure S4. *Nicotiana tabacum 35S::AHL15-GR* plants showing phase change from adult to juvenile and juvenile to adult morphology. 40 days old *Nicotiana tabacum* (a) *35S::AHL15-GR* adult plant developed on MS medium. (b) After 15 days of transfer to DEX containing medium, the leaves show transition phase while (c) after 40 days all the new leaves show juvenile characteristics. (d) *35S::AHL15-GR* plant on DEX containing MS medium shows juvenile morphology (e) after 15 days of transfer to non-DEX medium shows transition phase while (f) after 40 days the plant shows completely adult morphology.

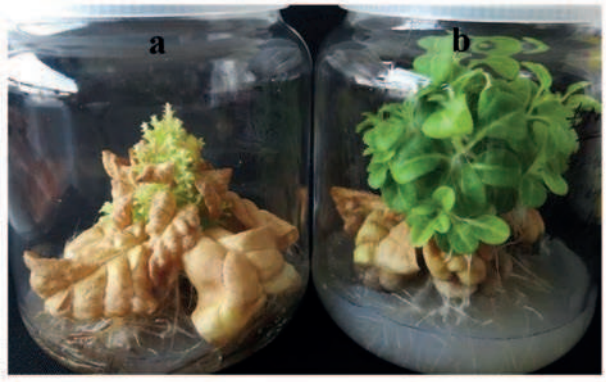

Figure S5. 4 months old *Nicotiana tabacum 35S:AHL15-GR* plants developed on MS medium. After 40 days the plants were transferred to DEX containing MS medium. After 15 days of induction, plant (b) is transferred back to MS medium without DEX showing the new shoots with juvenile characteristics and branched morphology while plant (a) left on inducible medium shows many small branches in the axil of the dried adult leaves with minute juvenile leaves.

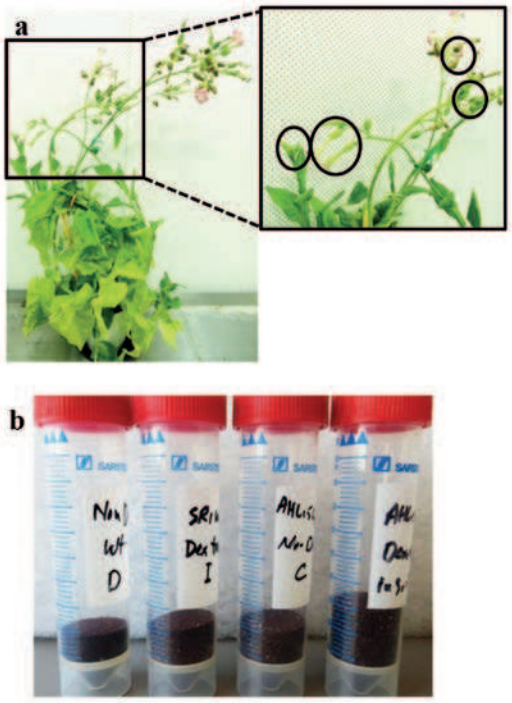

Figure S6. AHL15 induces continuous reproductive phase in *Nicotiana tabacum 35S::AHL15-GR* plants. (a) 3 months old DEX-treated *N. tabacum 35S::AHL15-GR* plant having flower buds, mature flowers, green fruits and ripened fruits (encircled in highlighted part) during the same phase of plant development. (b) Volume of total seeds collected from DEX-treated and non DEX-treated wild type and *35S::AHL15GR* plants. The right tube shows the volume of the seeds collected from only ripened fruits of DEX-treated *35S::AHL15-GR* plants.

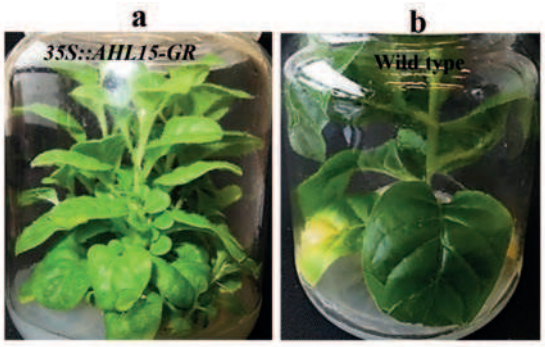

Figure S7. 3 months old DEX-treated *Nicotiana tabacum*. (a) *35S::AHL15-GR* plant shows breaking of lateral shoots dormancy while in (b) wild type plant the lateral shoot dormancy is maintained showing no effect of DEX on lateral shoot development.

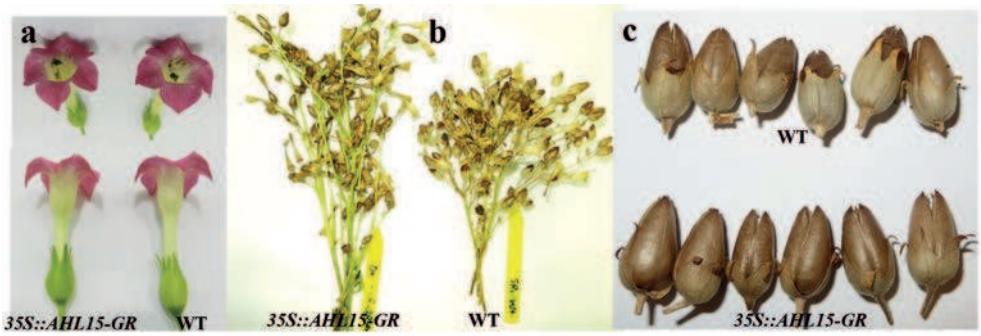

Figure S8. Flower, inflorescence and seed pods morphology comparison between wild type and *35S::AHL15-GR* tobacco plants. *Nicotiana tabacum* wild type and *35S::AHL15-GR* DEX-treated plants show similar (a,c) flower and seed pod morphology while *35S::AHL15-GR* inflorescence (b) is more dispersed compared to wild type.