The role of miR156 in rejuvenation in Arabidopsis thaliana

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The plant life cycle can be divided into three major phases (juvenile, adult, and reproductive) after seed germination, based on morphological and physiological differences. In contrast to juvenile plants, adult plants are capable of transitioning to the reproductive phase by responding to diverse floral induction signals (Bäurle and Dean 2006). Juvenile plants also differ from adult plants in terms of leaf morphology and regenerative capacity (Yu et al. 2015). The cues guiding juvenile-to-adult phase transition include conserved and intrinsic mechanisms as well as extrinsic inputs, such as nutrients, light, and temperature.

Rejuvenation refers to the process enabling plants to regain physiological and molecular characteristics lost after entering the adult phase (Greenwood 1987). Plant
rejuvenation can generally be achieved by repeated grafting of adult shoot tips onto juvenile rootstocks in vitro, severe pruning, and in vitro tissue culture (Greenwood 1987; Bon and Monteuuis 1991; Huang et al. 1992; Davenport 2006). Although the precise molecular mechanisms underlying plant rejuvenation remain unclear, several studies in Sequoia sempervirens have demonstrated that changes in hormone response, DNA methylation, genome arrangement, and induction of specific proteins are involved in rejuvenation (Sussex and Clutter 1960; Huang et al. 1996; Chang et al. 2010; Huang et al. 2012). Small RNA profiling reveals an increase in microRNA156 (miR156) during plant rejuvenation (Chen et al. 2012). miR156 maintains juvenile traits by repressing a group of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors (Wu and Poethig 2006; Chuck et al. 2007; Chen et al. 2010; Huijser and Schmid 2011; Xie et al. 2012; Poethig 2013; Wang and Wang 2015; Yu et al. 2015), and its levels gradually decrease after germination (Wu and Poethig 2006; Wang et al. 2009; Xu et al. 2018). Overexpression of miR156 prolongs juvenile phase, whereas inhibition of miR156 activity causes early appearance of adult traits (Wu et al. 2009). Importantly, miR156 is extremely conserved across land plants, suggesting that this miRNA may play a conserved role in juvenile-to-adult phase transition (Axtell and Bowman 2008) and contribute to the plant rejuvenation process.

Although constitutive overexpression of miR156 prolongs the juvenile phase and delays flowering in Arabidopsis thaliana (Wang et al. 2009; Yamaguchi et al. 2009), it remains unclear whether elevation of miR156 in the adult phase is sufficient to induce plant rejuvenation. To address this question, we generated an inducible A. thaliana line (named YE63) in which MIR156C is regulated by the synthetic chimeric transcription factor GVG (hormone binding domain of glucocorticoid receptor, DNA binding domain of yeast GAL4 and herpes viral protein VP16) and six copies of the GAL4 upstream activating sequence (UAS) (Figure S1A) (Aoyama and Chua 1997). Upon treatment with the GR ligand dexamethasone (DEX), GVG is translocated into the nucleus and activates miR156 expression through binding with 6×UAS. We treated seedlings of wild-type A. thaliana (Columbia accession [Col-0]) and five independent T2 transgenic lines with DEX for 24 h. Quantitative real-time PCR (qRT-PCR) revealed variable increases in miR156 expression level (Figure S1B) with accordant decreases in SPL expression (Figure S1C) in transgenic lines compared to...
the wild type. Since YE63 77# showed the highest fold induction, we used its T3 homozygous line (YE63 77-1#) for subsequent studies. It should be noted that the miR156 level in YE63 77-1# plants after induction was lower than that in p35S::MIR156 plants (Figure 1A). YE63 77-1# plants grew normally in the absence of DEX (mock) but exhibited a similar phenotype to p35S::MIR156 plants upon continuous treatment with DEX after germination under long days (Figure S2A-D). These results indicate that GVG is functional without leakage in YE63 77-1# plants.

Wild-type A. thaliana plants flower early under long days. Therefore, we conducted the rest of the experiments using plants grown under neutral days to extend the vegetative phase. We first explored whether high amounts of miR156 can induce juvenile leaf traits after plants are fully mature. The transition from juvenile to adult phase in A. thaliana is marked by the initiation of trichomes (leaf hairs) on the abaxial (lower) surface of the leaf, an increase in the length/width ratio of the leaf blade, production of serrations on the leaf margin, and a decrease in shoot regenerative capacity (Poethig 2003; Huijser and Schmid 2011; Poethig 2013; Yu et al. 2015; Zhang et al. 2015a; Zhang et al. 2015b; Wang et al. 2019). Eighteen-day-old YE63 77-1# plants produced abaxial trichomes and serrations on the 6th leaf, suggesting that these plants had entered the adult phase (Figure S3A and B). Scanning electron microscopy (SEM) further indicated that the shoot apical meristem of these plants was still in the vegetative phase (Figure S3C). Based on these pilot experiments, we chose 18-day-old YE63 77-1# plants for miR156 induction assays. Compared to mock plants, DEX-treated plants started to produce rosette leaves without abaxial trichomes from the 13th leaf, resembling p35S::MIR156 plants (Figure 1B). Similar reversion to the juvenile-phase phenotype was observed for the number of serrations (Figure 1C). Interestingly, although the induction of miR156 in adult phase led to downwardly curled leaf blades (Figure 1D; Figure S3D and 3E), the leaf length/width ratio of YE63 plants was slightly increased upon DEX treatment compared to that of the wild type (Figure 1D; Figure S3F; Figure S4A-4C). These results suggest that elevation of miR156 is not sufficient to restore all juvenile leaf traits.

We next examined whether induction of miR156 in the adult phase could delay flowering. The number of rosette leaves and days to flowering revealed no difference in flowering time between mock- and DEX-treated plants (Figure 1E). However,
DEX-treated plants produced similar cauline leaves and small flowers to p35S::MIR156 plants (Figure 1F-I). Thus, the induction of miR156 after the adult phase is fully established cannot delay flowering but is sufficient to regulate inflorescence development.

Hypocotyls from A. thaliana plants with low miR156 activity produce fewer adventitious roots than those from wild-type plants (Xu et al. 2016). Notably, the role of miR156 in adventitious root regeneration is evolutionarily conserved, with high miR156 expression also required for adventitious root formation in Medicago sativa and Malus xiaojinensis (Massoumi et al. 2016; Aung et al. 2017; Xu et al. 2017). To determine whether elevation of miR156 level in the adult phase is sufficient to promote adventitious root regeneration capacity, we cultured 6th leaf explants from YE63 77-1# plants (Figure S5A-D) on B5 medium without exogenous hormones (Liu et al. 2014). Consistent with a previous report (Xu et al. 2016), p35S::MIR156 leaf explants exhibited a high rooting rate (Figure 1J and K). Compared to mock-treated plants, increased levels of miR156 in 6th leaf explants of YE63 77-1# did not elevate rooting rate (Figure 1J and K; Figure S5), suggesting that miR156 is not able to promote adventitious root regeneration once adult phase is fully established.

Taken together, our results reveal that miR156 cannot fully induce plant rejuvenation in A. thaliana. Two possibilities could explain this observation. First, miR156 is not sufficient to reprogram the downstream signaling pathways once plants enter adult phase. Under this scenario, epigenetic remodeling might play an important role in the establishment of adult traits by SPLs. Once the adult phase is fully established, the epigenetic status of SPL-regulated genes is mitotically stable and irreversible. In agreement with this hypothesis, previous studies have reported changes in DNA methylation and genome arrangement during rejuvenation (Huang et al. 1996; Chang et al. 2010). The second possibility is that a parallel, unknown aging pathway exists in plants. Induction of miR156 in the adult phase cannot overcome the dominance of this pathway, whose key regulator remains to be identified. Whether expression of this gene is age regulated and, if so, how age regulates its abundance are completely unknown. Future identification of a suitable plant model for rejuvenation studies and subsequent elucidation of the molecular basis of this phenomenon will
sheds light on the nature of plant developmental plasticity and contribute to the next generation of plant biotechnology.

**MATERIALS AND METHODS**

**Plant materials**

For screening transgenic lines, *A. thaliana* (ecotype Col-0) plants were grown at 21°C (day)/19°C (night) under long days (16 h light/8 h dark). For rejuvenation experiments, *A. thaliana* plants were grown at 21°C (day)/19°C (night) under neutral days (12 h light/12 h dark). *p35S::MIR156* (Col-0 ecotype) was described previously (Wang et al. 2008). Binary constructs were delivered into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by a freeze–thaw method. Transgenic *A. thaliana* plants were generated by the floral dipping method (Clough and Bent 1998) and screened using 40 mg/ml hygromycin on 1/2 Murashige and Skoog (MS) plates.

**Constructs**

To generate *pGVG::MIR156C* vector (YE63), the genomic fragment containing *MIR156C* was PCR amplified and inserted into the binary vector LW413 behind 6×UAS. The vector map and DNA sequence are available upon request.

**Phenotype analyses**

For DEX treatment, 10 μM DEX solution with 0.014% (v/v) silwet was evenly sprayed onto *A. thaliana* plants. As a mock control, DEX solution was replaced with an equal volume of ethanol. For each plant, 1 ml DEX or mock solution was used.

The 13th fully expanded leaf was used for trichome and serration measurements. The length and width of mature leaves were measured to determine leaf shape. For flowering time, the number of rosette leaves and the number of days after germination to first floral bud were scored.

For root regeneration assay, seeds were germinated on 1/2 MS medium at 21°C under 16-h-light/8-h-dark conditions. The 6th leaves (3 mm in length) were detached and cultured on B5 medium without sucrose or exogenous hormones at 21°C under 16-h-light/8-h-dark conditions. Rooting rate was expressed as the percentage of

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explants with regenerated roots among a given number of explants at 12 days after culture (Chen et al. 2014). For DEX induction experiments, 10 μM DEX was added to the B5 medium; water containing an equal volume of ethanol was used as a mock control. DEX and mock B5 media were renewed every day for the first 4 days.

**Expression analyses**

Leaves were harvested and ground into fine powder with liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, 15596018), and 1 μg total RNA was treated with DNase I and used for cDNA synthesis with oligo (dT) primer (Fermentas, S0132). Mean expression levels were calculated from $2^{-\Delta\Delta C_{t}}$ values. The RT-qPCR primers for TUBULIN (TUB) have been described previously (Wang et al. 2009). Quantification of miR156 was performed according to a published protocol (Yu et al. 2013). Sequences of the specific primers are listed in Table S1.

**Scanning electron microscopy (SEM)**

Shoot apical meristems were fixed in FAA (50% [v/v] ethanol, 5% [v/v] acetic acid, and 3.7% [v/v] formaldehyde), dried to critical point, and then dissected under a stereomicroscope and mounted on SEM stubs. Mounted shoot apical meristems were coated with palladium–gold and examined using a JSM-6360LV SEM microscope (JEOL) with an acceleration voltage of 7 kV.

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**FIGURE LEGEND**

Figure 1. Induction of miR156 in the adult phase causes partial reversion to juvenile-phase traits in *A. thaliana*

**(A)** miR156 levels in 7-day-old YE63 77-1# seedlings treated with DEX or ethanol (mock). Average expression from two independent biological experiments (*n* = 20 for each experiment). Bars = SE. **P < 0.01; ns, not significant; Student’s t-test.**

**(B)** Abaxial trichome phenotype of the 13th rosette leaf from YE63 77-1# plants treated with DEX and p35S::MIR156 plants. Bar = 400 μm. (C) Serration phenotype of the 13th rosette leaf from YE63 77-1# and p35S::MIR156 plants. Bar = 1 cm. (D) Leaf shape in YE63 77-1# and p35S::MIR156. Bar = 2 cm. (E) Flowering time of YE63 77-1# plants. ns, Not significant. (F) Inflorescence phenotype of YE63 77-1# and p35S::MIR156. Bar = 1 cm. (G) Cauline leaf shape of YE63 77-1# and p35S::MIR156 plants. Bar = 1 cm. (H and I) Flower phenotype of YE63 77-1# and p35S::MIR156. Bar = 200 μm. (J) Rooting capacity of YE63 77-1# and p35S::MIR156 plants. Pictures taken at 20 days after culture (DAC). Bar = 1 cm. (K) Quantitative analysis of root regenerative
capacity of 6th leaf explants from YE63 77-1# and p35S::MIR156 at 12 DAC. Rooting rate is expressed as the percentage of leaf explants with regenerated roots. Data are from three or four independent experiments; \( n = 30\sim33 \) for each experiment. Bars = SD. **P < 0.01; ns, not significant; one-way ANOVA followed by Tukey’s multiple comparison test.