The *microRNA476a*/RFL module regulates adventitious root formation through a mitochondria-dependent pathway in *Populus*

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21 **Abstract**

22 Adventitious root (AR) formation at the base of stem cuttings determines the efficiency
 23 of clonal propagation for woody plants. Many endogenous and environmental factors
 24 influence AR formation. However, our knowledge about the regulation of AR
 25 development by mitochondrial metabolism in plants is very limited. Here we identified
 26 *Populus*-specific *miR476a* as a novel regulator of wound-induced adventitious rooting
 27 via orchestrating mitochondrial homeostasis in poplar. *MiR476a* exhibited inducible
 28 expression during AR formation and directly targets several *Restorer of Fertility like*
 29 (*RFL*) genes encoding mitochondrion-localized pentatricopeptide repeat proteins.
 30 Genetic modification of *miR476-RFL* expression revealed the *miR476/RFL*-mediated
 31 dynamic regulation of mitochondrial homeostasis on AR formation in transgenic
 32 poplar. Furthermore, mitochondrial perturbation via exogenous chemical inhibitor
 33 validated that the *miR476a/RFL*-directed AR formation depended on mitochondrial
 34 regulation though modulating the auxin pathway. Our results established a
 35 miRNA-directed mitochondrion-auxin signaling cascade required for AR development,
 36 providing novel insights into the understanding of mitochondrial regulation on plant
 37 developmental plasticity.

38

39 **Key words:** adventitious rooting; *miR476a*; mitochondria; auxin; *Populus*.

40

41 **Introduction**

42 Adventitious roots (ARs) post-embryonically develop from non-root tissues in plants
 43 including hypocotyls, leaves, shoots, and reproductive organs (Bellini et al., 2014).
 44 Shoot-borne ARs occur normally in many monocotyledons, especially cereals such as
 45 rice and maize, which are essential for establishment of a fibrous root system in these
 46 plants (Hochholdinger et al., 2004; Bellini et al., 2014). In many plant species, AR
 47 emergence is usually induced by environmental changes such as flooding and
 48 dark-light transitions (Lorbiecke and Sauter, 1999; Sorin et al., 2005), or artificial
 49 stimuli, including wounding and hormone treatment (Ahkami et al., 2009; Sukumar et
 50 al., 2013). Cutting propagation through detached branches or stem is widely used in
 51 horticulture and forest nurseries (Davis and Becwar, 2007). As the first developmental
 52 event after cutting, adventitious rooting determines explant survival and efficiency of
 53 clonal propagation (Legué et al., 2014). AR formation is a wounding-triggered plant
 54 regeneration known as *de novo* root organogenesis that occurs when detached organs
 55 meet suitable conditions (Xu and Huang, 2014). AR formation starts with specification
 56 of root founder cells that undergo a few events, including cell division, patterning for
 57 AR primordium establishment and AR outgrowth (Legué et al., 2014). Genetic and
 58 molecular studies suggest that adventitious rooting requires coordinated and dynamic
 59 regulation of hormonal crosstalk and transcription factors (Bellini et al., 2014).

60

61 Auxin is considered as the master player and hub of signaling network during AR
 62 formation (Lakehal and Bellini, 2019). Indole-3-acetic acid (IAA) and its synthetic
 63 analogs are widely applied in AR induction for vegetative propagation (Preece, 2003).
 64 Auxin-mediated regulatory framework of wound-induced AR formation has been
 65 established in *Arabidopsis* involving auxin local synthesis, polar transport and signal
 66 transduction (Bellini et al., 2014). YUCCA1/4-mediated auxin biosynthesis promotes
 67 the fate transition of rooting competent cells in leaf explants of *Arabidopsis* (Chen et al.,
 68 2016). A wound-triggered signaling cascade mediated by jasmonate (JA) signaling was
 69 recently identified to stimulate auxin biogenesis for adventitious rooting (Zhang et al.,
 70 2019). JA-induced ETHYLENE RESPONSE FACTOR 109 directly activates the
 71 transcription of *ANTHRANILATE SYNTHASE α1* encoding a tryptophan biosynthetic
 72 enzyme for auxin production (Zhang et al., 2019). Increased auxin polar transport
 73 labeled by radioactive IAA was detected around the position of excision where ARs
 74 develop, in line with AR deficient phenotypes resulting from impaired polar auxin
 75 transport (Sukumar et al., 2013). Wound-induced auxin local maximum triggers the
 76 expression of *WUSCHEL RELATED HOMEODOMAIN 11* and *12* via the auxin
 77 responsive elements located within their promoters (Liu et al., 2014c). WOX11 and 12
 78 act redundantly in specification of root founder cells, the first-step cell fate transition

79 for AR emergence (Liu et al., 2014c). Auxin-directed activation of *WOX5/7* is required
80 for cell patterning to establish AR primordia, the second step of cell fate transition
81 during adventitious rooting (Hu and Xu, 2016).

82

83 In addition to *Arabidopsis*, the central role of auxin in AR formation was also well
84 recognized in cereals (Orman-Ligeza et al., 2013; Bellini et al., 2014). *OsWOX11*, a
85 key transcription factor (TF) of shoot-borne root emergence in rice, acts under the
86 control of YUCCA-dependent auxin biosynthesis (Zhao et al., 2009; Zhang et al.,
87 2018). *OsGNOM1* encoding a guanine-nucleotide exchange TF in rice regulates AR
88 formation by modulating polar auxin transport (Liu et al., 2009). Gain-of-function of
89 *OsIAA23*, a repressor of auxin signaling, blocks AR initiation (Ni et al., 2011). Some
90 LOB and AP2 TFs were also identified as the key components in response to auxin
91 during AR formation in rice (Inukai et al., 2005; Kitomi et al., 2011).

92

93 In woody species, *Populus FBL1* encoding a homologous auxin receptor of TIR was
94 shown to regulate AR formation by interacting with Aux/IAA28 (Shu et al., 2019).
95 Similar to *Arabidopsis* and rice, *WOX5* and -11 also play crucial roles in the
96 adventitious rooting process of *Populus* (Xu et al., 2015; Li et al., 2018). Moreover, the
97 poplar AINTEGUMENTA LIKE1 (AIL1) and Response Regulator 13 (RR13) TFs

98 were identified to mediate transcriptional programs during AR formation in *Populus*
 99 (Ramirez-Carvajal et al., 2009; Rigal et al., 2012). However, molecular understanding
 100 of AR morphogenesis in trees is still limited (Legué et al., 2014), despite importance
 101 for vegetative propagation, a currently most common practice for tree breeding and
 102 commercialization.

103

104 Regulation of plant growth and development, such as AR formation, often involves
 105 components other than hormones and TFs, such as microRNAs (miRNAs), for a
 106 collaborative and more specific mode of regulation. MiRNAs are a class of endogenous
 107 small RNAs that regulate an array of plant developmental programs, including
 108 adventitious rooting (Chen, 2009; D'Ario et al., 2017). Aberrant functions of some key
 109 components in miRNA biogenesis pathway, such as ARGONAUTE 1 in *Arabidopsis*
 110 and CROWN ROOT DEFECT 1 in rice, give rise to defective AR formation (Sorin et
 111 al., 2005; Zhu et al., 2019). AR-associated miRNAs and their targets are known to be
 112 part of auxin-directed molecular framework for adventitious rooting (Bellini et al.,
 113 2014). In *Arabidopsis*, *AUXIN RESPONSE FACTOR (ARF) 6/8* and *17*
 114 antagonistically orchestrating AR formation are negatively regulated by *miR167* and
 115 *miR160*, respectively, by targeting their mRNAs for turnover (Gutierrez et al., 2009).
 116 Interactions of *miR167* and *miR160* with key TFs of the auxin signaling pathway are

117 required for fine-tuning AR developmental plasticity (Gutierrez et al., 2009). Similarly,
 118 *miR160-ARF17* was characterized to regulate AR formation in a *Populus* hybrid, *P.*
 119 *deltoides* × *P. euramericana* (Liu et al., 2019). *MiR393* regulates AR formation in rice
 120 through repressing the expression of *TIR1/AFB2* auxin receptors (Bian et al., 2012). A
 121 *miR156*-mediated *SPL3-MADS50* module was recently identified to orchestrate AR
 122 formation in rice through affecting auxin signaling (Shao et al., 2019).

123
 124 AR formation-associated miRNAs identified to date belong to the conserved miRNA
 125 families (Axtell and Bartel, 2005). There is a large number of nonconserved miRNAs
 126 in *P. trichocarpa*, of which functions are yet unknown (Lu et al., 2005). Of all the
 127 nonconserved miRNAs identified in *P. trichocarpa*, *miR476a* was found to have 20
 128 predicted target genes, all encoding pentatricopeptide repeat (PPR) proteins (Lu et al.,
 129 2005). PPRs are typically targeted to mitochondria or chloroplasts and bind and
 130 influence organellar transcript expressions, leading to important effects on organelle
 131 biogenesis and ultimately plant development (Schmitz-Linneweber and Small, 2008;
 132 Barkan and Small, 2014).

133
 134 Mitochondria are the energy producer in eukaryotic cells through coupling electron
 135 transport chain with oxidative phosphorylation for ATP generation (Millar et al., 2011).

136 Apart from energy production and biosynthetic function, mitochondria are increasingly
 137 defined as a hub integrating signaling cascades for plant development and stress
 138 responses (Liberatore et al., 2016). The best example of mitochondrial signaling is
 139 cytoplasmic male sterility (CMS), a pathway leading to microsporogenesis defects and
 140 pollen abortion in many plant species (Carlsson et al., 2008). A more recent study
 141 revealed that fission, a dynamic behavior of mitochondria, modulates mitochondrial
 142 status in tapetal cells and thereby contributes to pollen viability, providing a direct link
 143 between mitochondrial homeostasis and pollen development (Chen et al., 2019).
 144 Mitochondrial dynamic changes affecting seed germination were also well documented
 145 (Law et al., 2012; Paszkiewicz et al., 2017). What specific roles mitochondrial
 146 homeostasis plays during root formation is unknown. The association of miRNAs with
 147 organellar PPRs allows us to begin to understand such roles, leading to new insights
 148 into a combined regulation of miRNAs, TFs of auxin signaling and mitochondrial
 149 homeostasis for AR formation and development.

150

151 Here, we identified that *miR476a*, a member of the *miR476* family, functions as a novel
 152 regulator of wound-induced AR formation via orchestrating mitochondrial homeostasis
 153 in *P. tomentosa*. *MiR476a* directly targets several mitochondrion-localized PPRs that
 154 belong to *Restorer of Fertility like (RFL)* genes. Genetic modification of *miR476a-RFL*

155 expression and chemical perturbation of mitochondrial function revealed that the
 156 *miR476/RFL*-mediated dynamic regulation of mitochondrial homeostasis influenced
 157 AR formation via modulating the auxin pathway. Our results established a
 158 miRNA-directed mitochondrion-auxin cascade required for AR development in plants,
 159 especially in woody species.

160

161

162 **Results**

163 ***MiR476a* positively regulates wound-induced AR formation in *Populus tomentosa***

164 The *miR476* family in the annotated *Populus trichocarpa* genome comprises two
 165 members that display high identity in precursor and mature sequences (*PtmiR476a*
 166 and *-b*; Figure S1A). A pair of *miR476* homologs was also identified in *P. tomentosa*,
 167 and designated as *PtomiR476a* and *-b*. *PtomiR476s* exhibit high similarity in sequence
 168 and secondary structure to their *P. trichocarpa* counterparts (Figures S1A and S1B).
 169 We generated *PtomiR476a*-overexpressing poplar transgenic plants, and selected two
 170 lines (*miR476a*-OE-L1 and -L4) that had the highest transgene expression levels for
 171 further analyses (Figure S2A). Not only shoots but also a large number of roots were
 172 regenerated from the transgenic calli on shoot induction medium (SIM) (Figure S2B),
 173 suggesting that *miR476a* may enhance rooting capability, possibly even in the absence
 174 of rooting hormones. We then tested AR formation, the *de novo* root organogenesis,
 175 from wound-induced or cuttings of transgenic and wild type (WT) shoots and leaves in
 176 cultures without any hormones (Figures 1A to 1D). Shoot stem-derived ARs started to
 177 emerge 5 to 6 days after cutting (DAC) in both transgenic lines, but the emergence was
 178 delayed by ~2 days in WT (Figures 1A and 1C). The analysis of the cross-sections of
 179 stem bases undergoing AR formation revealed that AR primordia (ARPs) were already
 180 initiated at 3 DAC from cambial zone of *miR476a*-OE plants while visible until 5 DAC

Figure 1

Figure 1. *MiR476a* positively regulates adventitious rooting capacity in poplar. A and B, phenotypes of ARs emerging from stem (A) or leaf (B) explants in wild-type (WT) and independent *miR476a*-OE poplar lines (L1 and 4). DAC: days after cutting. Bars = 1 cm (A) and 500 μ m (B). C, quantification of the time for emergence of the first AR from stem and leaf explants. D, number of ARs derived from stem and leaf explants at 12 DAC. E, cross-sectioning and staining with toluidine blue of stem base at 3 and 5 DAC during AR formation in WT and the *miR476a*-OE line (L4). ARP: adventitious root primordium. Bars = 200 μ m. F, phenotypes of AR formation in WT and independent *pER8: miR476a*-STTM transgenic lines (L10 and L18) under mock and β -estradiol treatment at 12 DAC. Bar = 1 cm. G, quantification of AR number at 12 DAC corresponding to F. Error bars represent SD. For C and D, asterisks indicate statistically significant differences in Student's *t* test between WT and each transgenic line (*, $P < 0.05$; **, $P < 0.01$; $n = 4$). For F, asterisks indicate statistically significant differences in Student's *t* test between mock and β -estradiol treatment for each line (*, $P < 0.05$; $n = 3$).

181 in WT (Figure 1E). The *miR476a*-OE shoots developed 3 to 4 times more stem-derived

182 ARs than did WT (Figures 1A and 1D), consistent with the initiation of more ARPs in

183 *miR476a*-OE than in WT (Figure 1E). Similarly, faster and more ARs were derived

184 from *miR476a*-OE leaves than from WT leaves (Figures 1B to 1D).

185

186 We also observed ectopic development of ARs in upper internodes of *miR476a*-OE
187 plants cultivated either *in vitro* or in soil (Figure S2C), which do not occur in WT. The
188 *miR476a*-OE shoot cuttings cultivated hydroponically also developed more ARs and
189 from a more extended area than WT (Figure S2D). In potted soil, *miR476a*-OE plants
190 developed a denser root system with enhanced lateral root branching but impaired root
191 elongation compared with WT (Figures S2E, S2G and S2H). The total root biomass in
192 *miR476a*-OE plants was significantly higher than that in WT (Figure S2F). Therefore,
193 *miR476a* plays an important role in AR formation and some other key aspects of root
194 organogenesis.

195

196 To further validate the *miR476a*-mediated regulation on AR formation, Short Tandem
197 Target Mimic (STTM)-based knockdown (Bian et al., 2012) of *PtomiR476a* driven by
198 the *CaMV 35S* promoter was performed (Figure S3A). However, the transgenic calli
199 were difficult to induce roots on root induction medium (RIM) for generation of viable
200 transgenic plants (Figure S3B), suggesting that constitutive knockdown of *miR476a* is
201 lethal to poplar. Hence, we generated the *pER8:miR476a*-STTM transgenic plants that
202 would confer the knockdown of *PtomiR476a* driven by a β -estradiol inducible
203 promoter (Zuo et al., 2000) (Figures S3A and S3C). We selected two lines of

204 *miR476a*-STTM transgenics (L10 and L18) with the strongest knockdown for testing
205 *de novo* root organogenesis. Both lines displayed significantly retarded AR emergence
206 when exposed to β -estradiol (Figure 1F). Compared with the mock, the transgenic L10
207 failed to form any ARs at 12 DAC, and L18 was 80% less efficient in generating ARs
208 under the inducible knockdown of *miR476a* (Figure 1G). Both overexpression and
209 knockdown experiments reveal that *miR476a* is required for AR formation in poplar.

210

211 Dynamically inducible expression of *miR476a* during AR formation

212 To explain the *miR476a*-dependent AR phenotypes, tissue-specific and AR-associated
213 expression patterns of *miR476a* were assayed in *P. tomentosa* (Figure 2). Quantitative
214 PCR (qPCR) showed that *miR476a* was more abundantly expressed in roots, stems and
215 petiole relative to leaves (Figure 2A). Similar expression patterns were detected by
216 histologically staining with the GUS reporter driven by the *PtomiR476a* promoter
217 (Figure 2B). In stems, *PtomiR476a* was exclusively expressed in cambial cells (Figure
218 2B), where ARs usually initiate in poplar (Rigal et al., 2012). High expression of
219 *PtomiR476a* was also detected in both root tips (Figure 2Biv) and stele tissues (Figure
220 2Bv) of ARs. We next performed time-course analysis of the histological GUS activity
221 during wound-induced AR formation (Figures 2C to 2E). The *PtomiR476a* expression
222 remained low within 1 DAC at the base of stem cuttings, but drastically increased

Figure 2

Figure 2. Expression patterns of *miR476a* in poplar tissues and during AR formation. A, tissue-specific expression of *miR476a* assayed by qRT-PCR in poplar. L: leaf blade; S: stem; R: root; P: petiole. B, histological staining of tissues of the transgenic poplar line harboring GUS reporter gene driven by the promoter of *miR476a*. i: leaf blade and petiole; ii: cross-sections of stem base; iii: adventitious roots; iv: adventitious root tip; v: upper part of adventitious roots close to stem. Bars = 5 mm (i and iii), 500 μ m (ii), and 2 mm (iv and v). C, histological staining of stem bases close to cutting position of the GUS reporter line driven by the promoter of *miR476a* during AR formation. Bar = 5 mm. D, time-course quantitative assays of the GUS activity driven by the promoter of *miR476a* at stem bases during AR formation corresponding to C. E, histological staining of cross-sections of stem bases close to cutting position of the GUS reporter line driven by the *miR476a* promoter during AR formation. Bars = 500 μ m. Error bars represent SD. For D, asterisks indicate statistically significant differences with respect to 0 DAC (Student's *t* test; **, $P < 0.01$; $n = 3$).

223 reaching a maximum at 6 DAC where the activity was ~6 times of that at 1 DAC

224 (Figures 2C and 2D). At 8 DAC, the activity decreased (by 62% compared to the

225 maximum activity) along with the AR emergence (Figures 2C and 2D). GUS staining

226 of cross-sections of stem cuttings validated the progressively increased expression of

227 *miR476a* during AR formation (Figure 2E). The quantified promoter activity showed
228 that *miR476a* appeared to be more abundantly accumulated at the basal area of
229 emerged ARPs (Figure 2E). The inducible expression of *miR476a* during AR
230 formation is consistent with the positive role for *miR476a* in regulating adventitious
231 rooting capacity.

232 **A group of RFL-PPR genes are targeted by *miR476a* during AR formation**

233 Several *pentatricopeptide repeat* (PPR) genes were previously shown to be the
234 potential targets of *miR476s* in *P. trichocarpa* (Lu et al., 2005). Based on the updated *P.*
235 *trichocarpa* genome sequences (V3.0), we identified a total of 36 PPRs encoding
236 Restorer of Fertility Like (RFL) proteins (Fujii et al., 2011) and designated according to
237 their chromosomal locations (Figure S4). Using a similar prediction algorithm (Lu et
238 al., 2005), 11 putative *miR476a* targets with high reliability (score ≤ 1.5) were
239 identified from the 36 RFLs (Figures S4 and S5A). All 11 predicted *miR476a*-targeting
240 RFL genes belong to the same phylogenetic subclade (Figure S4) and their counterparts
241 were cloned from *P. tomentosa*. There are 3 or 4 unpaired bases between *PtomiR476a*
242 and the predicted binding sites harbored by these *PtoRFLs* except for *PtoRFL27*
243 (Figure S5B).

244

245 We next constructed degradome libraries from the WT and *miR476a*-OE lines for

Figure 3

Figure 3. A group of *RFL* genes mediate *miR476a*-dependent AR formation in poplar. **A**, expression levels of *RFL1*, 16, 17, 18, 26, and 34 in WT and *miR476a*-OE lines determined by qRT-PCR. Stem bases of seedlings cultivated *in vitro* were collected for RNA extraction. *Ubiquitin* was used as a reference gene. The values of WT were normalized to 1. **B**, time-course expression assays of *miR476a*-targeted *RFL* genes during AR formation by qRT-PCR. For each gene, expression levels are normalized to that of 0 DAC and transformed to log₂ values for heatmap analyses (leaf panel). Hierarchical clustering revealed three time-course expression patterns of these *RFL* genes during AR formation, represented by those of *RFL26* (purple), -34 (orange), and -18 (brown) in the right panel. Fc: fold of change. **C**, expression of *miR476a*-targeted *RFL* genes in *pER8: miR476a*-STTM line under mock and β-estradiol treatment. **D**, experimental validation of the *miR476a*-targeted cleavage of *RFL26*. The plasmid of *miR476a*-OE was transiently transformed into the protoplasts isolated from roots of transgenic *Arabidopsis* harboring ectopically expressed *RFL26*-GFP and *RFL26m*-GFP. *RFL26m* represents the mutated *RFL26* gene harboring the impaired *miR476a*-binding site as indicated in Figure S10A. Red fluorescence was emitted from mitochondrial dye mito-tracker Red (Mito-red). The fluorescence was detected via confocal microscope (Olympus) and quantified by FV10-ASW software (Olympus). Bar = 10 μm. **E** and **F**, phenotypes of ARs derived from stem (**E**) and leaf explants (**F**) of overexpressing *RFL26m* in the *miR476a*-OE line at 12 DAC. **G**, quantification of AR number of overexpressing *RFL26m* in the *miR476a*-OE line at 12 DAC. Error bars represent SD. For **A** and **G**, asterisks indicate statistically significant differences in Student's *t* test between WT and each transgenic line (*, *P* < 0.05; **, *P* < 0.01; n = 4). For **C**, asterisks indicate statistically significant differences with respect to mock for each time point (Student's *t* test; *, *P* < 0.05; **, *P* < 0.01; n = 5). For **D**, asterisks indicate statistically significant differences with respect to *RFL26* and *RFL26m* transformants, respectively (Student's *t* test; *, *P* < 0.05; n = 5).

246 single-ended Illumina sequencing to provide evidence for the *miR476a*-targeted *RFL*

247 cleavage (Figure S6A; Dataset S1). The degraded fragments, the cleavage of which

248 occurred within the predicted binding sites of *miR476a*, were detected for 6 of the 11

249 predicted targets, i.e. *RFL1*, -16, -17, -18, -26, and -34, in both libraries (Figure S6A).

250 These *RFL* transcripts contained 3 bases unpaired with *miR476a* within their predicted

251 binding sites (Figure S5B). Due to the identical sequences of the predicted *miR476a*

252 binding sites and flanking regions in *RFL1*, -16, -18, and -34, the fragments mapped to

253 these *RFLs* could not be differentiated (Figure S6A). The cleaved fragments derived

254 from these 6 *RFLs* accumulated in much higher frequencies in *miR476a*-OE plants than

255 in WT (Figure S6A). Consistently, all of the 6 *RFLs* displayed significantly attenuated

256 expression levels in *miR476a*-OE lines (Figure 3A). Other than these 6 *RFLs*, no

257 accumulation of the cleaved fragments was detected for the other 5 predicted targets

258 (*RFL19*, -27, -30, -32, and -33; Figure S6A). Moreover, the expression of these 5 *RFLs*

259 was not modulated in *miR476a*-OE lines (Figure S6B). These results provide strong

260 evidence that the 6 *PtoRFLs*, i.e. *PtoRFL1*, -16, -17, -18, -26, and -34, are most likely

261 the authentic targets of *Ptomir476a*.

262

263 Given the inducible expression of *miR476a* during AR formation, transcript profiles of

264 the *miR476a*-targeted *RFLs* were also determined during this process (Figure 3B).

265 Clustering of the time-course assay datasets revealed various expression patterns of the

266 *miR476a*-targeted *RFLs* during AR formation (Figure 3B). The first pattern shared by

267 *RFL1*, -16, -17, and -26 underwent dramatic increase of expression within 0.5 DAC

268 and then rapid decrease during the following 1 - 5 DAC. *RFL34* exhibited the highest
 269 expression at 1 DAC. In contrast, the expression of *RFL18* was continuously decreased
 270 throughout this process. The decreased expression of the *miR476a*-targeted *RFLs*
 271 between 1 and 6 DAC coincided with the increased accumulation of *miR476a* (Figures
 272 2C-E and 3C).

273

274 Since wounding is considered as a necessary stimulus for the initiation of ARs⁴⁸. We
 275 speculated that both wounding signals and *miR476a* might regulate the stage-specific
 276 expression patterns of these *RFLs*. To test this idea, a time-course assay was performed
 277 in wounded leaves that were not detached for adventitious rooting. The results revealed
 278 that the expression of *RFL1*, -16, -17, -26, and -34 was induced under wounding while
 279 that of *RFL18* was repressed (Figure S7A). The wounding responsiveness of these *RFL*
 280 genes mimicked the increase of their expression levels within 0.5-1 DAC during AR
 281 formation (Figures S7A and 3C). The qPCR assays in *pER8:miR476a*-STTM
 282 transgenic plants showed that the expression decrease of these *RFL* genes was
 283 significantly compromised when the knockdown of *miR476a* was triggered (Figure 3C).
 284 Therefore, the repressed expression of these *RFL* genes after 1 DAC largely depended
 285 on the *miR476a* expression during AR formation.

286 ***RFL26* mediates *miR476a*-dependent AR formation in *P. tomentosa***

287 Pfam analyses⁴⁹ showed that all the 6 validated *PtomiR476a* targets harbore 6 or 7
 288 canonical PPR motifs (Figure S8), demonstrating that these RFL proteins belong to the
 289 P-type PPR subfamily⁵⁰. Moreover, these RFL proteins displayed high probability of
 290 mitochondrial localization (Figure S8). Close phylogenetic relationship, conserved
 291 protein domains and common *miR476a*-directed transcript cleavage implied similar
 292 biological roles of these *RFL* genes. Therefore, we decided to focus on one target for a
 293 more thorough investigation. Since among the 6 validated targets, *RFL26* exhibited the
 294 highest frequency of cleavage by *miR476a* and the strongest transcript repression in
 295 *miR476a*-OE lines (Figures S6A and 3A), we selected it to further explore the
 296 *miR476a*-mediated genetic and molecular context of AR formation.

297
 298 RNA *in situ* hybridization showed that the *RFL26* transcripts were preferentially
 299 accumulated in cambial zone at 1 DAC while in ARP tissues at 6 DAC (Figure S9).
 300 The *RFL26-GFP* fusion was stably introduced into *Arabidopsis*, and the GFP signals in
 301 the root cells were overlapped with those derived from the mitochondrion-specific
 302 fluorescent dye (Figure S10A), validating the mitochondrial localization of RFL26.
 303 The root protoplasts harboring RFL26-GFP were isolated and transiently transfected
 304 with *miR476a* (Figure 3D). Fluorescence detection revealed the significantly reduced
 305 abundance of RFL26 protein when *miR476a* was introduced into cells (Figure 3D). In

306 contrast, RFL26 protein accumulation was not declined when the *miR476a*-binding site
 307 within the *RFL26* transcript was disrupted by mutagenesis (Figures 3D and S10B). As
 308 a control, RFL30, encoded by a *RFL* gene that could not be targeted by *miR476a*
 309 (Figure S6), did not exhibit the *miR476a*-inhibited accumulation (Figure S10C). These
 310 results suggested the *Ptomir476a*-directed post-transcriptional regulation of *PtoRFL26*
 311 encoding a mitochondrion-localized typical RFL-PPR protein.

312

313 To establish the genetic context of *miR476a*-RFL during AR formation, *RFL26m*, a
 314 *miR476a*-resistant *RFL26*, was introduced into the WT and *miR476a*-OE backgrounds
 315 (Figure S11A). Phenotypic analyses showed that *RFL26m* led to significantly delayed
 316 emergence and compromised number of ARs derived from stems and leaf explants,
 317 and even weaker rooting capacity than WT (Figures 3E to 3G and S11B). Functional
 318 complementation revealed the negative regulation of *miR476a*-targeted *RFL26* on AR
 319 formation.

320 **The *miR476a*-RFL module modulates functional homeostasis of mitochondria**

321 *RFL* genes identified in rice, rape and *Petunia* encode the mitochondrion-localized
 322 PPR proteins that suppress the expression of mitochondria-encoded genes responsible
 323 for cytoplasmic male sterility (Bentolila et al., 2002; Kazama et al., 2008; Uyttewaal et
 324 al., 2008). A number of mitochondrial-encoded genes displayed differential expression

Figure 4

Figure 4. Mitochondrial homeostasis and dynamics modulated by *miR476a/RFL*.

A and B, Quantitative assays of ATP (A) and NADH/NAD⁺ (B) in WT, *miR476a*-OE, and *RFL26m*-OE lines. Stem bases at 3 DAC were collected for measurements. C and D, Time-course quantification of ATP production (C) and NADH/NAD⁺ ratio (D) during AR formation in poplar. E, DAB staining of 3-DAC stem bases of WT, *miR476a*-OE, and *RFL26m*-OE lines. Scale bars = 0.5 cm. F, quantification of H₂O₂ content of 3-DAC stem bases of WT, *miR476a*-OE, and *RFL26m*-OE lines. Error bars represent SD. For A, B, and F, the values of WT are normalized to 1, and asterisks indicate statistically significant differences with respect to WT (Student's *t* test; *, *P* < 0.05; **, *P* < 0.01; *n* = 3).

325 levels in *miR476a*-OE and *RFL26m*-OE lines compared with WT (Figure S12),
 326 implying the functional modulation of mitochondria depending on the *miR476a*-RFL
 327 module. ATP content and NADH/NAD⁺ ratio were significantly increased in the
 328 *miR476a*-OE lines compared with WT, while decreased in *RFL26m*-OE lines (Figures

4A and 4B). To further uncover mitochondrial homeostasis mediated by *miR476a/RFL*, time-course measurement of ATP content was conducted during AR formation (Figures 4C and 4D). A reduction of ATP content within 0.5 or 1 DAC followed by a dramatic increase along with ARP emergence at 6 DAC was detected (Figure 4C). However, the reduction of ATP level was absent at the early stage of AR formation due to the overexpression of *miR476a*, and higher ATP content was detected during the whole period of AR formation in the *miR476a*-OE plants than WT (Figure 4C). In contrast, a decrease in ATP accumulation was present during AR formation in the *RFL26m*-OE plants (Figure 4C). Similar changes were detected for NADH/NAD⁺ ratio among *miR476a-RFL26m* transgenic lines (Figure 4D). In addition, DAB staining and quantitative measurement revealed that H₂O₂, a molecule of reactive oxygen species (ROS), displayed preferential accumulation in *RFL26m*-OE lines, while compromised in *miR476a*-OE plants (Figures 4E and 4F). Taken together, these results revealed the antagonistically modulated mitochondrial functions by *miR476a* and its target *RFL26* in *P. tomentosa*.

AR formation requires *miR476a/RFL*-mediated mitochondrial regulation

Given the dynamic expression and coordinated regulation on mitochondria during AR formation, we questioned whether *miR476a*-driven mitochondrial modulation is required for adventitious rooting capacity. To test this hypothesis, exogenous

Figure 5

Figure 5. *MiR476a*-mediated AR formation depends on mitochondrial homeostasis. A and B, rotenone-inhibited ATP production (A) and NADH/NAD⁺ (B) during AR formation in WT and *miR476a*-OE lines. Rotenone (50 μ M) was added for treatment at 1 DAC. C, AR phenotypes of WT and *miR476a*-OE lines under rotenone treatment at 8 and 12 DAC. Bars = 2 cm. D and E, quantification of the time for emergence of the first AR (D) and AR number (E) under rotenone treatment at 12 DAC in WT and *miR476a*-OE lines. Error bars represent SD. For D and E, asterisks indicate statistically significant differences with respect to mock (Student's *t* test; **, *P* < 0.01; n = 5).

348 application of rotenone, a chemical inhibitor of activity of mitochondrial respiratory
 349 chain complex (MRCC) I, was performed to determine the effect of mitochondrial
 350 respiration on AR development. ATP content was strongly inhibited and maintained at

low levels in both WT and *miR476a*-OE lines after exposure to rotenone at 1 DAC during AR formation (Figure 5A). A similar reduction was detected for NADH/NAD⁺ in response to rotenone treatment (Figure 5B). AR development was suppressed by rotenone from shoot explants of both WT and *miR476a*-OE lines (Figure 5C). Accordingly, 2-3 days were delayed for AR emergence from WT and *miR476a*-OE explants under rotenone treatment (Figure 5D). The decreases of AR number were also detected for both genotypes (73.8% for WT and 77.3% for *miR476a*-OE; Figure 5E). These results indicated that functional homeostasis of mitochondria compromised by exogenous chemical inhibitor retarded AR formation, mimicking the *RFL26*-resulting AR defects. Therefore, mitochondrial regulation is required for adventitious rooting capacity in poplar.

To examine whether mitochondrial regulation on adventitious rooting is conserved across woody and herbaceous plants, ATP quantification and mitochondrial perturbation were conducted using *Arabidopsis* leaf explants for AR formation. Similar to poplar, the *Arabidopsis* explants displayed 2-fold increase of ATP content at 4 DAC during AR formation (Figure S10A). The rotenone treatment resulted in dramatically retarded the emergence of ARs from leaf explants in *Arabidopsis* (Figures S10B and S10C). These results suggested conserved regulation of mitochondrial homeostasis on

370 adventitious rooting in herbaceous species.

371 ***MiR476a*-mediated AR formation depends on auxin transport and homeostasis**

372 To explore the *miR476a*-mediated molecular context of AR formation, comparative

373 transcriptomic profiling was conducted *via* RNAseq, showing 484 differential

374 expression genes (DGE) in *miR476a*-OE plants and WT (fold change ≥ 2 , false

375 discovery rate $\leq 1\%$; Figure S14A). Among them, 30 genes from different transcription

376 factor families were identified to be regulated by *miR476a* overexpression (Figure

377 S14B). These genes including *WUSCHEL-RELATED HOMEODOMAIN (WOX) 4/5/11*,

378 *AINTEGUMENTA LIKE (AIL) 1*, and D-type *Cyclin (CYCD) 3.1*, which are known to

379 positively regulate AR formation in poplar (Karlberg et al., 2011; Rigal et al., 2012;

380 Liu et al., 2014a), were preferentially expressed in *miR476a*-OE plants (Figure S14C).

381 In contrast, these genes were down-regulated in both β -estradiol-induced

382 *miR476a*-knockdown and *RFL26m*-OE lines (Figures S14D and S14E), indicating that

383 they function downstream of the *miR476a*/RFL module during AR formation in poplar.

384 The time-course assay of stem-derived AR formation revealed the increased transcript

385 accumulation of *WOX5a* until the emergence of ARPs at 6 DAC in WT (Figure S14F),

386 while its expression at high level in the *miR476a*-OE plants during the whole process

387 of AR formation but at low level in the *RFL26m*-OE plants (Figure S14F). In addition,

388 exogenous rotenone treatment could significantly suppress the expression of the *WOX*

389 genes, validating mitochondrial regulation on *WOX*-mediated AR formation (Figure
390 S14G).

391

392 A previous study in *P. tomentosa* showed that *PIN2* encodes a plasma
393 membrane-localized efflux carrier essential for auxin polar transport, while *PIN5s*
394 encode endoplasmic reticulum (ER) membrane-localized transporters affecting auxin
395 cellular homeostasis (Liu et al., 2014b). The expression of *PIN2* and *-5b* were
396 determined in *miR476a*- and *RFL26m*-OE lines (Figures 6A and 6B). Compared to WT,
397 both *PIN* genes were preferentially expressed in *miR476a*-OE plants while weakly in
398 *RFL26m*-OE plants (Figure 6A). Correspondingly, the expression levels of both *PINs*
399 were attenuated by the β -estradiol-induced *miR476a* knockdown (Figure 6B). The
400 modulated expression of *PIN2* and *-5b* raised the likelihood that auxin homeostasis and
401 polar transport function downstream of *miR476a*/RFL during AR formation.

402 Compromising auxin homeostasis via application of 1-N-naphthylphthalamic acid
403 (NPA), an inhibitor of auxin polar transport, was conducted to test this hypothesis. We
404 found that no ARs emerged at 12 DAC under NPA treatment for both WT and
405 *miR476a*-OE explants (Figure 6C). These results suggested that auxin polar transport is
406 required for the *miR476a*/RFL-directed mitochondrial regulation of AR formation.

407

Figure 6

Figure 6. *MiR476a*-mediated mitochondrial regulation on adventitious rooting requires auxin pathway.

A, expression levels of *PIN2* and *5b* in *miR476a*-OE and *RFL26m*-OE lines at 3-DAC stem bases assayed by qRT-PCR. B, expression levels of *PIN2* and *5b* in the *pER8:miR476a-STTM* line under mock and β -estradiol treatment. C, AR phenotypes of WT and *miR476a*-OE plants at 8 DAC under mock and 5 μ M 1-N-naphthylphthalamic acid (NPA) treatment. D, expression levels of *PIN2* and *5b* under 50 μ M rotenone treatment for 3 days during AR formation. E, quantitative assays of indole-3-acetic acid (IAA) in stem bases under 50 μ M rotenone treatment for 3 days during AR formation. F, fluorescent imaging of cross-sections of the *DR5-YFP* reporter line at 3 DAC under 50 μ M rotenone treatment. Red dashed circle indicates an area where YFP fluorescence is highly accumulated. PF: phloem fibers; Ca: cambium; Xy: xylem. Bars = 20 μ m. G, quantification of YFP fluorescence corresponding to H. The values of mock (i) are normalized to 1. Error bars represent SD. For A, asterisks indicate statistically significant differences with respect to WT (Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; $n = 5$). For B, D, E, and G, asterisks indicate statistically significant differences with respect to mock (Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; $n = 3$).

408 In consistence with the *miR476a*/RFL26-mediated regulation on *PIN2/5b*, the

409 expression of both *PINs* was suppressed by exogenous treatment of rotenone during

410 AR formation (Figure 6D). This implied probable mitochondrial modulation of auxin

411 transport and homeostasis, and IAA content was thereby examined in shoot explants
 412 for rooting under rotenone treatment (Figure 6E). Auxin content at stem bases of the
 413 explants on 3 DAC was reduced by 15.3% under rotenone treatment (Figure 6E),
 414 indicating negative effects of mitochondrial perturbation on auxin accumulation for
 415 rooting. Finally, the *DR5:YFP* reporter, a biosensor of in vivo auxin signaling, was
 416 introduced into poplar to investigate mitochondrial regulation on auxin pathway during
 417 AR formation (Figure 6F). Fluorescence detection of stem bases of shoot explants for
 418 AR formation revealed a preferential accumulation of auxin signaling at some position
 419 of cambial and neighboring zone at 3 DAC (Figure 6F). In contrast, the uneven
 420 distribution of auxin signaling at cambium disappeared in response to rotenone
 421 treatment (Figure 6F), and the fluorescence quantification showed the significantly
 422 rotenone-suppressed auxin signaling in comparison with the mock treatment (Figure
 423 6G). Therefore, functional perturbation of mitochondria restricts auxin pathway during
 424 AR formation.

425

426

427 **Discussion**

428 **MiRNA-directed dynamic posttranscriptional control of *RFL* genes synchronizes** 429 **mitochondrial status with adventitious rooting**

430 Mitochondria are the major organelle for primary metabolism and energy production in
431 plant non-photosynthetic organs (Millar et al., 2011). A variety of developmental
432 defects were identified in some mutants of genes encoding mitochondrion-localized
433 proteins (Millar et al., 2011). The emerging role of mitochondrial homeostasis and
434 dynamics has been characterized in a few developmental programs of *Arabidopsis*
435 including seed germination, leaf senescence, and pollen development (Chrobok et al.,
436 2016; Paszkiewicz et al., 2017; Chen et al., 2019). The loss-of-function of the
437 mitochondrial pyruvate dehydrogenase E1 subunits led to root morphogenesis defects
438 (Ohbayashi et al., 2019), suggesting the regulation of metabolic adjustment in
439 mitochondria on root development. However, molecular connection and action mode
440 enabling coordination of *de novo* root organogenesis and mitochondrial homeostasis
441 has not been established yet. Here we identified a miRNA-directed signaling pathway
442 that coordinates mitochondrial functional balance with adventitious rooting capacity in
443 *Populus*. *MiR476* was initially cloned in tension/compression-stressed woody tissues in
444 *P. trichocarpa* (Lu et al., 2005). We found the induced enrichment of *PtomiR476a* in
445 cambium and neighboring parenchyma cells where stem-derived ARs developed

(Figure 2). This dynamic expression pattern was consistent with the positive regulation of *miR476a* on adventitious rooting capacity addressed by genetic evidence (Figure 1). Noticeably, the induction of *miR476a* was absent at the early stage after wounding (before 1 DAC; Figure 2). Therefore, *miR476a* functions as a stage-specific regulator of AR formation.

Lu et al. (2005) showed *miR476a*-directed cleavage of a *PPR* gene via 5' RACE. We provided multiple lines of evidence that *miR476a* targeted a group of *RFL* genes for transcript turnover to allow suppression of their expression (Figures 3 and S6). In addition to genetic complementation (Figures 3D to 3F), spatiotemporal patterns of *miR476a* and its *RFL* targets addressed their antagonistic regulation on adventitious rooting (Figures 2 and 3). Functional status of mitochondria characterized by energy production was activated by *miR476a* whereas suppressed by *RFLs* (Figures 4A and 4B). We found a stage-specific modulation of mitochondrial homeostasis during wound-induced AR formation in poplar (Figures 4D and 4E). The wound-induced expression of *RFLs* accounts for the decreased mitochondrial functions within 1 DAC while the *miR476a*-attenuated accumulation of the *RFL* transcripts contributes to the increase of mitochondrial functions thereafter (Figure 4). *MiR476a/RFL*-mediated mitochondrial modulation allows us to divide the wound-induced AR formation into

465 two sequential phases, including wound response and root induction. Our results
466 revealed that mitochondrial functional homeostasis is dynamically coordinated with
467 wound-induced AR formation by the *miR476a*-directed posttranscriptional control of
468 *RFLs* encoding mitochondria-localized proteins in poplar.

469

470 **Mitochondrial regulation of energy homeostasis is crucial for adventitious rooting**

471 Due to the sessile lifestyle of plants, mitochondrial functionality for energy production
472 requires dynamic flexibility depending on variable cellular functions (Schwarzlander
473 and Finkemeier, 2013). For instance, mitochondria are immediately reactivated upon
474 imbibition to support germination program of seeds for organogenesis of a new
475 seedling (Paszkiwicz et al., 2017). Higher respiratory rate of mitochondria has been
476 detected for root meristem than the other root tissues (Kuroiwa et al., 1992; Fujie et al.,
477 1993). AR formation is a process of cell fate reprogramming for root organogenesis in
478 parallel with active cell proliferation and differentiation (Legué et al., 2014; Pizarro
479 and Díaz-Sala, 2018). A demand for high energy may exist to support these cellular
480 behaviors, hinted by dynamic carbohydrate allocation at the base of cuttings during AR
481 formation (Akhkami et al., 2009). Our results indicated that AR induction requires the
482 elevated energy supply of mitochondria by the *miR476a*-restricted *RFL* expression
483 (Figure 4). The genetic evidence was validated by pharmacological inhibition of

484 mitochondrial functionality, which mimicked the *RFL*-resulting perturbation of energy
485 production, and arrested *miR476a*-directed AR formation (Figure 5). The evidence of
486 genetically or chemically modulated mitochondrial functionality suggested the key role
487 of mitochondria-directed energy homeostasis in AR formation.

488

489 Similar to poplar, the elevated mitochondria-producing energy was detected for AR
490 formation in *Arabidopsis*, the inhibition of which also impaired adventitious rooting
491 capacity (Figure S13). These results revealed the conserved mitochondrial regulation
492 on AR formation across herbaceous and woody plant species. However, *miR476* was
493 considered as a *Populus*-specific miRNA family, due to its absence in the genome of
494 *Arabidopsis* (Lu et al., 2005). It is thereby hypothesized that, although the conserved
495 demand for mitochondria-generating energy, a variable action mode may function for
496 coordinating mitochondrial homeostasis with adventitious rooting in *Arabidopsis*.

497

498 **Mitochondrial actions on AR formation requires retrograde regulation on auxin**
499 **transport and homeostasis**

500 Nucleus-organelle communication require bi-directional signals called anterograde and
501 retrograde (de Souza et al., 2017). Retrograde signals confer feedback regulation from
502 organelles including mitochondria on transcriptional responses in nucleus(Wagner et

al., 2018). In addition to the miRNA-directed mitochondrial homeostasis, our results also showed that retrograde regulation on the auxin pathway delivers physiological alternations of mitochondria to direct AR formation (Figure 6). The *Arabidopsis mas* (*More Axillary Shoots*) mutant lacking the mitochondrial FTSH4 AAA-protease exhibits the compromised auxin accumulation or responsiveness, supporting a mechanistic link between mitochondrial functions and auxin (Zhang et al., 2014). Independently, using a chemical screening approach, two mitochondrion perturbing compounds sharing a common 3-(2-furyl) acrylate substructure were identified to inhibit auxin signaling (Kerchev et al., 2014). We found the modulated expression of *PIN2/5* encoding auxin transporters at the bases of cuttings in response to genetically or chemically orchestrated mitochondrial functionality (Figure 6). Due to exclusively inducible expression during AR formation, *PIN2/5* genes were considered to regulate polar transport and homeostasis of auxin for AR formation (Liu et al., 2014b). Importantly, exogenous treatment of auxin transport inhibitor and detection of auxin content and signaling under chemical perturbation of mitochondria validated the requirement of the retrograde regulation on auxin pathway (Figure 6).

Despite mitochondrial regulation on auxin homeostasis, the substances functioning as retrograde signals during AR formation are still unknown. ROS production in

mitochondria is considered as a candidate signal of mitochondrial energy signaling (Schwarzlander and Finkemeier, 2013; Wagner et al., 2018). In the case of the *fish4* mutant, H₂O₂, a species of ROS, was identified to mediate mitochondrial regulation on auxin catabolism (Zhang et al., 2014). We also found *miR476a*-suppressing but *RFL*-promoting accumulation of H₂O₂ along with energy modulation during AR formation (Figure 4C). However, more lines of evidence are still required to elucidate the role of ROS in mitochondrial retrograde regulation during adventitious rooting.

Collectively, we proposed a model of the *miR476a*/*RFL*-directed mitochondrial regulation on adventitious rooting capacity in *Populus* that is defined as two sequential and interconnected stages (Figure 7). Wound at the bases of cutting explants induces the expression of several *RFL* genes encoding mitochondria-localized PPR proteins, and thereby restricts mitochondrial energy production that is unfavorable to AR initiation. Subsequently, the enrichment of *miR476a* defines the secondary phase for rooting induction. *miR476a* directs the cleavage of the *RFL* transcripts for degradation, thereby suppressing the amount of the mitochondria-targeted RFL proteins. The *miR476a*-dependent suppression of RFLs causes the augmented mitochondrial status for energy supply indicated by increased ATP and NADH/NAD⁺ production whereas decreased ROS generation. The retrograde signals from mitochondria activate the

Figure 7

Figure 7. A proposed model for *miR476a*/RFL-dependent AR formation in *Populus*. The *miR476a*/RFLs-mediated mitochondrial functional modulation allows to divide the process of wound-induced AR formation into two sequential phases, including wounding response and root induction. At the first stage, wound signals at the bases of cutting explants induce the expression of several RFL genes encoding mitochondria-localized PPR proteins, and thereby restrict mitochondrial energy production that is unfavorable to AR initiation. At the second stage, the induction of *miR476a* results in the cleavage of the RFL transcripts for degradation, thus suppressing the amount of RFL proteins targeted into mitochondria. *MiR476a*-dependent suppression of RFLs causes the augmented mitochondrial status for energy supply indicated by increased ATP and NADH/NAD⁺ production whereas decreased ROS generation. The mitochondrial retrograde signals activate the expression of *PIN2/5b* auxin transporters, which modulated polar transport and homeostasis of auxin at rooting competent cells, and promote AR formation through elevating the expression of AR-associated key genes.

541 nuclear expression of *PIN2/5b* encoding auxin transporters. *PIN2/5b*-modulated polar

542 transport and homeostasis contribute to auxin maxima at rooting competent cells, and

543 thereby drive AR formation via promoting the expression of AR-associated key genes.

544 Our findings provide new insights into the mechanism by which a miRNA-directed

545 signaling pathway dynamically coordinates mitochondrial functionality with

546 adventitious rooting capacity in *Populus*.

547

548 **Materials and methods**

549 **Gene cloning and plasmid construction**

550 The precursors of *PtomiR476a* and *-b* were amplified from genomic DNA of *P.*

551 *tomentosa* using the primer pairs *Pre-PtomiR476a*-OE-*Bg/II*-fw/*SpeI*-rv and

552 *Pre-PtomiR476b*-fw/rv (Table S1), respectively, and ligated into the pMD19 vector via

553 TA-cloning for sequencing. The *miR476a* precursor was then subcloned into the

554 pCAMBIA1302 vector driven by the *CaMV 35S* promoter via *Bg/II*/*SpeI* restriction

555 sites. The STTM-based knockdown plasmid of *miR476a* was constructed as previously

556 described (Bian et al., 2012; He et al., 2018). An oligonucleotide fragment for STTM

557 containing the sequences of the *miR476a*-binding site with an insertion of AGA and an

558 88-bp spacer sequence was de novo synthesized (Figure S3A), and then inserted into

559 the pCXSN vector under the control of the *CaMV 35S* promoter via *Bam*HI restriction

560 sites (Chen et al., 2009). For inducible knockdown, the STTM fragment was amplified

561 using the primers *PtomiR476a*-STTM-*XhoI*-fw/rv (Table S3), and ligated into the

562 pER8 vector encoding a β -estradiol-inducible expression system (Zuo et al., 2000) via

563 *XhoI* restriction sites. A 2973-bp promoter sequence upstream of the *miR476a*

564 precursor was amplified from genomic DNA of *P. tomentosa* via the primer pair

565 *Pro-PtomiR476a*-fw/rv (Table S3), and constructed into *Xcm*I-digested pCXGUS-P via
 566 TA-cloning to drive the expression of GUS (Chen et al., 2009). The full-length coding
 567 sequences of *RFL1*, -16, -17, -18, -19, -26, -27, -30, -32, -33, and -34 were amplified
 568 from the *P. tomentosa* cDNA using primer pairs *PtoRFL*-CDS-fw/rv (Table S1),
 569 respectively, and constructed into pCXSN for sequencing via TA-cloning. For GFP
 570 tagging, the coding sequences of *PtoRFL26* and *PtoRFL30* without the stop codon
 571 were subcloned into pCAMBIA1302 with the hygromycin-resistant gene using the
 572 primer pairs CDS-*Nco*I-fw/noTAA-*Nco*I-rv for *PtoRFL26* and 30 (Table S1),
 573 respectively. For overexpression, the coding sequence of *PtoRFL26* was constructed
 574 into a modified pCAMBIA1302 with the kanamycin-resistant gene under the control of
 575 the *CaMV 35S* promoter. The *DR5-YFP* construct was modified with the replacement
 576 of *GFP* by *YFP* from the plasmid of *DR5-GFP* (He et al., 2018).

577 **Genetic transformation, root phenotyping and chemical treatment**

578 *P. tomentosa* was stably transformed using agrobacterium-mediated infiltration of leaf
 579 disks as described previously (Jia et al., 2010). PCR genotyping was conducted with
 580 the primers of hygromycin/kanamycin-resistant genes for screening positive transgenic
 581 plants. Microcutting-propagated transgenic seedlings were transplanted into soil in pots,
 582 and cultivated in a greenhouse at 20-25°C with the light of 10 000 lux under a 16 h: 8 h,
 583 day: night cycle. For stem-derived AR phenotyping, shoot segments of 3-4 cm with

584 two or three young leaves were cut from sterilized WT and transgenic seedlings and
 585 cultivated on sterile WPM (woody plant medium) without any phytohormones (Jia et
 586 al., 2010) at 25°C with 16 h of light of 5,000 lx and 8 h of dark. For phenotyping ARs
 587 derived from leaf explants, leaves similar in size were cut from 4-week-old sterilized
 588 seedlings and incubated on WPM for rooting at 25°C with 16 h of light of 5,000 lx and
 589 8 h of dark. For examining AR formation under hydroponic condition, the upper part of
 590 2-month-old WT and *miR476a*-OE plants with 5-6 leaves was cut off and transferred to
 591 Hoagland solution for rooting.

592 **Crosssectioning and GUS staining**

593 Stem bases from shoot explants were sectioned with a razor blade, and then stained
 594 with 0.05% (w/v) toluidine blue for 5 min. The cross-sections were observed and
 595 captured using a light microscope (Zeiss). For GUS staining, cross-sections were fixed
 596 in acetone for 1 h at 20°C, and then washed twice in double distilled H₂O. The
 597 cross-sections were soaked in GUS staining solution (0.5 M Tris, pH 7.0, 10% Triton
 598 X-100 with 1mM X-Gluc (5-bromo-4- chloro-3-indolyl-D-glucuronide)) for 15 min at
 599 37°C in the dark. After reaction, Chl was removed by use of 75% ethanol three times at
 600 65°C. The Chl-free stained stems were observed under an Olympus 566 SZX16
 601 microscope (Tokyo, Japan) and documented using an Olympus DP73 camera.

602 **RNA in situ hybridization**

For preparing probes, 223-bp gene-specific cDNA fragments were amplified for *PtoRFL26* using the primer pair *PtoRFL26*-in situ-fw/rv (Table S1). The probes were labeled using a DIG RNA Labeling Kit (Roche). Section pretreatment, hybridization and immunological detection were performed as described (Sang et al., 2012).

qRT-PCR

Total RNA was extracted from tissues of 2-month-old *P. tomentosa* plants using a Plant RNeasy Mini Kit (Qiagen). The cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Quantitative real time polymerase chain reaction (qRT-PCR) was performed using SYBR Premix ExTaq (Takara, Japan) in a TP800 Real-Time PCR machine (Takara, Japan). The poplar *Ubiquitin* gene was used as the reference gene as an internal standard. The primers used for qRT-PCR are listed in Table S1.

Degradome sequencing

Degradome libraries were constructed using RNA isolated from shoot cuttings of WT and *miR476a*-OE lines, respectively, for rooting at 3 DAC as previously described (Ma et al., 2010). Briefly, 200ng polyA-enriched RNAs were annealed with biotinylated random primers, and then those with monophosphate at 5' end ligated to 5' adaptors, followed by reverse transcription and PCR amplification. The libraries were single-end sequenced using an Illumina HiSeq2500 at Biomarker Technologies (Beijing, China)

622 following the manufacturer's recommended protocol. Approximately 20 M single-end
623 tags of 50 bp in length were produced for each library.

624

625 For data analyses, the tags with low quality and matched to other noncoding RNA such
626 as rRNA, tRNA, and small nuclear RNA were removed. Subsequently, remained tags
627 were aligned to *P. trichocarpa* v3.0 genome, allowing no more than one mismatch.
628 Targets were identified using CleaveLand (version 4.3). The tags that could be mapped
629 to PPR genes of *P. trichocarpa* were aligned to the 11 cloned *RFL* genes of *P.*
630 *tomentosa* including *PtoRFL1*, 16, 17, 18, 19, 26, 27, 30, 32, 33, and 34.

631 **Quantification of ATP content, NAHD/NAD⁺, and H₂O₂**

632 Shoot cuttings at 3 DAC during AR formation were sampled for quantifying ATP
633 content, NADH/NAD⁺ and H₂O₂. Quantification of ATP and H₂O₂ content was
634 conducted using ATP Assay Kit (S0026) and Hydrogen Peroxide Assay Kit (S0038)
635 both produced by Beyotime Biotechnology (Shanghai, China). NADH/NAD⁺ was
636 assayed using NAD(H) Assay Kit (Nanjing Jiancheng Bioengineering Institute,
637 Nanjing, China). The experiments were performed according to the manufacturer's
638 manuals.

639 **RNA sequencing**

640 Total RNA was isolated from 5 DAC shoot cuttings of WT and *miR476a*-OE plants

641 using RNeasy Plant Mini Kit (QIAGEN). Messenger RNA was purified and used for
642 Illumina sequencing according to the guide of the Illumina mRNA sequencing sample
643 preparation. Reads were mapped to *Populus trichocarpa* v3.0 genome using Bowtie2
644 and HISAT allowing up to two mismatches and only uniquely mapped reads were
645 retained. Expression level of loci was determined by fragment per kilobase per million
646 mapped reads (FPKM). Differentially expressed genes (DEGs) were defined as FPKM
647 change in WT and *miR476a*-OE plants of more than 2 folds and FDR < 0.01. The
648 DEGs were then conducted for functional classification using the website of agriGO
649 (<http://bioinfo.cau.edu.cn/agriG>).

650 **Gene accessions**

651 GeneBank accession numbers of the *P. tomentosa* genes are: *PtoRFL1* (MN242831),
652 *PtoRFL16* (MN242830), *PtoRFL17* (MN242832), *PtoRFL18* (MN242833), *PtoRFL19*
653 (MN242834), *PtoRFL26* (MN242835), *PtoRFL27* (MN242836), *PtoRFL30*
654 (MN242837), *PtoRFL32* (MN242838), *PtoRFL33* (MN242839), and *PtoRFL34*
655 (MN242840).

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663 **Author contributions**

664 K.L., C.X. and H.X. designed the work; C.X., Y.T., X.F., L., H.X., H.S., X.W., J.H.,
665 and D.F. performed experiments and data analyses; C.X. drafted the manuscript; K.L.
666 and V.C. revised the manuscript. All authors approved the final version of the
667 manuscript for publication. ^[1]_{SEP}

668 **Conflicts of interest**

669 The authors have no conflicts of interest to declare.

670 **Supplemental information**

671 Figure S1. Sequence alignment and secondary structure prediction of poplar *miR476s*.
672 Figure S2. Additional root phenotypes of *miR476a*-overexpressing transgenic lines.
673 Figure S3. Generation of STTM-based *miR476a* knockdown transgenic poplar lines.
674 Figure S4. Phylogenetic analysis of the *RFL-PPR* subfamily from *P. trichocarpa*.
675 Figure S5. Predicted binding sites of *miR476a* harbored in poplar *RFL* genes.
676 Figure S6. Degradome sequencing and expression of non-*miR476a*-targeted *RFLs*.
677 Figure S7. Expression of the *miR476a*-targeted *RFL* genes under wounding treatment.
678 Figure S8. Domain analysis and mitochondrial localization prediction of the PPR
679 proteins encoded by the *miR476a*-targeted *RFL* genes.
680 Figure S9. RNA in situ hybridization of *RFL26* at 1 and 6 DAC during AR formation

681 in poplar.

682 Figure S10. Validation of mitochondrial localization and *miR476a*-directed degradation

683 of RFL proteins.

684 Figure S11. Generation and AR phenotypes of *RFL26m*-overexpressing poplar lines.

685 Figure S12. Expression levels of mitochondrial-encoded genes in *miR476a*-OE (A) and

686 *RFL26m*-OE (B) lines.

687 Figure S13. ATP content assays and mitochondrial perturbation during AR formation

688 in Arabidopsis.

689 Figure S14. Comparative transcriptomic analysis between WT and *miR476a*-OE lines.

690 Dataset S1. Sequences of degraded fragments identified by degradome sequencing.

691 Dataset S2. Differentially expressed genes in the RNAseq-based transcriptomic

692 analysis of *miR476a*-OE vs WT.

693 Table S1. Sequences of oligonucleotide primers and probes used in this study.

Figure legends

Figure 1. *MiR476a* positively regulates adventitious rooting capacity in poplar.

A and B, phenotypes of ARs emerging from stem (A) or leaf (B) explants in wild-type (WT) and independent *miR476a*-OE poplar lines (L1 and 4). DAC: days after cutting. Bars = 1 cm (A) and 500 μ m (B). C, quantification of the time for emergence of the first AR from stem and leaf explants. D, number of ARs derived from stem and leaf explants at 12 DAC. E, cross-sectioning and staining with toluidine blue of stem base at 3 and 5 DAC during AR formation in WT and the *miR476a*-OE line (L4). ARP: adventitious root primordium. Bars = 200 μ m. F, phenotypes of AR formation in WT and independent *pER8: miR476a*-STTM transgenic lines (L10 and L18) under mock and β -estradiol treatment at 12 DAC. Bar = 1 cm. G, quantification of AR number at 12 DAC corresponding to F. Error bars represent SD. For C and D, asterisks indicate

statistically significant differences in Student's *t* test between WT and each transgenic line (*, $P < 0.05$; **, $P < 0.01$; $n = 4$). For F, asterisks indicate statistically significant differences in Student's *t* test between mock and β -estradiol treatment for each line (*, $P < 0.05$; $n = 3$).

Figure 2. Expression patterns of *miR476a* in poplar tissues and during AR formation.

A, tissue-specific expression of *miR476a* assayed by qRT-PCR in poplar. L: leaf blade; S: stem; R: root; P: petiole. B, histological staining of tissues of the transgenic poplar line harboring GUS reporter gene driven by the promoter of *miR476a*. i: leaf blade and petiole; ii: cross-sections of stem base; iii: adventitious roots; iv: adventitious root tip; v: upper part of adventitious roots close to stem. Bars = 5 mm (i and iii), 500 μ m (ii), and 2 mm (iv and v). C, histological staining of stem bases close to cutting position of the GUS reporter line driven by the promoter of *miR476a* during AR formation. Bar = 5 mm. D, time-course quantitative assays of the GUS activity driven by the promoter of *miR476a* at stem bases during AR formation corresponding to C. E, histological staining of cross-sections of stem bases close to cutting position of the GUS reporter line driven by the *miR476a* promoter during AR formation. Bars = 500 μ m. Error bars represent SD. For D, asterisks indicate statistically significant differences with respect to 0 DAC (Student's *t* test; **, $P < 0.01$; $n = 3$).

Figure 3. A group of *RFL* genes mediate *miR476a*-dependent AR formation in poplar.

A, expression levels of *RFL1*, 16, 17, 18, 26, and 34 in WT and *miR476a*-OE lines determined by qRT-PCR. Stem bases of seedlings cultivated in vitro were collected for RNA extraction. *Ubiquitin* was used as a reference gene. The values of WT were normalized to 1. B, time-course expression assays of *miR476a*-targeted *RFL* genes

during AR formation by qRT-PCR. For each gene, expression levels are normalized to that of 0 DAC and transformed to log2 values for heatmap analyses (leaf panel). Hierarchical clustering revealed three time-course expression patterns of these *RFL* genes during AR formation, represented by those of *RFL26* (purple), -34 (orange), and -18 (brown) in the right panel. Fc: fold of change. C, expression of *miR476a*-targeted *RFL* genes in *pER8: miR476a*-STTM line under mock and β -estradiol treatment. D, experimental validation of the *miR476a*-targeted cleavage of *RFL26*. The plasmid of *miR476a*-OE was transiently transformed into the protoplasts isolated from roots of transgenic Arabidopsis harboring ectopically expressed *RFL26*-GFP and *RFL26m*-GFP. *RFL26m* represents the mutated *RFL26* gene harboring the impaired *miR476a*-binding site as indicated in Figure S10A. Red fluorescence was emitted from mitochondrial dye mito-tracker Red (Mito-red). The fluorescence was detected via confocal microscope (Olympus) and quantified by FV10-ASW software (Olympus). Bar = 10 μ m. E and F, phenotypes of ARs derived from stem (E) and leaf explants (F) of overexpressing *RFL26m* in the *miR476*-OE line. Bars = 2 cm. G, quantification of AR number of overexpressing *RFL26m* in the *miR476*-OE line at 12 DAC. Error bars represent SD. For A and G, asterisks indicate statistically significant differences in Student's *t* test between WT and each transgenic line (*, $P < 0.05$; **, $P < 0.01$; $n = 4$). For C, asterisks indicate statistically significant differences with respect to mock for each time point (Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; $n = 5$). For D, asterisks indicate statistically significant differences with respect to *RFL26* and *RFL26m* transformants, respectively (Student's *t* test; *, $P < 0.05$; $n = 5$).

Figure 4. Mitochondrial homeostasis and dynamics modulated by *miR476a/RFL*.

A and B, Quantitative assays of ATP (A) and NADH/NAD⁺ (B) in WT, *miR476a*-OE,

and RFL26m-OE lines. Stem bases at 3 DAC were collected for measurements. C and D, Time-course quantification of ATP production (C) and NADH/NAD⁺ ratio (D) during AR formation in poplar. E, DAB staining of 3-DAC stem bases of WT, miR476a-OE, and RFL26m-OE lines. Scale bars = 0.5 cm. F, quantification of H₂O₂ content of 3-DAC stem bases of WT, miR476a-OE, and RFL26m-OE lines. Error bars represent SD. For A, B, and F, the values of WT are normalized to 1, and asterisks indicate statistically significant differences with respect to WT (Student's *t* test; *, *P* < 0.05; **, *P* < 0.01; n = 3).

Figure 5. *MiR476a*-mediated AR formation depends on mitochondrial homeostasis.

A and B, rotenone-inhibited ATP production (A) and NADH/NAD⁺ (B) during AR formation in WT and *miR476a*-OE lines. Rotenone (50 μM) was added for treatment at 1 DAC. C, AR phenotypes of WT and *miR476a*-OE lines under rotenone treatment at 8 and 12 DAC. Bars = 2 cm. D and E, quantification of the time for emergence of the first AR (D) and AR number (E) under rotenone treatment at 12 DAC in WT and *miR476a*-OE lines. Error bars represent SD. For D and E, asterisks indicate statistically significant differences with respect to mock (Student's *t* test; **, *P* < 0.01; n = 5).

Figure 6. *MiR476a*-mediated mitochondrial regulation on adventitious rooting requires auxin pathway.

A, expression levels of *PIN2* and *5b* in *miR476a*-OE and *RFL26m*-OE lines at 3-DAC stem bases assayed by qRT-PCR. B, expression levels of *PIN2* and *5b* in the *pER8:miR476a*-STTM line under mock and β-estradiol treatment. C, AR phenotypes of WT and *miR476a*-OE plants at 8 DAC under mock and 5 μM 1-N-naphthylphthalamic acid (NPA) treatment. D, expression levels of *PIN2* and *5b* under 50μM rotenone treatment

for 3 days during AR formation. E, quantitative assays of indole-3-acetic acid (IAA) in stem bases under 50 μ M rotenone treatment for 3 days during AR formation. F, fluorescent imaging of cross-sections of the *DR5-YFP* reporter line at 3 DAC under 50 μ M rotenone treatment. Red dashed circle indicates an area where YFP fluorescence is highly accumulated. Pf: phloem fibers; Ca: cambium; Xy: xylem. Bars = 20 μ m. G, quantification of YFP fluorescence corresponding to H. The values of mock (i) are normalized to 1. Error bars represent SD. For A, asterisks indicate statistically significant differences with respect to WT (Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; $n = 5$). For B, D, E, and G, asterisks indicate statistically significant differences with respect to mock (Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; $n = 3$).

Figure 7. A proposed model for *miR476a/RFL*-dependent AR formation in *Populus*.

The *miR476a/RFLs*-mediated mitochondrial functional modulation allows to divide the process of wound-induced AR formation into two sequential phases, including wounding response and root induction. At the first stage, wound signals at the bases of cutting explants induce the expression of several *RFL* genes encoding mitochondria-localized PPR proteins, and thereby restrict mitochondrial energy production that is unfavorable to AR initiation. At the second stage, the induction of *miR476a* results in the cleavage of the *RFL* transcripts for degradation, thus suppressing the amount of *RFL* proteins targeted into mitochondria. *MiR476a*-dependent suppression of *RFLs* causes the augmented mitochondrial status for energy supply indicated by increased ATP and NADH/NAD⁺ production whereas decreased ROS generation. The mitochondrial retrograde signals activate the expression of *PIN2/5b* auxin transporters, which modulated polar transport and homeostasis of auxin at rooting competent cells, and promote AR formation through

elevating the expression of AR-associated key genes.

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