# Red light controls adventitious root regeneration by modulating hormone homeostasis in *Picea abies* seedlings

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**Word count: 6261**

**Short title:** Light quality and adventitious rooting

**Highlight:**

Blue and red light have an opposite effect on adventitious root initiation in Norway spruce hypocotyl, red light having a promoting effect by modulating hormone homeostasis.

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**Abstract**

Vegetative propagation relies on the capacity of plants to regenerate *de novo* adventitious roots (ARs), a quantitative trait controlled by the interaction of endogenous factors such as hormones and environmental cues among which light plays a central role. However, the physiological and molecular components mediating light cues during AR initiation (ARI) remain largely elusive. We explored the effect of light spectral quality on ARI in de-rooted Norway spruce seedlings as well as on hormone metabolism with sensitive mass spectrometry-based methods. We coupled this to gene expression analysis to identify potential signaling pathways and to extensive anatomical characterization to investigate ARI at the cellular level. We showed that in contrast to white light and blue light, red light promoted ARI likely by reducing jasmonate (JA) and JA-isoleucine biosynthesis and repressing the accumulation of isopentyl-adenine-type cytokinins and abscisic acid. We confirmed that exogenously applied JA and/or CK inhibit ARI, and found that they possibly act in the same pathway. The negative effect of JA was confirmed at the histological level. We showed that JA represses the early events of ARI. In conclusion**,** RL promotes ARI by repressing the accumulation of the wound-induced phytohormones JA and CK.

55 **Introduction**:

56 Vegetative (or clonal) propagation through stem cuttings is widely used in breeding
57 programs for rapid fixation of elite genotypes. Successful clonal propagation requires
58 *de novo* regeneration of adventitious roots (ARs), a process governed by a multitude of
59 developmentally-programmed and environmentally-induced cues including wounding,
60 light and nutrient availability (Bellini *et al.*, 2014; Steffens & Rasmussen, 2016).
61 Because AR regeneration is a quantitative trait, the rooting capacity of cuttings varies
62 among individuals within species, populations, or even clones (Abarca & Díaz-Sala,
63 2009). Phytohormones, mainly auxin, jasmonate (JA), cytokinins (CKs), ethylene and
64 abscisic acid (ABA), play major roles in AR formation. It has been shown that there is
65 interplay among these phytohormones in complex networks and loops that ensure
66 proper AR development (Lakehal & Bellini, 2019). For example, in *Arabidopsis*
67 *thaliana*, it has been shown that auxin signaling controls AR formation by modulating
68 JA homeostasis in the hypocotyl (Gutierrez *et al.*, 2012; Lakehal *et al.*, 2019a) and that
69 JA cooperates with CKs to repress AR formation (Lakehal *et al.*, 2020a). Moreover, JA
70 signaling induces the expression of *DIOXYGENASE FOR AUXIN OXIDATION 1*, a
71 key enzyme catalysing the irreversible degradation of auxin, to ensure robust crosstalk
72 between auxin and JA signaling pathways (Lakehal *et al.*, 2019b). However, the extent
73 to which these complex molecular networks are evolutionarily conserved among plant
74 species is poorly understood, especially for trees. Although it remains difficult to
75 address the molecular mechanisms controlling AR formation in perennial species, the
76 rapid development of sequencing technologies giving access to most genome and
77 transcriptome sequences now offers the possibility of further exploring the degree of
78 evolutionary conservation or divergence of the known molecular networks.

79 Light is one of the most important external cues controlling plant growth and
80 development. It is perceived by highly sophisticated sensing machinery which mediates
81 a plethora of physiological and developmental processes, *inter alia* photosynthesis,
82 photomorphogenesis, flowering time and tropisms (de Wit *et al.*, 2016). Depending on
83 light intensity and spectral quality, the cues perceived trigger specific downstream
84 transcriptional signatures enabling a plant to adjust its growth and development as well
85 as its response to the environment. Phytohormones play central roles in integrating and
86 translating cues into developmentally coherent outputs and responses (de Wit *et al.*,
87 2016). In particular, light intensity and spectral quality have long been manipulated as
88 tools in vegetative propagation practices in order to stimulate the rooting ability of

recalcitrant species. For example, treating donor plants of difficult-to-root *Eucalyptus globulus* species with far-red-enriched light stimulated the rooting capacity of microcuttings (Ruedell *et al.*, 2015). This stimulation was possibly due to upregulation of the expression of *AUXIN RESPONSE FACTOR6-like (ARF6-like)* and *ARF8-like* genes at the cutting site by far-red light. These transcriptional regulators have been shown to promote ARI in Arabidopsis hypocotyls (Gutierrez *et al.*, 2009, 2012) and in *Populus* stem cuttings (Cai *et al.*, 2019). Far-red-enriched light also induced the expression of a putative indole 3-acetic acid (IAA) biosynthetic gene**,** *YUCCA3-like,* and two putative auxin efflux carriers genes**,** *PIN-FORMED 1-like (PIN1-like)* and *PIN-FORMED 2-like (PIN2-like)***,** in *Eucalyptus globulus* (Ruedell *et al.*, 2015). These data suggest that far-red-enriched light might act on the auxin biosynthesis, transport and/or signaling pathways to stimulate AR formation in *Eucalyptus globulus.* Keeping leafy stem cuttings of *Petunia hybrida* (Klopotek *et al.*, 2016) or *Dianthus caryophyllus* (Agulló-Antón *et al.*, 2011) in dark conditions prior to light exposure improved their rooting capacity. The effect of light on Norway spruce (*Picea abies*) growth and morphogenesis has also been documented (Strömquist & Eliasson, 1979; Mortensen & Sandvik, 1988; Bollmark & Eliasson, 1990; Mølmann *et al.*, 2006; Burescu *et al.*, 2015; OuYang *et al.*, 2015) and it has been shown that high-irradiance white light inhibited AR in Norway spruce stem cuttings (Strömquist & Eliasson, 1979).

Despite the importance of light in regulating AR formation, the physiological and molecular mechanisms involved are still unknown. Here, we used de-rooted Norway spruce seedlings as a model system to explore the role of light in the control of AR initiation in conifers. We found that constant red light promotes this process**,** possibly through downregulation of JA signaling and repression of cytokinin and abscisic acid (ABA) accumulation compared to constant white light conditions. It appears that the cooperative role of JA and CK signaling in the repression of ARI is evolutionarily conserved**,** as it represses very early events in the ARI process in Norway spruce hypocotyls.

## Materials and methods

### Plant material

Commercial seeds from Norway spruce (*Picea abies* L. Karst) were provided by Svenska Skogsplantor (https://www.skogsplantor.se/; Sweden) and kept at a temperature of 4 °C prior to use. The seeds were soaked in tap water for 24 h, in the dark at 4 °C**.** They were then sown in fine wet vermiculite as described in Ricci *et al.* (2008) and allowed to germinate and grow in a growth chamber. The seedlings were watered twice a week with tap water and left to grow for three weeks (Supplementary Fig. S1A). The growth conditions were as follow: 16 h light with 75 µmol /$m^2$/s (400 to 700 nm, Fig. S2A); 8 h dark; 20°C day temperature and 18°C night temperature. The white light was provided by 36W/840 Super 80 Cool White TL-D tubes (Philips, https://www.philips.se/).

### Test of effects of different light conditions on adventitious rooting of de-rooted seedlings

Twenty-one days after sowing, the seedling hypocotyls were severed 2 cm below the apex (Supplementary Fig. S1B) and placed in 24 ml vials filled with distilled water (Supplementary Fig. S1C). Three vials containing 5 seedlings each, constituting technical replicates**,** were prepared.

In order to test different white light conditions, vials containing de-rooted seedlings were kept in either long-day conditions in the growth chamber as described above (Supplementary Fig. S2A) or in a Percival growth cabinet equipped with cool white fluorescent tubes (F17T8/TL741, 17 Watt; Philips U.S.A: Percival Scientific, Perry Iowa, USA) (Supplementary Fig. S2B) or in a Percival plant growth cabinet (E-30NL/*floraLED*) equipped with six CLF *floraLED* modules (CLF PlantClimatics GmbH; Emersacker, Germany) providing white, red or blue light (Supplementary Fig. S2C,E,F). Under these latter conditions the light was constant and referred to as constant White Light (cWL), constant Blue Light (cBL) or constant Red Light (cRL), and the temperature was constant and set at 20 °C.

In order to remove the blue region of the spectrum from the white LEDs we added a yellow filter, which almost totally abolished the blue peak at 460 nm (Supplementary Fig. S2D).

156 The cBL (460 nm) intensity was set at 9 µmol/m$^2$/s (Supplementary Fig. S2E), and cRL
157 (660 nm) was set at an intensity of 9 µmol /m$^2$/s (Supplementary Fig. S2F).
158 The vials were refilled daily with distilled water to replace the water lost by
159 evapotranspiration. The number of roots per cutting and the number of rooted cuttings
160 were monitored for 30 days after cutting (DAC) and all the experiments were repeated
161 at least three times.
162
163 **Hormone treatments**
164 Auxin treatment:
165 Five hypocotyl cuttings per vial, with three technical replicates**,** were cultured in 24 ml
166 of distilled water supplemented with indole butyric acid (IBA), indole acetic acid (IAA)
167 or 1-Naphthalene Acetic Acid (NAA) at final concentrations of 1 or 5 µM, or without
168 supplementation as mock treatment.
169 Jasmonate (JA) treatment:
170 Five hypocotyl cuttings per vial, with three technical replicates**,** were cultured in 24 ml
171 of distilled water supplemented with JA at final concentrations of 2 µM, 10 µM or 20
172 µM**,** or without supplementation as mock treatment.
173 Cytokinin treatment:
174 Five hypocotyl cuttings per vial, with three technical replicates**,** were cultured in 24 ml
175 of distilled water supplemented with 6-benzylaminopurine (BAP) at a final
176 concentration of 0.1 µM or 0.01 µM, or without supplementation.
177 BAP was also used in combination with 1 µM IBA, 2 µM or 5 µM JA.
178 In all these experiments vials were covered with parafilm in order to limit the
179 evaporation of hormone solutions during the rooting period**;** small holes allowed the
180 cuttings to be inserted into the vials.
181
182 **Histology**
183 Five mm sections of hypocotyl cuttings were dissected from three-week-old seedlings
184 at T0**,** just before transferring the cuttings to rooting conditions, then 3, 5, 10, 13 and
185 15 days after cutting. Similar material was taken from de-rooted seedlings treated with
186 1 µM IBA or 20 µM JA. Hypocotyl samples were vacuum infiltrated with fixation
187 medium, 37% formalin, 5% acetic acid, 100% ethanol **,** for 20 seconds and left for 24
188 hours at room temperature. The samples were then washed in fixation solvent (70 %

189 ethanol) for 10 minutes and transferred into 70 % EtOH until required for use. Samples
190 were then gradually dehydrated in an ethanol series (80 %, 90 % , 96 % for 2h each and
191 100 % overnight at room temperature). The 100 % EtOH was gradually replaced by
192 HistoChoice tissue fixative (VWR Life, https://us.vwr.com/) in three steps of 1:3, 1:1,
193 3:1 (EtOH: HistoChoice ratio) and two 1 h steps with pure HistoChoice. The
194 HistoChoice fixative was gradually replaced with Paraplast Plus for tissue embedding
195 (Sigma-Aldrich, https://www.sigmaaldrich.com/), over 6 days. Serial sections (10 µm)
196 were obtained with a Zeiss HM 350 rotary microtome (https://www.zeiss.com/).
197 Longitudinal and transverse sections were stained with safranin (Sigma-Aldrich,
198 https://www.sigmaaldrich.com/) and alcian blue (Sigma-Aldrich,
199 https://www.sigmaaldrich.com/) in a ratio of 1:2; using methods from (Hamann *et al.*,
200 2011).
201
202 **Sources of sequence data and phylogenetic tree construction**
203 The *AtMYC2* (At1g32640), *AtJAZ3* (At3g17860), *AtJAZ10* (At5g13220), *AtAOC1*
204 (At3g25760) and *AtCOI1* (At2g39940) *Arabidopsis thaliana* gene coding sequences
205 (CDS) from TAIR (https://www.arabidopsis.org) were used as queries in BLASTN
206 searches for CDS predicted with high confidence from *P. abies* (http://congenie.org/;
207 Nystedt *et al.*, 2013) to identify potential orthologs in the *Picea abies* genome. All
208 sequence information is provided in Supplementary Fig. S4. To find the putative
209 Arabidopsis orthologs in *Picea abies*, a BLAST alignment of the coding sequences of
210 selected genes was generated using "Genome tools" from the Congenie database with
211 default settings. Subsequently, the sequences were used to construct phylogenetic trees
212 with the tools available at "http://www.phylogeny.fr". The phylogenetic analysis was
213 based on the neighbor-joining method (with 1000 bootstrap replicates). The putative
214 orthologs were named according to the Congenie database.
215
216 **Primer design, efficiency and qRT-PCR analysis**
217 Candidate genes were identified by BLASTN searches against the spruce database
218 (http://congenie.org) using Arabidopsis protein coding nucleotide sequences. The
219 Norway spruce genes that showed the highest sequence similarity with Arabidopsis
220 genes were further considered for primer design and gene expression analysis. Primers
221 for the selected candidate genes and reference genes to be used for qRT-PCR were
222 designed using Primer3web version 4.1.0 (http://primer3.ut.ee)**;** the primer sequences

223 are listed in Table **S1**. Specificity of the primers was analyzed by Sanger sequencing of
224 amplified PCR products and cDNA was used as a template for PCR amplification from
225 each primer pair. For each primer pair, PCR reaction efficiency estimates were
226 calculated from a standard curve generated from a serial dilution of cDNA. Based on
227 the average cycle threshold (Ct) values for each five-fold dilution, a standard curve was
228 generated using linear regression. PCR efficiency (E) was derived from the equation:
229 Efficiency % = (10 (-1/slope) - 1) ×100%. PCR conditions were as follows: 95°C for 1
230 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Finally, dissociation
231 curves were generated by increasing the temperature from 65 to 95°C, stepwise by
232 0.3°C every 10 s. For gene expression analysis by quantitative PCR (qPCR), total RNA
233 was isolated from the base of spruce hypocotyls (5 mm long from 36 to 38 hypocotyl
234 cuttings) using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich;
235 https://www.sigmaaldrich.com/). cDNA was synthesized using an iScript™ cDNA
236 Synthesis Kit (Bio-Rad; https://www.bio-rad.com/en-se/) following DNase treatment.
237 Expression was quantified by the LightCycler® 480 System (Roche, Basel,
238 Switzerland) using LightCycler® 480 SYBR Green I Master (Roche, Basel,
239 Switzerland). For each sample**,** three technical replicates from two independent
240 biological replicates were analyzed. The $\Delta\Delta^{CT}$ method was used to measure the relative
241 amount of transcript from each gene. Expression values were normalized to the
242 expression of two reference genes, *PaEIF4A1* and *PaUBC28***,** and calibrated with the
243 control value arbitrarily set to 1.
244 In order to check whether expression of the selected putative orthologs *PaMYC2-like,*
245 *PaAOC1-like*, *PaJAZ3-like* and *PaJAZ10-like* could be induced by jasmonate, 3-week-
246 old whole Norway spruce seedlings were treated with 50 µM of JA or mock treatment
247 for 3 h. Total RNA was prepared and qPCR experiments performed as described above.
248
249 **Hormone profiling experiments**
250 For hormone quantification**,** 5 mm hypocotyl segments were dissected at the base of
251 de-rooted hypocotyls 6, 24, 48, 72 and 120 hours after they had been transferred to
252 cWL, cBL or cRL, in order to provide an average of 20 mg of fresh weight.
253 Quantification of cytokinin metabolites was performed according to the method
254 described by (Svačinová et al., 2012), including modifications described by (Antoniadi
255 et al., 2015). Samples (20 mg fresh weight, FW) were homogenized and extracted in 1
256 ml of modified Bieleski buffer (60% MeOH, 10% HCOOH and 30% $H_2O$) together

257 with a cocktail of stable isotope-labeled internal standards (0.25 pmol of CK bases
258 added per sample). The extracts were applied **to** an Oasis MCX column (30 mg/1 ml,
259 Waters) conditioned with 1 ml each of 100% MeOH and $H_2O$, equilibrated sequentially
260 with 1 ml of 50% (v/v) nitric acid, 1 ml of $H_2O$, and 1 ml of 1M HCOOH, and washed
261 with 1 ml of 1M HCOOH and 1 ml 100% MeOH. Analytes were then eluted in two
262 steps using 1 ml of 0.35M $NH_4OH$ aqueous solution and 2 ml of 0.35M $NH_4OH$ in 60%
263 (v/v) MeOH. The eluates were then evaporated to dryness *in vacuo* and stored at -20°C.
264 Cytokinin levels were determined by ultra-high performance liquid chromatography-
265 electrospray tandem mass spectrometry (UHPLC-MS/MS) using stable isotope-labeled
266 internal standards as a reference (Rittenberg and Foster, 1940). Separation was
267 performed on an Acquity UPLC® System (Waters, Milford, MA, USA) equipped with
268 an Acquity UPLC BEH Shield RP18 column (150x2.1 mm, 1.7 µm; Waters), and the
269 effluent was introduced into the electrospray ion source of a triple quadrupole mass
270 spectrometer Xevo™ TQ-S MS (Waters). Six independent biological replicates were
271 performed.
272 Endogenous levels of free IAA, ABA, SA and JA**,** as well as the conjugated form of
273 JA, JA-Ile, in 20 mg of hypocotyls were determined according to the method described
274 in (Floková *et al.*, 2014). The phytohormones were extracted using an aqueous solution
275 of methanol (10% MeOH/$H_2O$, v/v). To validate the LC-MS method, a cocktail of
276 stable isotope-labeled standards was added with the following composition: 5 pmol of
277 [$^{13}C_6$]IAA, 10 pmol of [$^{2}H_6$]JA, [$^{2}H_2$]JA-Ile and 20 pmol of [$^{2}H_4$]SA (all from
278 Olchemim Ltd, Czech Republic) per sample. The extracts were purified using Oasis
279 HLB columns (30 mg/1 ml, Waters, Milford, MA, USA) and targeted analytes were
280 eluted using 80% MeOH. Eluate containing neutral and acidic compounds was gently
281 evaporated to dryness under a stream of nitrogen. Separation was performed on an
282 Acquity UPLC® System (Waters, Milford, MA, USA) equipped with an Acquity
283 UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm; Waters, Milford, MA, USA), and the
284 effluent was introduced into the electrospray ion source of a triple quadrupole mass
285 spectrometer Xevo™ TQ-S MS (Waters, Milford, MA, USA).
286
287 **Statistical analysis**
288 For statistical analyses, the SPSS 20 software package was used
289 (https://www.ibm.com/support/pages/spss-statistics-20). Student's t-tests were
290 performed to evaluate the effects of different light qualities on hormone contents.

Repeated measures ANOVAs were used to test the effects of time and hormone concentrations under constant red light.

294 **Results:**
295

296 **Auxin is not sufficient to stimulate adventitious rooting in Norway spruce**
297 **hypocotyl cuttings kept under white light**

298 To investigate the role of light in adventitious root initiation (ARI) in Norway spruce,
299 we used de-rooted Norway spruce seedlings as a model system. Three-week-old
300 seedlings grown under white light condition were harvested and de-rooted as described
301 in Materials and methods (Fig. S1). They were first kept in hormone free (HF) distilled
302 water, under three different white light (WL) regimes as described in Materials and
303 methods (irradiance 69 to 75 $\mu M/m^2/s^{-1}$**;** Supplementary Fig. S2A,B,C). In these
304 conditions no AR developed at the base of the hypocotyls regardless of the photoperiod
305 or the source of white light. Since we obtained the same results in all WL conditions,
306 we continued to use constant WL (cWL) provided by LEDs (Supplementary Fig. S1D
307 and Supplementary Fig. S2C) as control conditions for the rest of the study.
308 It is well known that auxin can stimulate adventitious rooting in several plant species
309 (Lakehal & Bellini, 2019), therefore we tested whether different types of auxin could
310 stimulate ARI under cWL. Three-week-old de-rooted seedlings were treated with 1 or
311 5 µM of Indole 3-Acetic Acid (IAA), 1-Naphthalene 3-Acetic Acid (1-NAA) or Indole
312 3-Butyric Acid (IBA), but none of the auxins could induce ARI under cWL conditions
313 (Fig. 1A). These results indicate that auxin is not sufficient to induce ARI in de-rooted
314 Norway spruce seedlings kept under cWL.
315

316 **Constant red light (cRL) and constant blue light (cBL) have opposite effects on**
317 **ARI in de-rooted Norway spruce hypocotyls**

318 Next, we wondered whether a particular region of the light spectrum had a negative
319 effect on ARI. To test this hypothesis, three-week old de-rooted seedlings were kept in
320 HF distilled water under either cWL (Fig. S1D and Fig. S2C), constant blue light (cBL)
321 (Fig. S1f and Fig. S2D) or constant red light (cRL) (Fig. S1E and Fig. S2E).
322 Interestingly, AR developed only when seedlings were kept under cRL conditions (Fig.
323 1B-D). In these conditions 100% of the cuttings developed at least 1 AR by 30 days
324 after cutting (DAC) (Fig. 1B). These results raised the possibility that in cWL, the blue
325 light part of the spectrum has a negative effect on ARI. To test this hypothesis**,**
326 hypocotyl cuttings were kept under cWL but a yellow filter was added to remove the
327 blue wavelength region (Fig. S2F). In these conditions 83% of the cuttings developed

328 at least 1 AR (Fig. 1B).
329 These results indicate that cRL promotes ARI, whereas cBL inhibits this process, and
330 the absence of AR under cWL could be due to the presence of blue light.
331
332 **Constant red light induces ARI by reducing endogenous levels of jasmonate,**
333 **cytokinins, and abscisic acid**
334 We have previously shown that ARI in intact etiolated Arabidopsis hypocotyls is under
335 the control of IAA and JA signaling pathways (Gutierrez *et al.*, 2012). More recently
336 we showed that JA and CK cooperatively repress this process (Lakehal *et al.*, 2020a).
337 We therefore hypothesized that light might also modulate phytohormone signaling
338 and/or homeostasis pathways to control ARI in Norway spruce hypocotyls. To test this
339 hypothesis, we quantified the endogenous contents of different hormones known to
340 either inhibit ARI**,** such as JA, abscisic acid (ABA) or CKs (Steffens *et al.*, 2006;
341 Ramírez-Carvajal *et al.*, 2009; Gutierrez *et al.*, 2012; McAdam *et al.*, 2016; Mao *et al.*,
342 2019) or promote ARI**,** such as IAA or salicylic acid (SA) (Gutierrez *et al.*, 2012).
343 Quantifications were performed for hypocotyl samples taken at time 0 (T0)**,** i.e. just
344 before being transferred in HF distilled water, and at the base of hypocotyl cuttings
345 maintained under different light qualities 6, 24, 48 and 72 hours after cutting (Fig. 2;
346 Supplemental data sets 1 and 2).
347 Under cWL conditions, we observed a 40% increase in the free IAA level 6 h after
348 cutting compared to the level at T0 **(**Supplemental data set 1), but thereafter the IAA
349 content remained mostly constant over time, with the exception of a slight increase 24
350 h after cutting (Fig. 2A; Supplemental data set 1). There was no effect of wounding on
351 the endogenous content of SA**,** as shown in Supplemental data set 1**,** and the SA content
352 remained constant up to 48 h after cutting (Fig. 2B). A slight increase could be observed
353 72 h after cutting (Fig. 2B, Supplemental data set 1). Based on these results, we
354 concluded that the inability to initiate ARs under cWL could not be explained by a
355 reduction in auxin content, and this is in agreement with the fact that exogenous
356 application of IAA or other auxins could not stimulate ARI under these conditions (Fig.
357 1A)
358 We have previously shown that JA and its bioactive form Jasmonoyol-Isoleucine (JA-
359 Ile) inhibit ARI in Arabidopsis hypocotyls (Gutierrez *et al.*, 2012; Lakehal *et al.*,
360 2020a). Here, as expected in the case of wounding, JA and JA-Ile were induced 6 h
361 after cutting in all light conditions (Supplemental data set 1). Under cWL conditions,

362 we observed a 93 % increase in both JA and JA-Ile 6 h after cutting and transfer to cWL
363 conditions (Supplemental data set 1). Both JA and JA-Ile contents subsequently
364 decreased over time (Fig. 2C,D; Supplemental data set 1). Interestingly, although we
365 observed a significant decrease (72%) in total CK content 6 h after cutting compared to
366 the content at T0 (Supplemental data set 2), the total CK content then increased again
367 and CKs accumulated over time at the base of the hypocotyls (Fig. 2). This increase in
368 CKs was mostly due to the accumulation of isopentyl-adenine-type (iP-type) cytokinins
369 including the precursors iP riboside 5′-monophosphate (iPRMP) and iP ribosides (iPR)
370 (Fig. 2E-G; Supplemental data set 2). Similarly, we observed a 78 % decrease in the
371 endogenous content of ABA 6 h after cutting compared to the content at T0
372 (Supplemental data set 2), but it increased again over time (Fig. 2h).
373 Interestingly**,** in cRL conditions, although the free IAA content was significantly
374 reduced compared to that in cWL 24 h after cutting and continued to decrease over time
375 (Fig. 2A), cutting developed ARs. This could be explained by a significant reduction in
376 the amount of JA, Ja-Ile, CKs and ABA compared to cWL at all time points (Fig. 2C-
377 H). In contrast to what was observed in cWL, the amounts of CKs and ABA remained
378 constant over time, except for iP which continued to decrease over time (Fig. 2G).
379 Although the interaction between light spectral quality and hormone homeostasis is
380 complex, these results suggest that the positive effect of cRL on ARI cannot be
381 explained by the modification of IAA homeostasis**;** rather it is a consequence of a
382 decrease in negative regulators such as JA, JA-Ile, and iP.
383 Surprisingly, although cBL had the same effect as cRL on the endogenous levels of
384 hormones (Fig. 2A-H), ARs could not develop. These results suggest that cBL inhibits
385 adventitious rooting through another pathway yet to be identified. In the remainder of
386 this study we therefore investigated only the role of cRL on ARI.
387
388 **Exogenously applied JA and CK inhibit ARI under cRL**
389 To get physiological insights into the possible crosstalk between IAA, JA and CKs
390 during cRL-induced AR development, we treated the de-rooted seedlings exogenously
391 with either auxins, JA, or CK alone or in combination.
392 First, we showed that the three types of auxin (IAA, NAA and IBA) enhanced AR
393 formation under cRL conditions in a dose-dependent manner (Fig. 3A-C). IBA, which
394 is often used to induce rooting in recalcitrant species (reviewed in Geiss *et al.*, 2018;
395 Stevens *et al.*, 2018), appeared to be the most efficient auxin (Fig. 3A). When applied

396 exogenously, both JA and 6-Benzylaminopurine (6-BAP) inhibited AR development in
397 a dose-dependent manner (Fig. 3D,E) and they both repressed the positive effect of
398 exogenous IBA (Fig. 3F,G). These results suggest that JA and CK act downstream of
399 auxin signaling to repress ARI, as has been described for intact Arabidopsis hypocotyls
400 (Gutierrez *et al.*, 2012; Lakehal *et al.*, 2020a). When JA and CK were combined, no
401 additive or synergistic effect was observed (Fig. 3H), suggesting that they act in the
402 same pathway. These results are in agreement with our previous data that showed that
403 CK signaling is induced by JA to repress AR formation in intact Arabidopsis
404 hypocotyls (Lakehal *et al.*, 2020a).
405
406 **JA signaling is downregulated in de-rooted Norway spruce hypocotyls kept under**
407 **cRL compared to cWL conditions**
408 The reduction in content of JA and JA-Ile under cRL compared to cWL (Fig. 2B)
409 prompted us to check whether the expression of genes involved in JA biosynthesis or
410 JA signaling was affected in hypocotyls of seedlings kept under cRL compared to cWL
411 conditions. We first searched the Norway spruce genome (Nystedt *et al.*, 2013) for
412 putative orthologs of the key Arabidopsis genes in JA signaling or biosynthesis using
413 http://congenie.org/. We looked for putative orthologs of the JA-Ile receptor
414 *CORONATINE INSENSITIVE 1 (AtCOI1)* (Xie *et al.*, 1998), *AtMYC2* transcription
415 factors (Lorenzo *et al.*, 2004), the *JASMONATE ZIM-DOMAIN3 (AtJAZ3) and*
416 *AtJAZ10* transcriptional repressors (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*,
417 2007) and *ALLENE OXYDE CYCLASE (AtAOC*), which encodes a key enzyme in the
418 JA biosynthesis pathway (Stenzel *et al.*, 2012). We found that the Norway spruce
419 genome contains eleven putative *PaCOI1-like* genes (Fig. S3A), five putative
420 *PaMYC2-like* genes (Fig. S3B), one and three putative orthologs of *PaJAZ3-like* and
421 *PaJAZ10-like* respectively (Fig. S3C) and two putative *PaAOC-like genes* (Fig. S3D).
422 The full-length coding sequences are reported in Fig. S4. When more than one putative
423 ortholog was identified we chose that most closely related to the Arabidopsis one for
424 further experiments.
425 We first confirmed that expression of *PaMYC2-like* (MA_15962g0010), *PaJAZ3-like*
426 (MA_6326g0010), *PaJAZ10-like* (MA_10229741g0010) and *PaAOC-like*
427 (MA_56386g0010) was induced by exogenously applied JA (Fig. 4A). We next
428 checked the expression of these genes, together with *PaCOI1-like*, at the base of
429 hypocotyl cuttings kept under cWL or cRL for 6 h, 24 h, 48 h or 72 h after cutting (Fig.

430 4B). The relative amounts of *PaMYC2-like*, *PaJAZ3-like*, and *PaAOC-like* transcripts
431 were slightly reduced in cRL compared to those observed in cWL (Fig. 4B). These
432 results are in agreement with the reduced content of JA-Ile, the active form of JA, in
433 cRL compared to cWL (23% less in cRL compared to cWL) (Fig. 2F and Supplemental
434 data set 2). Twenty-four hours after cutting, *PaMYC2-like, PaJAZ10-like*, and *PaCOI1-*
435 *like* were slightly upregulated in cRL compared to cWL (Fig. 4b), results which also
436 coincided with a slightly higher concentration of JA-Ile (20% more in cRL compare to
437 cWL) (Fig. 2F). Later on, by 48h and 72h**,** the JA responsive genes were downregulated
438 (Fig. 4)**,** and this coincided with a greater decrease in JA and JA-Ile contents in cRL
439 compared to cWL (Fig. 2F; Supplemental data set 2). In conclusion, when de-rooted
440 hypocotyls were kept in cRL conditions, the endogenous contents of JA and JA-Ile
441 decreased faster than in cWL**,** resulting in downregulation of JA signaling in cRL
442 compared to cWL conditions. According to our previous results (Gutierrez *et al.*, 2012;
443 Lakehal *et al.*, 2020a)**,** this most likely contributes to an improvement in adventitious
444 root development.
445
446 **Exogenously applied JA inhibits ARI in Norway spruce hypocotyl in cRL**
447 Our data indicated that JA signaling was rapidly downregulated (within 6 hours) in the
448 hypocotyls of cuttings kept under cRL compared to cWL**,** suggesting that JA signaling
449 repressed an early event in AR formation. To assess the repressive effect of JA at the
450 cellular level, we analyzed and compared the anatomy of hypocotyls kept under cRL,
451 in hormone free distilled water or in the presence of 1 µM IBA or 20 µM JA, at different
452 time points after cutting (Fig. 5 and 6). On day 0 (i.e. immediately after cutting the
453 hypocotyl of three-week-old Norway spruce seedlings), the hypocotyl had a primary
454 structure consisting of a single layer of epidermis, and six to seven cortex cell layers
455 composed of isodiametric parenchyma cells (Fig. 5A). We could guess at the presence
456 of the endodermis layer although it was not always morphologically distinctive (Fig.
457 5A). The central cylinder consisted of seven to eight layers of parenchyma cells which
458 were smaller compared to the cortex parenchyma cells. The vascular system was
459 composed of a continuous procambium and differentiated elements of the protophloem
460 and protoxylem. The pith region showed small isodiametric parenchyma cells (Fig.
461 5A).

462 Three days after cutting, in HF distilled water and in the presence of 1 μM IBA, a few
463 cells in the cambial zone and in the adjacent phloem cells developed dense cytoplasm
464 and large nuclei indicating the initiation of the first cell divisions (Fig. 6A,B). In
465 contrast, no obvious histological modifications were observed in the presence of 20 μM
466 JA, and the histological organization remained unchanged compared to day 0 (Fig. 6C).
467 By five days after cutting, periclinal and anticlinal divisions were clearly observed in
468 the cambial zone and in the outermost layers of the phloem region (Fig. 6D). In
469 longitudinal section these cells were seen to be organized into vertical files external,
470 but adjacent, to the vascular cylinder (Fig. 5B). In the presence of 1 μM IBA it was
471 clearly apparent that the number of cell divisions was higher than under HF conditions
472 (Fig. 6E), whereas no divisions were observed in the presence of 20 μM JA (Fig. 6F).
473 Ten days after cutting, in HF conditions, cambial derivatives divided and formed radial
474 rows of tracheal elements around the xylem, but no AR meristemoids were observed
475 (Fig. 6G), while in the presence of 1 μM IBA several AR root meristemoids were visible
476 (Fig. 5C and Fig. 6H); no cell divisions were observed in the presence of 20 μM JA
477 (Fig. 6I). It was only by thirteen days after cutting that AR meristemoids began to form
478 at the periphery of the trachea elements in HF conditions (Fig. 6J), while in the presence
479 of IBA, AR had already emerged (Fig. 5D and Fig. 6K); still no cell division activity
480 was observed in the presence of JA (Fig. 6L). In HF, the first emergence of ARs was
481 observed only 15 days after cutting (Fig. 5E). These data clearly indicate that
482 exogenously applied JA repressed the very early stage of ARI, whereas auxin not only
483 promoted but also speeded up the process.
484

485 **Discussion**

486 When optimizing conditions for the development of roots from cuttings during
487 vegetative propagation, it has long been considered that light should be taken into
488 account as an important factor. Since the early eighties, studies related to the effect of
489 light on AR formation in woody plant cuttings have been pursued (Druart *et al.*, 1982;
490 Hammerschlag, 1987; Klopotek *et al.*, 2010). Several studies have addressed the effect
491 of light quality and/or intensity on the rooting of cuttings (Jarvis & Shaheed, 1987;
492 Baraldi *et al.*, 1988; Fuernkranz *et al.*, 1990; Fett-Neto *et al.*, 2001; Iacona & Muleo,
493 2010; Daud *et al.*, 2013), but the exact molecular and genetic mechanisms underlying
494 the role of light as well as the downstream targets of light signals during ARI remain
495 poorly understood. In this study, we showed that under cWL conditions de-rooted
496 Norway spruce hypocotyls were unable to produce AR and that this was unlikely to be
497 due solely to an increase in endogenous CK levels, as suggested by (Strömquist &
498 Eliasson, 1979; Bollmark & Eliasson, 1990) since exogenously applied auxin was
499 unable to stimulate AR development. This result led us to hypothesize that specific
500 wavelengths within the white spectrum could be responsible for this strong inhibition,
501 and we therefore grew seedlings under white or monochromatic LEDs. In these
502 conditions, we showed that AR developed under cRL even in the absence of exogenous
503 auxin application, while they did not develop under cBL.

504 The cRL-mediated ARI is possibly due to a faster reduction in JA and JA-Ile contents
505 compared to cWL. cRL also repressed the accumulation of CKs and ABA which was
506 observed under cWL. Reduction in the amounts of the three negative regulators to
507 below a certain threshold led to a de-repression of ARI.

508 The reduced amount of JA and JA-Ile in the base of cuttings could be partially explained
509 by downregulation of *de novo* JA biosynthesis**,** since we observed a reduced amount of
510 the JA precursor *cis*-12-oxo-phytodienoic acid (*cis*-OPDA) under cRL compared to
511 cWL conditions 24, 48 and 72 h after cutting (Supplemental data set **2**). This result is
512 supported by the downregulation of expression of *PaAOC-like*, a key gene in the JA
513 biosynthesis pathway. The link between light and the JA signaling pathway has been
514 proposed in several contexts, but how it affects JA biosynthesis is not yet known.
515 Interestingly, several reports have revealed mechanistic insights into the roles of RL or
516 red/far-red light ratio in the control of plant development or in adjusting growth-defense
517 tradeoffs (Kazan & Manners, 2011). For example, (Campos *et al.*, 2016) found that
518 mutating the RL-receptor *Phytochrome B* (*PhyB*) suppressed the stunted rosette

519 phenotype of the *JazQ* mutant, which harbors five null mutations in JAZ family
520 members. These data suggest that *PhyB*-mediated light signaling interacts with JA
521 signaling to control growth in Arabidopsis. Under our conditions, we found that the
522 expression levels of *PaMYC2-like*, a master transcriptional regulator of JA signaling,
523 were slightly but significantly downregulated under cRL compared to cWL. This result
524 could be explained either by a direct effect of RL on transcriptional regulation or by the
525 reduced amount of the bioactive form JA-Ile present under these conditions. The latter
526 assumption is supported by the fact that exogenous application of JA strongly induced
527 the expression of *PaMYC2-like,* an observation consistent with what has been reported
528 in Arabidopsis. The reduced amounts of JA and JA-Ile and the downregulation of the
529 expression of *MYC2-like* under cRL conditions led to a reduced JA response**,** as
530 indicated by downregulation of the steady state level of expression of the well-known
531 JA responsive genes (*PaJAZ10-like* and *PaJAZ3-like*).
532 Although the negative role of JA on ARI has been demonstrated in the model plant
533 Arabidopsis, its exact role in other species previously remained unclear (reviewed in
534 (Lakehal *et al.*, 2020b). Our results showed that JA is a negative regulator of ARI in
535 de-rooted Norway spruce hypocotyl and that it acts during the very early stages of
536 initiation**,** as indicated by anatomical characterization. We showed that under cRL,
537 certain cells, most likely cambial cells, have the competence to form ARs in a similar
538 way to that described for other conifers such as *Pinus taeda*, *P. contorta* and *P. radiata*
539 (Diaz-Sala *et al.*, 1996; Lindroth *et al.*, 2001B,a; Ricci *et al.*, 2008). When we exposed
540 the de-rooted hypocotyls to exogenous auxin, these cells exhibited rapid cell division
541 and re-orientation of division planes to organize the AR meristem**;** in contrast when
542 exogenous JA was added**,** no cell divisions were observed. In addition, we showed that
543 JA repressed the positive effect of auxin when the compounds were applied together,
544 supporting the conclusion that, as in Arabidopsis, JA acts downstream of auxin in the
545 control of ARI in de-rooted Norway spruce hypocotyls. Moreover, we showed that, like
546 JA, CK inhibits ARI and counteracts the action of auxin, but when combined with JA
547 no additive or synergistic effect was observed, which is in agreement with our recent
548 results showing that cytokinins act downstream of JA to repress ARI in Arabidopsis
549 hypocotyls (Lakehal *et al.*, 2020a).
550 Our data also showed that total CKs accumulated at the base of cuttings when the de-
551 rooted seedlings were exposed to cWL, whereas their amount remained constant over
552 time under cRL conditions. Under cWL, the increasing amounts of the iP-type CKs

553 could be due to *de novo* biosynthesis since we also observed an increased level of the
554 precursors iPR and iPRMP over time. (Bollmark & Eliasson, 1990) showed that de-
555 rooted Norway spruce hypocotyls kept under high irradiance WL (270 $\mu E.m^{-2}.s^{-1}$) could
556 not develop ARs, probably because of the accumulation of CKs. Nevertheless, the
557 effect of WL may be more complex**,** since under our conditions, although the WL
558 irradiance was much lower (75 $\mu E.m^{-2}.s^{-1}$), CKs still accumulated. In our conditions, as
559 a response to wounding, JA and JA-Ile accumulated at the base of hypocotyls 6 h after
560 cutting, and their content started decreasing thereafter, whereas iP-type CKs began to
561 accumulate 48 hours after cutting. Recently we showed that MYC2-mediated JA
562 signaling promotes the accumulation of CKs through control of expression of the
563 *APETALA2/ETHYLENE RESPONSE FACTOR 115* transcription factor in Arabidopsis
564 hypocotyls (Lakehal *et al.*, 2020a). We therefore propose that in Norway spruce
565 hypocotyls, wound induced accumulation of JA and JA-Ile triggers *de novo* cytokinin
566 biosynthesis which results in inhibition of rooting. Interestingly, under constant cRL**,**
567 the more rapid reduction in JA and JA-Ile contents and subsequent downregulation of
568 MYC2-mediated JA signaling may prevent the induction of *de novo* CK biosynthesis
569 and this would allow ARI, although we also cannot rule out a direct effect of cRL on
570 the repression of CK accumulation.
571 In conclusion**,** we propose that de-rooted Norway spruce seedlings do not develop AR
572 under WL**,** on the one hand because they accumulate CKs and ABA which are
573 repressors of ARI and on the other because of the presence of light in the blue region
574 of the spectrum which has a negative effect independently of JA, CKs and ABA.
575 Understanding the role of blue light requires further investigation. In contrast**,** we
576 showed that red light has a positive effect on ARI**,** most likely because it represses the
577 JA signaling pathway and the accumulation of CKs.
578
579
580

**Supplemental data**

The following supplemental data are available

**Supplementary Fig. S1:** Method for *de novo* root organogenesis under different light conditions

**Supplementary Fig. S2:** Spectral emission curves for the various light sources used in this study

**Supplementary Fig. S3:** Phylogenetic trees

**Supplementary Fig. S4:** Coding sequences used for the phylogenetic analysis

**Supplementary Table S1** Primers used for qPCR experiments in this study

**Accession numbers:**

*AtMYC2* (At1g32640), *AtJAZ3* (At3g17860), *AtJAZ10* (At5g13220), *AtAOC1* (At3g25760), *AtCOI1* (At2g39940), *PaMYC2-like* (MA_15962g0010), *PaJAZ3-like* (MA_6326g0010), *PaJAZ10-like* (MA_10229741g0010) and *PaAOC-like* (MA_56386g0010)

**Acknowledgements:**

The authors sincerely thank Marta Derba Maceluch from the UPSC Microscopy platform for her advice and help with histological characterization.

This research was supported by the Ministry of Higher Education and Scientific Research in Iraq (to SA), by grants from the Swedish research councils FORMAS, VR, VINNOVA, Kempestiftelserna, and the Carl Tryggers Stiftelse (to CB), and by grants from the Ministry of Education, Youth and Sports of the Czech Republic (European Regional Development Fund-Project "Plants as a tool for sustainable global development" No. CZ.02.1.01/0.0/0.0/16_019/0000827), and the Czech Science Foundation (Project No. 19-00973S) (to ON)

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771

772 **Figure Legends:**

773

774 **Fig. 1. Constant blue light inhibits, while constant red light promotes, ARI in de-**
775 **rooted Norway spruce hypocotyls**

776 (A) Percentage of rooting in hypocotyl cuttings kept under constant white light (cWL).
777 Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in
778 hormone free (HF) distilled water, or in the presence of 1 or 5 μM indole-3-acetic acid
779 (IAA), indole-3-butyric acid (IBA) or 1-naphthalene acetic acid (NAA), under cWL.
780 (B) Percentage of rooting in hypocotyl cuttings kept under cWL, cWL with a yellow
781 filter (YF), constant red light (cRL) or constant blue light (cBL).
782 Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in
783 hormone free (HF) distilled water under the different light conditions.
784 (C) Average number of ARs on Norway spruce hypocotyls kept under cWL, cBL or
785 cRL. Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in
786 hormone free (HF) distilled water. The hypocotyl cuttings were observed every day to
787 count emerging AR primordia and emerged ARs as in (**d**). Fifteen seedlings were
788 analyzed for each condition.
789 (D) Different stages of AR development in de-rooted 3-week-old Norway spruce
790 seedling in distilled water, under cRL. DAC: days after cut; scale bar = 2 mm.

791

792 **Fig. 2. Light spectral quality controls the homeostasis of endogenous contents of**
793 **different hormones, which positively or negatively affect ARI**

794 (A) to (H) Three-week-old Norway spruce seedlings were grown under long day
795 conditions as described in Materials and methods. De-rooted seedlings were then kept
796 in constant cWL, cBL, or cRL for 6, 24, 48 and 72 hours. For each time point in each
797 condition 5 mm hypocotyls were taken and pooled for hormone quantification. Five
798 independent biological replicates were collected for analysis of IAA, ABA, SA and JA
799 contents, and additional five biological replicates were collected for cytokinin
800 quantification.
801 (A) free IAA content; (B) free salicylic acid (SA) content; (C) free jasmonic acid (JA)
802 content; (D) jasmonate-isoleucine (JA-Ile) content; (E) content of total cytokinins
803 (CKs); (F) total isopentyl-adenine type (iP-type) CKs; (G) isopentyl-adenine (iP)
804 content; (H) Free abscisic acid (ABA) content.

805 Values are the means with standard deviation (SD) of 5 biological replicates. Asterisks
806 indicate statistically significant differences under light conditions cRL versus cWL and
807 cBL versus cWL in t-test; *, **, and *** correspond to P-values of (0.05 > p > 0.01,
808 0.01 > p > 0.001, and p < 0.001 respectively; *n = 5*); Dashes indicate statistically
809 significant difference 6 h versus 24 h, 48 h or 72 h in t-test; #, ##, ### correspond to P-
810 values of (0.05 > p > 0.01, 0.01 > p > 0.001, and p < 0.001, respectively; n = 5; FW,
811 fresh weight.
812
813 **Fig. 3. Auxin enhances RL-induced adventitious rooting, whereas CKs and JA**
814 **repress this process**
815 Three-week-old Norway spruce seedlings were de-rooted and kept under cRL for 30
816 days in hormone free (HF) distilled water, or in the presence of 1 or 5 µM IBA (A); or
817 in the presence of 1 or 5 µM NAA (B); or in the presence of 1 or 5 µM IAA (C); or in
818 the presence of jasmonate (JA) (D); or in the presence of benzyl adenine (BAP) (E); or
819 in the presence of both JA and IBA (F); or in the presence of both BAP and IBA (G);
820 or in the presence of both JA and BAP (H).
821 (A) Both concentrations of exogenously applied IBA significantly increased the
822 average number of ARs over time (repeated measures ANOVA; P < 0.0001).
823 (B) Both concentration of exogenously applied NAA significantly increased the
824 average number of adventitious roots over time (repeated measures ANOVA; P <
825 0.0001).
826 (C) Exogenously applied IAA did not have any significant effect compared to HF
827 conditions (Repeated measures ANOVA; P = 0.073).
828 (D) Exogenously applied JA had a significant negative effect on the average number of
829 ARs over time (repeated measures ANOVA; P < 0.0001). This negative effect was
830 concentration dependent (repeated measures ANOVA; P < 0.0001).
831 (E) Exogenously applied BAP significantly reduced the number of ARs compared to
832 HF conditions (repeated measures ANOVA; P < 0.0001).
833 (F) When JA was applied together with IBA, it significantly repressed the positive
834 effect of IBA. The combination 1 µM IBA + 2 µM JA significantly reduced the number
835 of roots compared to those in HF conditions by 24 days after cut (DAC) and 30 DAC
836 (t-test, degree of freedom df = 14; p-value = 0.02 and 0.001 respectively). The

837 combination 1 μM IBA + 20 μM JA significantly reduced the average AR number
838 compared to HF conditions (t-test, df = 14; p-value < 0.01).
839 (G) When BAP was applied together with IBA, it repressed the positive effect of IBA.
840 The combination 1 μM IBA + 0.01 μM BAP significantly reduced the average number
841 of ARs compared to the 1 μM IBA treatment. (13, 15, 18, 24 and 30 DAC; t-test, df =
842 14; p-value < 0.009, 0.007, 0.003, 0.0001And 0.02 respectively). The combination 1
843 μM IBA + 0.1 μM BAP significantly inhibited rooting induced by 1μM IBA at
844 (13,15,18,24 and 30 DAC (t-test, df = 14; p-value < 0.008,0.007,0.0009, 0.000000009
845 and 0.0009 respectively);
846 (H) Both JA and BA repressed AR compared to the HF control, but no significant
847 additive or synergistic effect was observed when JA and BAP were applied together.
848 The combination 0.01 μM BAP + 2 μM JA significantly inhibited rooting compared to
849 the HF control at 24 and 30 DAC (t-test, df = 14; p-value < 0.0007 and 0.003
850 respectively), but the result was not significantly different from JA or BAP alone; The
851 combination of 0.01 μM BAP + 5μM JA significantly inhibited rooting compared to
852 the HF controls at 24 and 30 DAC (t-test, df = 14; p-value < 0.00004 and 0.0000003
853 respectively), but with no significant difference from JA or BAP alone.
854 (A to H) Results are expressed as average number of ARs per cutting. Error bars
855 indicate standard error (SE). For each treatment 15 hypocotyls were used, and the
856 experiment was repeated twice.
857
858 **Fig. 4. JA signaling is downregulated in hypocotyl cuttings kept under constant**
859 **red light (cRL) compared to hypocotyl cuttings kept under constant white light**
860 **(cWL)**
861 (A) Relative transcript amount of putative JA-responsive genes *PaMYC2-like*, *PaJAZ3-*
862 *like*, *PaJAZ10-like* and *PaAOC-like* in three-week-old Norway spruce seedling
863 hypocotyls treated for 3 h with 50 μM JA or with a mock treatment. Values are relative
864 (on a log10 scale) to the values for mock treated seedlings which were arbitrarily set to
865 1. Error bars indicate ±SE obtained from two independent biological replicates, with
866 three technical replicates of each. A *t*-test was carried out, and values indicated by
867 asterisks differed significantly from the mock values ($P < 0.001$, $n \geq 80$).
868 (B) Relative transcript amounts of putative JA-responsive genes *PaMYC2-like*,
869 *PaJAZ3-like*, *PaJAZ10-like* and *PaAOC-like*, and the putative JA receptor *PaCOI1-*

870 *like*, at the base of three-week-old de-rooted hypocotyls kept under cRL or cWL for 6,
871 24, 48 and 72 h. The values are relative (on a log2 scale) to the values obtained from
872 seedlings kept under cWL, which were arbitrarily set to 1. Error bars indicate ±SE
873 obtained from two independent biological replicates, with three technical replicates of
874 each. A *t*-test was carried out, and asterisks indicate values that differed significantly
875 from the control values ($P < 0.001$, $n \geq 80$).
876
877 **Fig. 5. Histological analysis of AR development in Norway spruce hypocotyl**
878 **cuttings under cRL**
879 (A) Cross section through the base of a 21-day-old hypocotyl cutting just before transfer
880 to rooting conditions.
881 (B) Longitudinal section through the base of a hypocotyl cutting kept in hormone free
882 (HF) conditions for 5 days. Arrowheads indicate localized cell divisions, probably at
883 the points of origin of future ARs.
884 (C) Longitudinal section through the base of a hypocotyl cutting kept in the presence
885 of 1 µM IBA for 10 days. Arrowheads indicate the presence of meristemoids.
886 (D) Longitudinal section through the base of a hypocotyl cutting kept in the presence
887 of 1 µM IBA for 13 days. Arrows indicate an adventitious root primordium (ARP) and
888 an emerged adventitious root (AR).
889 (E) Cross section through the base of a hypocotyl cutting kept in HF conditions for 15
890 days. Arrows indicate an ARP and an emerged AR.
891 e, epidermis; Co, cortex; Pa, parenchyma; Ph, phloem; en, endodermis; c, cambium
892 region; P, pith; x, xylem. In all panels scale bars = 20 µm.
893
894 **Fig. 6. Histological analysis of ARI in Norway spruce hypocotyl cuttings under**
895 **cRL in the presence of IBA, JA or HF.**
896 (A, D, G, J) Cross sections were prepared from the base of de-rooted Norway spruce
897 hypocotyl cuttings, kept under cRL in hormone free (HF) distilled water for 3, 5, 10 or
898 13 days. In (A) the arrows show early cell activation, *i.e.* small cells with dense
899 cytoplasm and large nuclei. In (D) arrows indicate anticlinal and periclinal division. In
900 (G) arrows show the presence of tracheal elements. In (j) arrows indicate meristemoids
901 developing at the periphery of the tracheal elements.
902 (B, E, H, K) Cross sections were taken from the base of de-rooted Norway spruce
903 hypocotyl cuttings, kept under cRL in the presence of 1 µM IBA for 3, 5, 10 or 13 days.

904 In (B) arrows indicate cell divisions; In (H) arrows indicate the presence of
905 meristemoids. In (K) arrows indicate emerged adventitious roots (ARs).
906 (C, F, I, L) Cross sections were prepared from the base of de-rooted Norway spruce
907 hypocotyl cuttings, kept under cRL in the presence of 20 μM JA for 3, 5, 10 or 13 days.
908 e, epidermis; Co, cortex; Ph, phloem; en, endodermis; c, cambium region; P, pith; x,
909 xylem. In all panels scale bars = 20 μm.
910

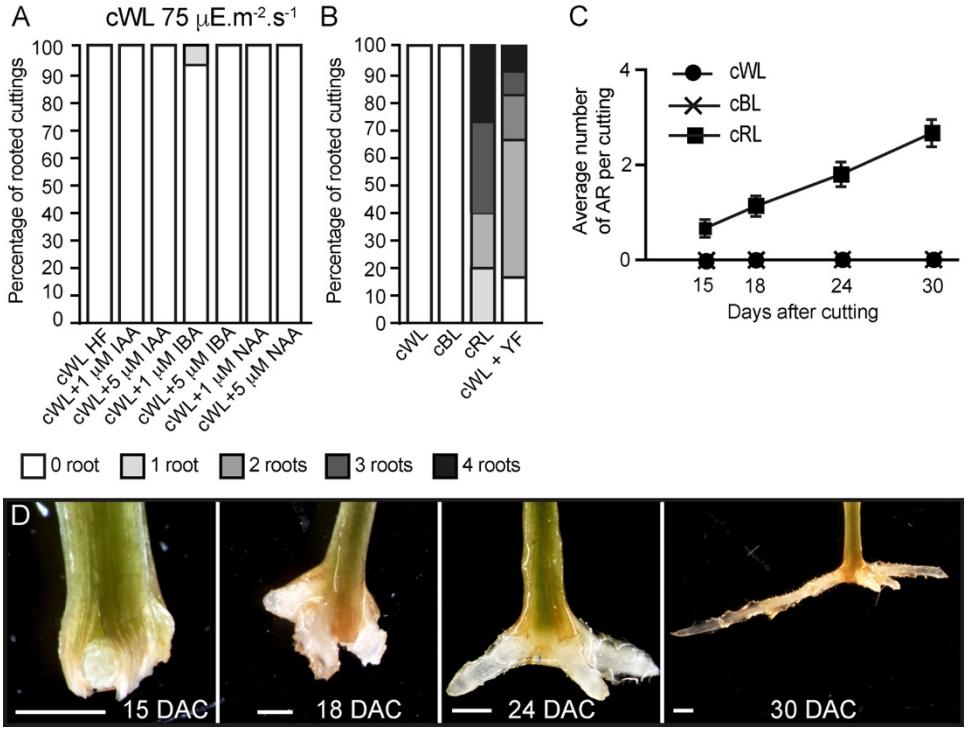


**Fig. 1. Constant blue light inhibits, while constant red light promotes, ARI in de-rooted Norway spruce hypocotyls**

(A) Percentage of rooting in hypocotyl cuttings kept under constant white light (cWL). Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in hormone free (HF) distilled water, or in the presence of 1 or 5 µM indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or 1-naphthalene acetic acid (NAA), under cWL.

(B) Percentage of rooting in hypocotyl cuttings kept under cWL, cWL with a yellow filter (YF), constant red light (cRL) or constant blue light (cBL).

Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in hormone free (HF) distilled water under the different light conditions.

(C) Average number of ARs on Norway spruce hypocotyls kept under cWL, cBL or cRL. Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in hormone free (HF) distilled water. The hypocotyl cuttings were observed every day to count emerging AR primordia and emerged ARs as in (**d**). Fifteen seedlings were analyzed for each condition.

(D) Different stages of AR development in de-rooted 3-week-old Norway spruce seedling in distilled water, under cRL. DAC: days after cut; scale bar = 2 mm.

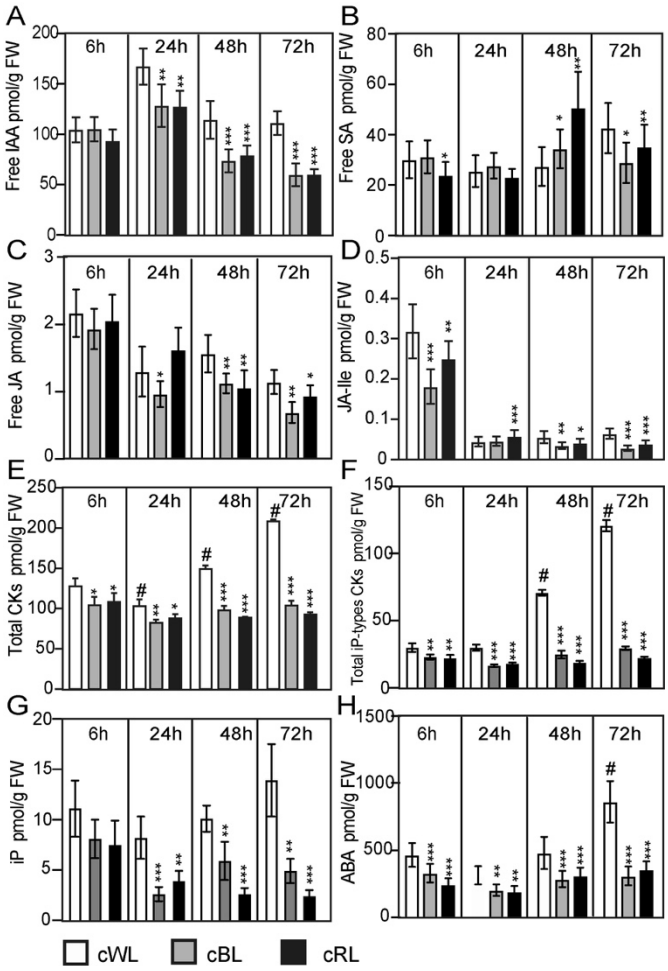


**Fig. 2. Light spectral quality controls the homeostasis of endogenous contents of different hormones, which positively or negatively affect ARI**

(A) to (H) Three-week-old Norway spruce seedlings were grown under long day conditions as described in Materials and methods. De-rooted seedlings were then kept in constant cWL, cBL, or cRL for 6, 24, 48 and 72 hours. For each time point in each condition 5 mm hypocotyls were taken and pooled for hormone quantification. Five independent biological replicates were collected for analysis of IAA, ABA, SA and JA contents, and additional five biological replicates were collected for cytokinin quantification.

(A) free IAA content; (B) free salicylic acid (SA) content; (C) free jasmonic acid (JA) content; (D) jasmonate-isoleucine (JA-Ile) content; (E) content of total cytokinins (CKs); (F) total isopentyl-adenine type (iP-type) CKs; (G) isopentyl-adenine (iP) content; (H) Free abscisic acid (ABA) content.

Values are the means with standard deviation (SD) of 5 biological replicates. Asterisks indicate statistically significant differences under light conditions cRL versus cWL and cBL versus cWL in t-test; *, **, and *** correspond to P-values of ($0.05 > p > 0.01$, $0.01 > p > 0.001$, and $p < 0.001$ respectively; $n = 5$); Dashes indicate statistically significant difference 6 h versus 24 h, 48 h or 72 h in t-test; #, ##, ### correspond to P-values of ($0.05 > p > 0.01$, $0.01 > p > 0.001$, and $p < 0.001$, respectively; n = 5; FW, fresh weight.

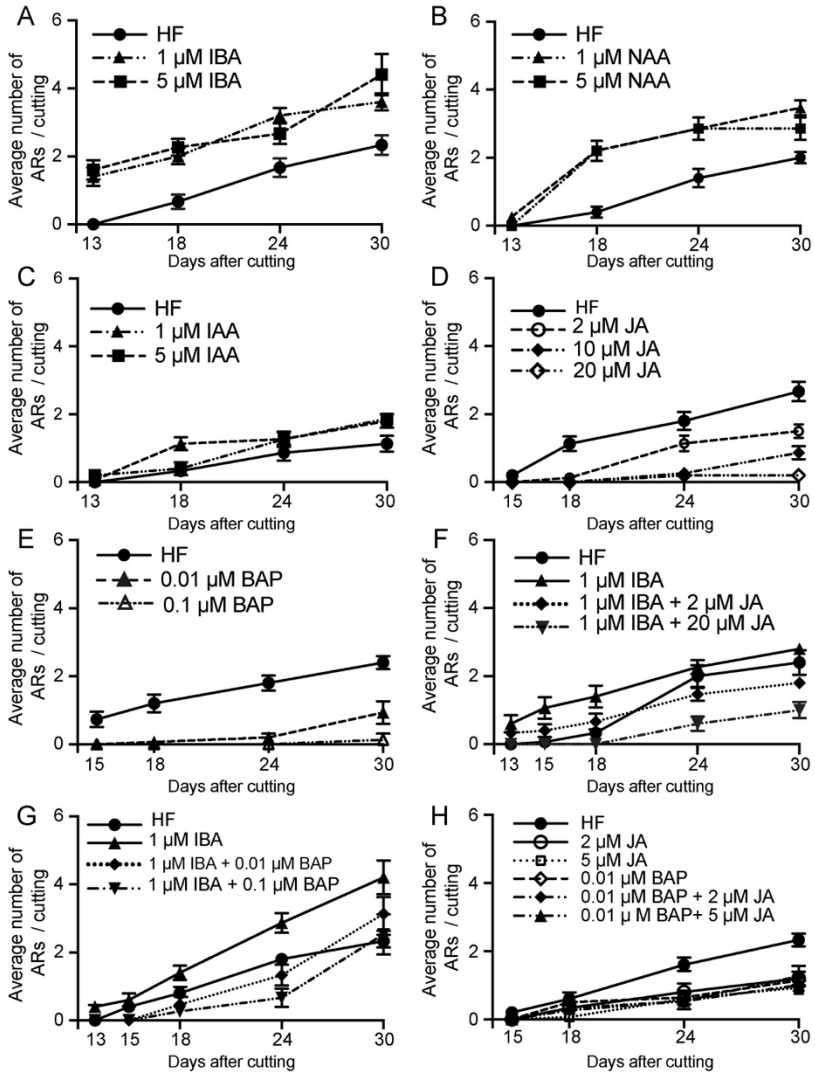


**Fig. 3. Auxin enhances RL-induced adventitious rooting, whereas CKs and JA repress this process**

Three-week-old Norway spruce seedlings were de-rooted and kept under cRL for 30 days in hormone free (HF) distilled water, or in the presence of 1 or 5 µM IBA (A); or in the presence of 1 or 5 µM NAA (B); or in the presence of 1 or 5 µM IAA (C); or in the presence of jasmonate (JA) (D)*;* or in the presence of benzyl adenine (BAP) (D); or in the presence of both JA and IBA (F); or in the presence of both BAP and IBA (G); or in the presence of both JA and BAP (H).

(A) Both concentrations of exogenously applied IBA significantly increased the average number of ARs over time (repeated measures ANOVA; $P < 0.0001$).

(B) Both concentration of exogenously applied NAA significantly increased the average number of adventitious roots over time (repeated measures ANOVA; $P < 0.0001$).

(C) Exogenously applied IAA did not have any significant effect compared to HF conditions (Repeated measures ANOVA; $P = 0.073$).

(D) Exogenously applied JA had a significant negative effect on the average number of ARs over time (repeated measures ANOVA; $P < 0.0001$). This negative effect was concentration dependent (repeated measures ANOVA; $P < 0.0001$).

(E) Exogenously applied BAP significantly reduced the number of ARs compared to HF conditions (repeated measures ANOVA; $P < 0.0001$).
(F) When JA was applied together with IBA, it significantly repressed the positive effect of IBA. The combination 1 μM IBA + 2 μM JA significantly reduced the number of roots compared to those in HF conditions by 24 days after cut (DAC) and 30 DAC (t-test, degree of freedom df = 14; p-value = 0.02 and 0.001 respectively). The combination 1 μM IBA + 20 μM JA significantly reduced the average AR number compared to HF conditions (t-test, df = 14; p-value < 0.01).
(G) When BAP was applied together with IBA, it repressed the positive effect of IBA. The combination 1 μM IBA + 0.01 μM BAP significantly reduced the average number of ARs compared to the 1 μM IBA treatment. (13, 15, 18, 24 and 30 DAC; t-test, df = 14; p-value < 0.009, 0.007, 0.003, 0.0001and 0.02 respectively). The combination 1 μM IBA + 0.1 μM BAP significantly inhibited rooting induced by 1μM IBA at (13,15,18,24 and 30 DAC (t-test, df = 14; p-value < 0.008,0.007,0.0009, 0.000000009 and 0.0009 respectively);
(H) Both JA and BA repressed AR compared to the HF control, but no significant additive or synergistic effect was observed when JA and BAP were applied together. The combination 0.01 μM BAP + 2 μM JA significantly inhibited rooting compared to the HF control at 24 and 30 DAC (t-test, df = 14; p-value < 0.0007 and 0.003 respectively), but the result was not significantly different from JA or BAP alone; The combination of 0.01 μM BAP + 5μM JA significantly inhibited rooting compared to the HF controls at 24 and 30 DAC (t-test, df = 14; p-value < 0.00004 and 0.0000003 respectively), but with no significant difference from JA or BAP alone.
(A to H) Results are expressed as average number of ARs per cutting. Error bars indicate standard error (SE). For each treatment 15 hypocotyls were used, and the experiment was repeated twice.

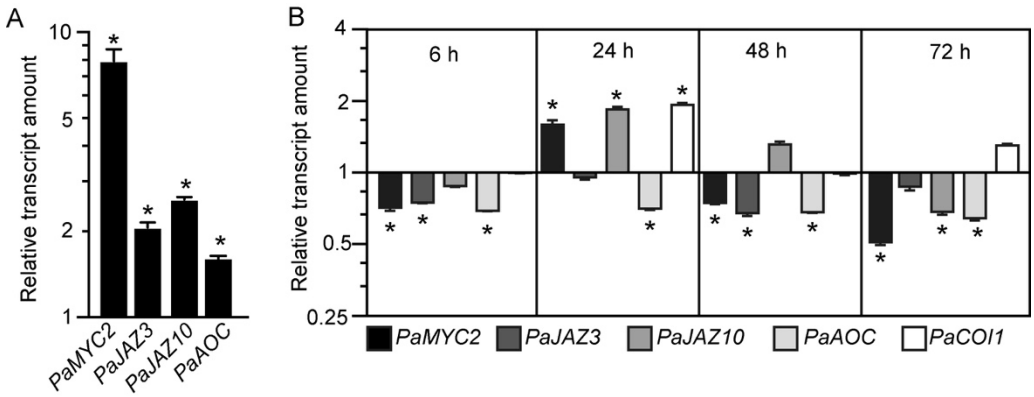


**Fig. 4. JA signaling is downregulated in hypocotyl cuttings kept under constant red light (cRL) compared to hypocotyl cuttings kept under constant white light (cWL)**

(A) Relative transcript amount of putative JA-responsive genes *PaMYC2-like*, *PaJAZ3-like*, *PaJAZ10-like* and *PaAOC-like* in three-week-old Norway spruce seedling hypocotyls treated for 3 h with 50 µM JA or with a mock treatment. Values are relative (on a log10 scale) to the values for mock treated seedlings which were arbitrarily set to 1. Error bars indicate ±SE obtained from two independent biological replicates, with three technical replicates of each. A *t*-test was carried out, and values indicated by asterisks differed significantly from the mock values ($P < 0.001$, $n \geq 80$).

(B) Relative transcript amounts of putative JA-responsive genes *PaMYC2-like*, *PaJAZ3-like*, *PaJAZ10-like* and *PaAOC-like*, and the putative JA receptor *PaCOI1-like*, at the base of three-week-old de-rooted hypocotyls kept under cRL or cWL for 6, 24, 48 and 72 h. The values are relative (on a log2 scale) to the values obtained from seedlings kept under cWL, which were arbitrarily set to 1. Error bars indicate ±SE obtained from two independent biological replicates, with three technical replicates of each. A *t*-test was carried out, and asterisks indicate values that differed significantly from the control values ($P < 0.001$, $n \geq 80$).

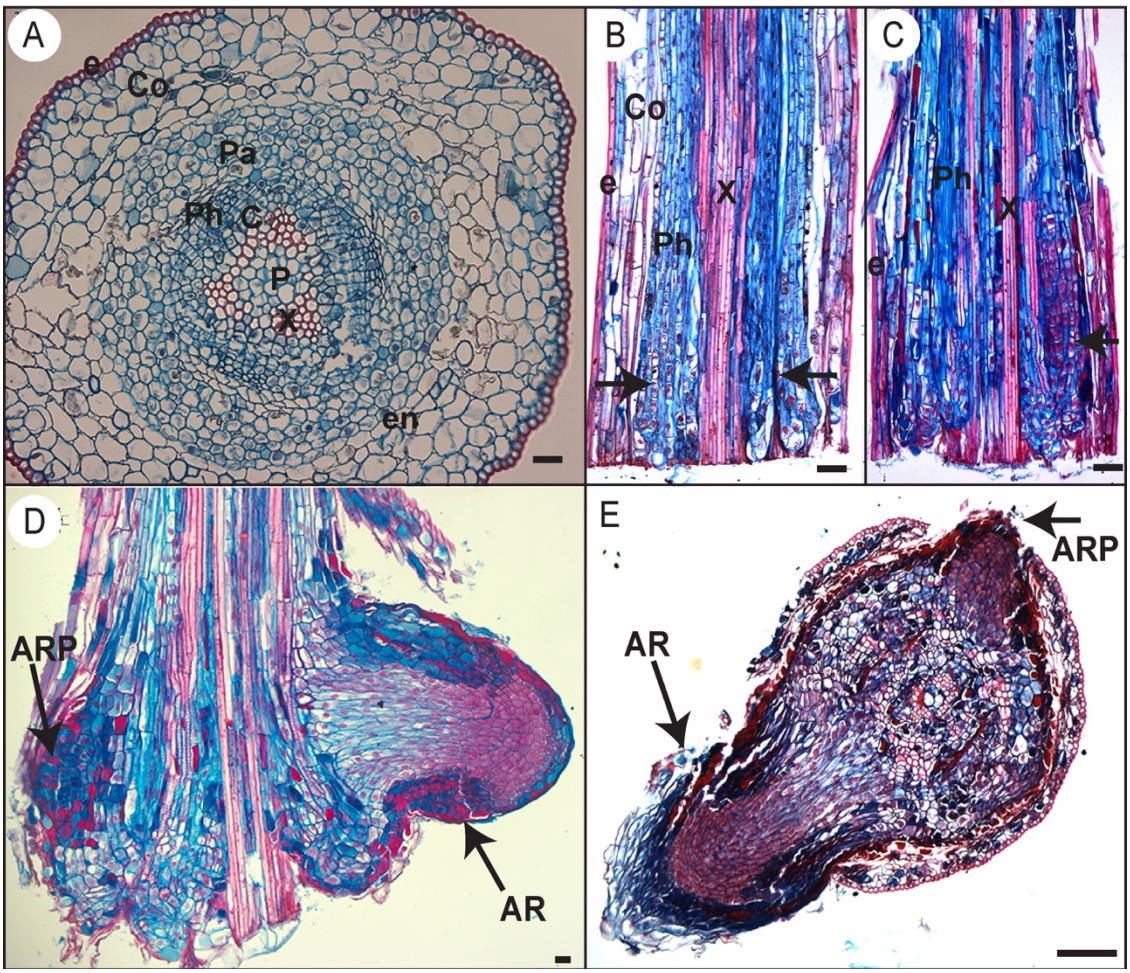


**Fig. 5. Histological analysis of AR development in Norway spruce hypocotyl cuttings under cRL**

(A) Cross section through the base of a 21-day-old hypocotyl cutting just before transfer to rooting conditions.

(B) Longitudinal section through the base of a hypocotyl cutting kept in hormone free (HF) conditions for 5 days. Arrowheads indicate localized cell divisions, probably at the points of origin of future ARs.

(C) Longitudinal section through the base of a hypocotyl cutting kept in the presence of 1 µM IBA for 10 days. Arrowheads indicate the presence of meristemoids.

(D) Longitudinal section through the base of a hypocotyl cutting kept in the presence of 1 µM IBA for 13 days. Arrows indicate an adventitious root primordium (ARP) and an emerged adventitious root (AR).

(E) Cross section through the base of a hypocotyl cutting kept in HF conditions for 15 days. Arrows indicate an ARP and an emerged AR.

e, epidermis; Co, cortex; Pa, parenchyma; Ph, phloem; en, endodermis; c, cambium region; P, pith; x, xylem. In all panels scale bars = 20 µm.

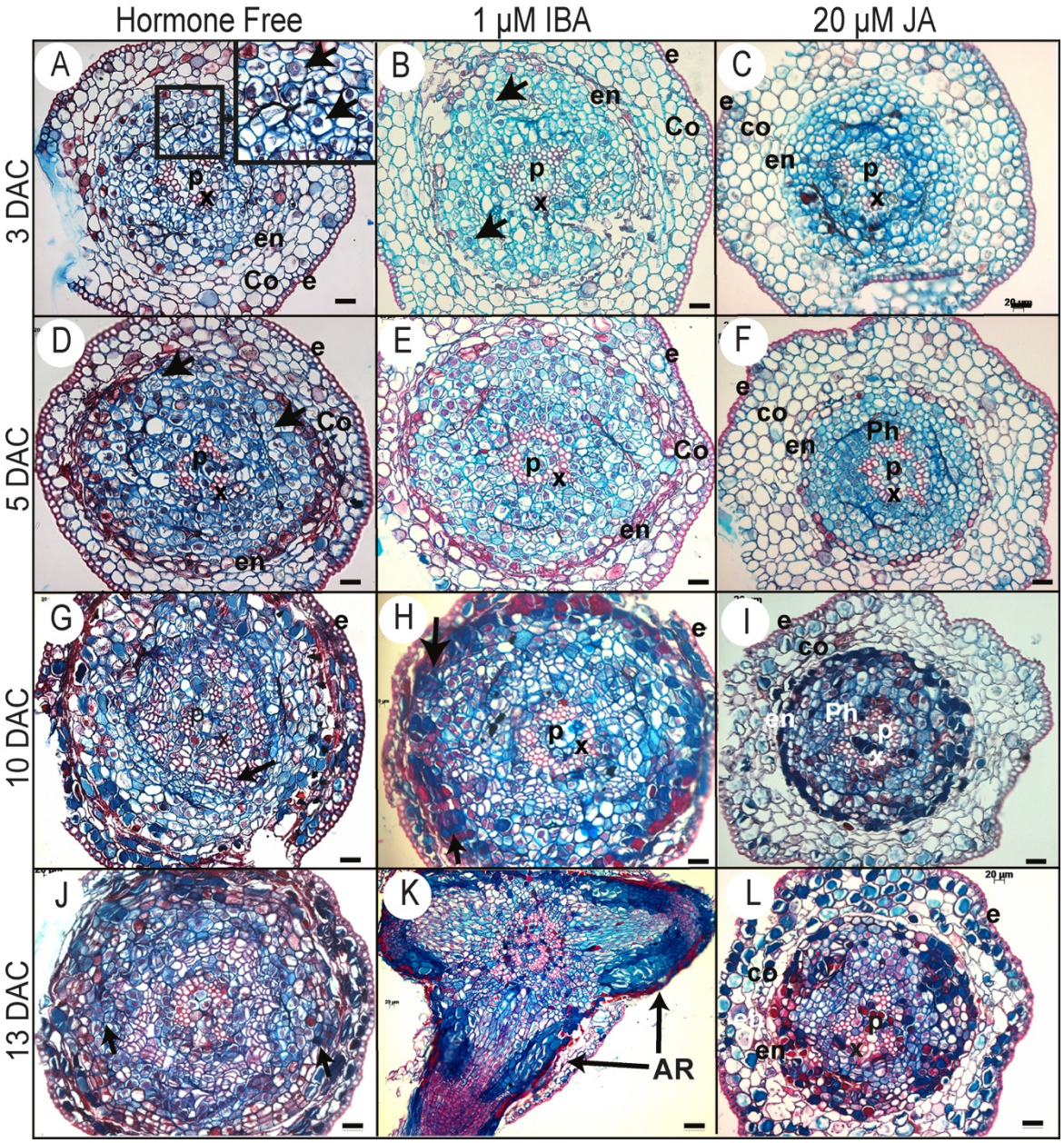


**Fig. 6. Histological analysis of ARI in Norway spruce hypocotyl cuttings under cRL in the presence of IBA, JA or HF.**

(A, D, G, J) Cross sections were prepared from the base of de-rooted Norway spruce hypocotyl cuttings, kept under cRL in hormone free (HF) distilled water for 3, 5, 10 or 13 days. In (A) the arrows show early cell activation, *i.e.* small cells with dense cytoplasm and large nuclei. In (D) arrows indicate anticlinal and periclinal division. In (G) arrows show the presence of tracheal elements. In (j) arrows indicate meristemoids developing at the periphery of the tracheal elements.

(B, E, H, K) Cross sections were taken from the base of de-rooted Norway spruce hypocotyl cuttings, kept under cRL in the presence of 1 µM IBA for 3, 5, 10 or 13 days. In (B) arrows indicate cell divisions; In (H) arrows indicate the presence of meristemoids. In (K) arrows indicate emerged adventitious roots (ARs).

(C, F, I, L) Cross sections were prepared from the base of de-rooted Norway spruce hypocotyl cuttings, kept under cRL in the presence of 20 µM JA for 3, 5, 10 or 13 days.

e, epidermis; Co, cortex; Ph, phloem; en, endodermis; c, cambium region; P, pith; x, xylem. In all panels scale bars = 20 µm.