Light controls *de novo* adventitious root regeneration by modulating jasmonate and cytokinin homeostasis in Norway spruce hypocotyls

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Summary

- Vegetative propagation relies on the capacity of plants to regenerate adventitious roots (ARs) *de novo*. Light plays a central role in this process; however the physiological and molecular components mediating light cues remain largely elusive, especially in conifers. Here, we explore the effect of light spectral quality on AR initiation (ARI) in de-rooted Norway spruce seedlings.
- We used light emitting diodes (LEDs) to study the effects of different light spectra on ARI, hormone metabolism and gene expression. We used sensitive mass spectrometry-based methods, and, since the Norway spruce genome sequence recently became available, we coupled this to gene expression analysis. We performed extensive anatomical characterization to investigate ARI at the cellular level.
- We showed that in contrast to constant white light (WL) and constant blue light (cBL), constant red light (cRL) promoted ARI by reducing jasmonate (JA) and JA-isoleucine contents and repressing the accumulation of isopentyl-adenine-type cytokinins and abscisic acid, which are known to inhibit AR development. We found that JA and CKs possibly act in the same pathway to repress ARI. Anatomical analysis showed that JA represses the early events of ARI.
- In conclusion, cRL promotes ARI by repressing the accumulation of the wound-induced phytohormones JA and CK.

Key words :

Picea abies, adventitious roots, light spectral quality, blue light, red light, cytokinins, jasmonate

54 **Introduction**:

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

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

88 recalcitrant species. For example, treating donor plants of difficult-to-root *Eucalyptus*
89 *globulus* species with far-red-enriched light stimulated the rooting capacity of
90 microcuttings (Ruedell *et al.*, 2015). This stimulation was possibly due to upregulation
91 of the expression of *AUXIN RESPONSE FACTOR6-like (ARF6-like)* and *ARF8-like*
92 genes at the cutting site by far-red light. These transcriptional regulators have been
93 shown to promote ARI in Arabidopsis hypocotyls (Gutierrez *et al.*, 2009, 2012) and in
94 *Populus* stem cuttings (Cai *et al.*, 2019). Far-red-enriched light also induced the
95 expression of a putative indole 3-acetic acid (IAA) biosynthetic gene**,** *YUCCA3-like,*
96 and two putative auxin efflux carriers genes**,** *PIN-FORMED 1-like (PIN1-like)* and
97 *PIN-FORMED 2-like (PIN2-like)***,** in *Eucalyptus globulus* (Ruedell *et al.*, 2015). These
98 data suggest that far-red-enriched light might act on the auxin biosynthesis, transport
99 and/or signaling pathways to stimulate AR formation in *Eucalyptus globulus.* Keeping
100 leafy stem cuttings of *Petunia hybrida* (Klopotek *et al.*, 2016) or *Dianthus caryophyllus*
101 (Agulló-Antón *et al.*, 2011) in dark conditions prior to light exposure improved their
102 rooting capacity. The effect of light on Norway spruce (*Picea abies*) growth and
103 morphogenesis has also been documented (Strömquist & Eliasson, 1979; Mortensen &
104 Sandvik, 1988; Bollmark & Eliasson, 1990; Mølmann *et al.*, 2006; Burescu *et al.*, 2015;
105 OuYang *et al.*, 2015) and it has been shown that high-irradiance white light inhibited
106 AR in Norway spruce stem cuttings (Strömquist & Eliasson, 1979).
107 Despite the importance of light in regulating AR formation, the physiological and
108 molecular mechanisms involved are still unknown. Here, we used de-rooted Norway
109 spruce seedlings as a model system to explore the role of light in the control of AR
110 initiation in conifers. We found that constant red light promotes this process**,** possibly
111 through downregulation of JA signaling and repression of cytokinin and abscisic acid
112 (ABA) accumulation compared to constant white light conditions. It appears that the
113 cooperative role of JA and CK signaling in the repression of ARI is evolutionarily
114 conserved**,** as it represses very early events in the ARI process in Norway spruce
115 hypocotyls.
116
117 **Materials and methods**
118
119 **Plant material**
120 Commercial seeds from Norway spruce (*Picea abies* L. Karst) were provided by
121 Svenska Skogsplantor (https://www.skogsplantor.se/; Sweden) and kept at a

122 temperature of 4 °C prior to use. The seeds were soaked in tap water for 24 h, in the
123 dark at 4 °C**.** They were then sown in fine wet vermiculite as described in Ricci *et al.*
124 (2008) and allowed to germinate and grow in a growth chamber. The seedlings were
125 watered twice a week with tap water and left to grow for three weeks (Fig. **S1a**). The
126 growth conditions were as follow: 16 h light with 75 µmol /m^2/s (400 to 700 nm, Fig.
127 **S2a**); 8 h dark; 20°C day temperature and 18°C night temperature. The white light was
128 provided by 36W/840 Super 80 Cool White TL-D tubes (Philips,
129 https://www.philips.se/).
130
131 **Test of effects of different light conditions on adventitious rooting of de-rooted**
132 **seedlings**
133 Twenty-one days after sowing, the seedling hypocotyls were severed 2 cm below the
134 apex (Fig. **S1b**) and placed in 24 ml vials filled with distilled water (Fig. **S1c**). Three
135 vials containing 5 seedlings each, constituting technical replicates**,** were prepared.
136 In order to test different white light conditions, vials containing de-rooted seedlings
137 were kept in either long-day conditions in the growth chamber as described above (Fig.
138 S2a) or in a Percival growth cabinet equipped with cool white fluorescent tubes
139 (F17T8/TL741, 17 Watt; Philips U.S.A: Percival Scientific, Perry Iowa, USA) (Fig.
140 **S2b**) or in a Percival plant growth cabinet (E-30NL/*floraLED*) equipped with six CLF
141 *floraLED* modules (CLF PlantClimatics GmbH; Emersacker, Germany) providing
142 white, red or blue light (Fig. **S2c,e,f**). Under these latter conditions the light was
143 constant and referred to as constant White Light (cWL), constant Blue Light (cBL) or
144 constant Red Light (cRL), and the temperature was constant and set at 20 °C.
145 In order to remove the blue region of the spectrum from the white LEDs we added a
146 yellow filter, which almost totally abolished the blue peak at 460 nm (Fig. **S2d**).
147 The cBL (460 nm) intensity was set at 9 µmol/m^2/s (Fig. **S2e**), and cRL (660 nm) was
148 set at an intensity of 9 µmol /m^2/s (Fig**. S2f**).
149 The vials were refilled daily with distilled water to replace the water lost by
150 evapotranspiration. The number of roots per cutting and the number of rooted cuttings
151 were monitored for 30 days after cutting (DAC) and all the experiments were repeated
152 at least three times.

153

154 **Hormone treatments**

155 Auxin treatment:

156 Five hypocotyl cuttings per vial, with three technical replicates**,** were cultured in 24 ml
157 of distilled water supplemented with indole butyric acid (IBA), indole acetic acid (IAA)
158 or 1-Naphthalene Acetic Acid (NAA) at final concentrations of 1 or 5 µM, or without
159 supplementation as mock treatment.

160 Jasmonate (JA) treatment:

161 Five hypocotyl cuttings per vial, with three technical replicates**,** were cultured in 24 ml
162 of distilled water supplemented with JA at final concentrations of 2 µM, 10 µM or 20
163 µM**,** or without supplementation as mock treatment.

164 Cytokinin treatment:

165 Five hypocotyl cuttings per vial, with three technical replicates**,** were cultured in 24 ml
166 of distilled water supplemented with 6-benzylaminopurine (BAP) at a final
167 concentration of 0.1 µM or 0.01 µM, or without supplementation.

168 BAP was also used in combination with 1 µM IBA, 2 µM or 5 µM JA.

169 In all these experiments vials were covered with parafilm in order to limit the
170 evaporation of hormone solutions during the rooting period**;** small holes allowed the
171 cuttings to be inserted into the vials.

172

173 **Histology**

174 Five mm sections of hypocotyl cuttings were dissected from three-week-old seedlings
175 at T0**,** just before transferring the cuttings to rooting conditions, then 3, 5, 10, 13 and
176 15 days after cutting. Similar material was taken from de-rooted seedlings treated with
177 1 µM IBA or 20 µM JA. Hypocotyl samples were vacuum infiltrated with fixation
178 medium, 37% formalin, 5% acetic acid, 100% ethanol **,** for 20 seconds and left for 24
179 hours at room temperature. The samples were then washed in fixation solvent (70 %
180 ethanol) for 10 minutes and transferred into 70 % EtOH until required for use. Samples
181 were then gradually dehydrated in an ethanol series (80 %, 90 % , 96 % for 2h each and
182 100 % overnight at room temperature). The 100 % EtOH was gradually replaced by
183 HistoChoice tissue fixative (VWR Life, https://us.vwr.com/) in three steps of 1:3, 1:1,
184 3:1 (EtOH: HistoChoice ratio) and two 1 h steps with pure HistoChoice. The
185 HistoChoice fixative was gradually replaced with Paraplast Plus for tissue embedding
186 (Sigma-Aldrich, https://www.sigmaaldrich.com/), over 6 days. Serial sections (10 µm)
187 were obtained with a Zeiss HM 350 rotary microtome (https://www.zeiss.com/).

188 Longitudinal and transverse sections were stained with safranin (Sigma-Aldrich,
189 https://www.sigmaaldrich.com/) and alcian blue (Sigma-Aldrich,
190 https://www.sigmaaldrich.com/) in a ratio of 1:2; using methods from (Hamann *et al.*,
191 2011).
192
193 **Sources of sequence data and phylogenetic tree construction**
194 The *AtMYC2* (At1g32640), *AtJAZ3* (At3g17860), *AtJAZ10* (At5g13220), *AtAOC1*
195 (At3g25760) and *AtCOI1* (At2g39940) *Arabidopsis thaliana* gene coding sequences
196 (CDS) from TAIR (https://www.arabidopsis.org) were used as queries in BLASTN
197 searches for CDS predicted with high confidence from *P. abies* (http://congenie.org/;
198 Nystedt *et al.*, 2013) to identify potential orthologs in the *Picea abies* genome. All
199 sequence information is provided in Fig. **S4.** To find the putative Arabidopsis orthologs
200 in *Picea abies*, a BLAST alignment of the coding sequences of selected genes was
201 generated using "Genome tools" from the Congenie database with default settings.
202 Subsequently, the sequences were used to construct phylogenetic trees with the tools
203 available at "http://www.phylogeny.fr". The phylogenetic analysis was based on the
204 neighbor-joining method (with 1000 bootstrap replicates). The putative orthologs were
205 named according to the Congenie database.
206
207 **Primer design, efficiency and qRT-PCR analysis**
208 Candidate genes were identified by BLASTN searches against the spruce database
209 (http://congenie.org) using Arabidopsis protein coding nucleotide sequences. The
210 Norway spruce genes that showed the highest sequence similarity with Arabidopsis
211 genes were further considered for primer design and gene expression analysis. Primers
212 for the selected candidate genes and reference genes to be used for qRT-PCR were
213 designed using Primer3web version 4.1.0 (http://primer3.ut.ee)**;** the primer sequences
214 are listed in Table **S1**. Specificity of the primers was analyzed by Sanger sequencing of
215 amplified PCR products and cDNA was used as a template for PCR amplification from
216 each primer pair. For each primer pair, PCR reaction efficiency estimates were
217 calculated from a standard curve generated from a serial dilution of cDNA. Based on
218 the average cycle threshold (Ct) values for each five-fold dilution, a standard curve was
219 generated using linear regression. PCR efficiency (E) was derived from the equation:
220 Efficiency % = (10 (-1/slope) - 1) ×100%. PCR conditions were as follows: 95°C for 1
221 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Finally, dissociation

222 curves were generated by increasing the temperature from 65 to 95°C, stepwise by
223 0.3°C every 10 s. For gene expression analysis by quantitative PCR (qPCR), total RNA
224 was isolated from the base of spruce hypocotyls (5 mm long from 36 to 38 hypocotyl
225 cuttings) using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich;
226 https://www.sigmaaldrich.com/). cDNA was synthesized using an iScript™ cDNA
227 Synthesis Kit (Bio-Rad; https://www.bio-rad.com/en-se/) following DNase treatment.
228 Expression was quantified by the LightCycler® 480 System (Roche, Basel,
229 Switzerland) using LightCycler® 480 SYBR Green I Master (Roche, Basel,
230 Switzerland). For each sample**,** three technical replicates from two independent
231 biological replicates were analyzed. The $\Delta\Delta^{CT}$ method was used to measure the relative
232 amount of transcript from each gene. Expression values were normalized to the
233 expression of two reference genes, *PaEIF4A1* and *PaUBC28***,** and calibrated with the
234 control value arbitrarily set to 1.
235 In order to check whether expression of the selected putative orthologs *PaMYC2-like,*
236 *PaAOC1-like*, *PaJAZ3-like* and *PaJAZ10-like* could be induced by jasmonate, 3-week-
237 old whole Norway spruce seedlings were treated with 50 µM of JA or mock treatment
238 for 3 h. Total RNA was prepared and qPCR experiments performed as described above.
239
240 **Hormone profiling experiments**
241 For hormone quantification**,** 5 mm hypocotyl segments were dissected at the base of
242 de-rooted hypocotyls 6, 24, 48, 72 and 120 hours after they had been transferred to
243 cWL, cBL or cRL, in order to provide an average of 20 mg of fresh weight.
244 Quantification of cytokinin metabolites was performed according to the method
245 described by (Svačinová et al., 2012), including modifications described by (Antoniadi
246 et al., 2015). Samples (20 mg fresh weight, FW) were homogenized and extracted in 1
247 ml of modified Bieleski buffer (60% MeOH, 10% HCOOH and 30% H_2O) together
248 with a cocktail of stable isotope-labeled internal standards (0.25 pmol of CK bases
249 added per sample). The extracts were applied **to** an Oasis MCX column (30 mg/1 ml,
250 Waters) conditioned with 1 ml each of 100% MeOH and H_2O, equilibrated sequentially
251 with 1 ml of 50% (v/v) nitric acid, 1 ml of H_2O, and 1 ml of 1M HCOOH, and washed
252 with 1 ml of 1M HCOOH and 1 ml 100% MeOH. Analytes were then eluted in two
253 steps using 1 ml of 0.35M NH_4OH aqueous solution and 2 ml of 0.35M NH_4OH in 60%
254 (v/v) MeOH. The eluates were then evaporated to dryness *in vacuo* and stored at -20°C.
255 Cytokinin levels were determined by ultra-high performance liquid chromatography-

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

284 **Results:**

285

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

288 To investigate the role of light in adventitious root initiation (ARI) in Norway spruce,
289 we used de-rooted Norway spruce seedlings as a model system. Three-week-old
290 seedlings grown under white light condition were harvested and de-rooted as described
291 in Materials and methods (Fig. **S1**). They were first kept in hormone free (HF) distilled
292 water, under three different white light (WL) regimes as described in Materials and
293 methods (irradiance 69 to 75 $\mu M/m^2/s^{-1}$**;** Fig. **S2a,b,c**). In these conditions no AR
294 developed at the base of the hypocotyls regardless of the photoperiod or the source of
295 white light. Since we obtained the same results in all WL conditions, we continued to
296 use constant WL (cWL) provided by LEDs (Fig**. S1d** and Fig. **S2c**) as control
297 conditions for the rest of the study.

298 It is well known that auxin can stimulate adventitious rooting in several plant species
299 (Lakehal & Bellini, 2019), therefore we tested whether different types of auxin could
300 stimulate ARI under cWL. Three-week-old de-rooted seedlings were treated with 1 or
301 5 µM of Indole 3-Acetic Acid (IAA), 1-Naphthalene 3-Acetic Acid (1-NAA) or Indole
302 3-Butyric Acid (IBA), but none of the auxins could induce ARI under cWL conditions
303 (Fig. **1a**). These results indicate that auxin is not sufficient to induce ARI in de-rooted
304 Norway spruce seedlings kept under cWL.

305

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

308 Next, we wondered whether a particular region of the light spectrum had a negative
309 effect on ARI. To test this hypothesis, three-week old de-rooted seedlings were kept in
310 HF distilled water under either cWL (Fig. **S1d** and Fig**. S2c**), constant blue light (cBL)
311 (Fig. **S1f** and Fig**. S2d**) or constant red light (cRL) (Fig. **S1e** and Fig. **S2e**).
312 Interestingly, AR developed only when seedlings were kept under cRL conditions (Fig.
313 **1 b, c, d**). In these conditions 100% of the cuttings developed at least 1 AR by 30 days
314 after cutting (DAC) (Fig. **1b**). These results raised the possibility that in cWL, the blue
315 light part of the spectrum has a negative effect on ARI. To test this hypothesis**,**
316 hypocotyl cuttings were kept under cWL but a yellow filter was added to remove the
317 blue wavelength region (Fig. **S2f**). In these conditions 83% of the cuttings developed

318 at least 1 AR (Fig. **1b**).
319 These results indicate that cRL promotes ARI, whereas cBL inhibits this process, and
320 the absence of AR under cWL could be due to the presence of blue light.
321
322 **Constant red light induces ARI by reducing endogenous levels of jasmonate,**
323 **cytokinins, and abscisic acid**
324 We have previously shown that ARI in intact etiolated Arabidopsis hypocotyls is under
325 the control of IAA and JA signaling pathways (Gutierrez *et al.*, 2012). More recently
326 we showed that JA and CK cooperatively repress this process (Lakehal *et al.*, 2020a).
327 We therefore hypothesized that light might also modulate phytohormone signaling
328 and/or homeostasis pathways to control ARI in Norway spruce hypocotyls. To test this
329 hypothesis, we quantified the endogenous contents of different hormones known to
330 either inhibit ARI**,** such as JA, abscisic acid (ABA) or CKs (Steffens *et al.*, 2006;
331 Ramírez-Carvajal *et al.*, 2009; Gutierrez *et al.*, 2012; McAdam *et al.*, 2016; Mao *et al.*,
332 2019) or promote ARI**,** such as IAA or salicylic acid (SA) (Gutierrez *et al.*, 2012).
333 Quantifications were performed for hypocotyl samples taken at time 0 (T0)**,** i.e. just
334 before being transferred in HF distilled water, and at the base of hypocotyl cuttings
335 maintained under different light qualities 6, 24, 48 and 72 hours after cutting (Fig. **2;**
336 Supplemental data sets **1** and **2**).
337 Under cWL conditions, we observed a 40% increase in the free IAA level 6 h after
338 cutting compared to the level at T0 **(**Supplemental data set **1**), but thereafter the IAA
339 content remained mostly constant over time, with the exception of a slight increase 24
340 h after cutting (Fig. **2a**; Supplemental data set **1**). There was no effect of wounding on
341 the endogenous content of SA**,** as shown in Supplemental data set**1,** and the SA content
342 remained constant up to 48 h after cutting (Fig. **2b**). A slight increase could be observed
343 72 h after cutting (Fig. **2b**, Supplemental data set **1**). Based on these results, we
344 concluded that the inability to initiate ARs under cWL could not be explained by a
345 reduction in auxin content, and this is in agreement with the fact that exogenous
346 application of IAA or other auxins could not stimulate ARI under these conditions (Fig.
347 **1a**)
348 We have previously shown that JA and its bioactive form Jasmonoyol-Isoleucine (JA-
349 Ile) inhibit ARI in Arabidopsis hypocotyls (Gutierrez *et al.*, 2012; Lakehal *et al.*,
350 2020a). Here, as expected in the case of wounding, JA and JA-Ile were induced 6 h
351 after cutting in all light conditions (Supplemental data set **1**). Under cWL conditions,

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

386 exogenously**,** both JA and 6-Benzylaminopurine (6-BAP) inhibited AR development in
387 a dose-dependent manner (Fig. **3d,e**) and they both repressed the positive effect of
388 exogenous IBA (Fig. **3f,g**). These results suggest that JA and CK act downstream of
389 auxin signaling to repress ARI, as has been described for intact Arabidopsis hypocotyls
390 (Gutierrez *et al.*, 2012; Lakehal *et al.*, 2020a). When JA and CK were combined, no
391 additive or synergistic effect was observed (Fig. **3h**), suggesting that they act in the
392 same pathway. These results are in agreement with our previous data that showed that
393 CK signaling is induced by JA to repress AR formation in intact Arabidopsis
394 hypocotyls (Lakehal *et al.*, 2020a).
395
396 **JA signaling is downregulated in de-rooted Norway spruce hypocotyls kept under**
397 **cRL compared to cWL conditions**
398 The reduction in content of JA and JA-Ile under cRL compared to cWL (Fig. **2b**)
399 prompted us to check whether the expression of genes involved in JA biosynthesis or
400 JA signaling was affected in hypocotyls of seedlings kept under cRL compared to cWL
401 conditions. We first searched the Norway spruce genome (Nystedt *et al.*, 2013) for
402 putative orthologs of the key Arabidopsis genes in JA signaling or biosynthesis using
403 http://congenie.org/. We looked for putative orthologs of the JA-Ile receptor
404 *CORONATINE INSENSITIVE 1 (AtCOI1)* (Xie *et al.*, 1998)*, AtMYC2* transcription
405 factors (Lorenzo *et al.*, 2004)**,** the *JASMONATE ZIM-DOMAIN3 (AtJAZ3) and*
406 *AtJAZ10* transcriptional repressors (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*,
407 2007) and *ALLENE OXYDE CYCLASE (AtAOC*), which encodes a key enzyme in the
408 JA biosynthesis pathway (Stenzel *et al.*, 2012). We found that the Norway spruce
409 genome contains eleven putative *PaCOI1-like* genes (Fig. **S3a**), five putative *PaMYC2-*
410 *like* genes (Fig. **S3b**), one and three putative orthologs of *PaJAZ3-like* and *PaJAZ10-*
411 *like* respectively (Fig. **S3**c) and two putative *PaAOC-like genes* (Fig. **S3d**). The full-
412 length coding sequences are reported in Fig. S4. When more than one putative ortholog
413 was identified we chose that most closely related to the Arabidopsis one for further
414 experiments.
415 We first confirmed that expression of *PaMYC2-like* (MA_15962g0010), *PaJAZ3-like*
416 (MA_6326g0010), *PaJAZ10-like* (MA_10229741g0010) and *PaAOC-like*
417 (MA_56386g0010) was induced by exogenously applied JA (Fig. 4a). We next checked
418 the expression of these genes**,** together with *PaCOI1-like***,** at the base of hypocotyl
419 cuttings kept under cWL or cRL for 6 h, 24 h, 48 h or 72 h after cutting (Fig. **4b**). The

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

453 and large nuclei indicating the initiation of the first cell divisions (Fig. **6 a,b**). In
454 contrast, no obvious histological modifications were observed in the presence of 20 μM
455 JA, and the histological organization remained unchanged compared to day 0 (Fig. **6c**).
456 By five days after cutting, periclinal and anticlinal divisions were clearly observed in
457 the cambial zone and in the outermost layers of the phloem region (Fig. **6d**). In
458 longitudinal section these cells were seen to be organized into vertical files external**,**
459 but adjacent**,** to the vascular cylinder (Fig. **5b**). In the presence of 1 μM IBA it was
460 clearly apparent that the number of cell divisions was higher than under HF conditions
461 (Fig. **6e**)**,** whereas no divisions were observed in the presence of 20 μM JA (Fig. **6f**).
462 Ten days after cutting, in HF conditions, cambial derivatives divided and formed radial
463 rows of tracheal elements around the xylem**,** but no AR meristemoids were observed
464 (Fig. **6g**)**,** while in the presence of 1 μM IBA several AR root meristemoids were visible
465 (Fig. **5c** and Fig. **6h**); no cell divisions were observed in the presence of 20 μM JA (Fig.
466 **6i**)
467 It was only by thirteen days after cutting that AR meristemoids began to form at the
468 periphery of the trachea elements in HF conditions (Fig. **6j**)**,** while in the presence of
469 IBA**,** AR had already emerged (Fig. **5d** and Fig. **6k**)**;** still no cell division activity was
470 observed in the presence of JA (Fig. **6l**). In HF, the first emergence of ARs was
471 observed only 15 days after cutting (Fig. **5e**). These data clearly indicate that
472 exogenously applied JA repressed the very early stage of ARI, whereas auxin not only
473 promoted but also speeded up the process.
474
475 **Discussion**
476 When optimizing conditions for the development of roots from cuttings during
477 vegetative propagation, it has long been considered that light should be taken into
478 account as an important factor. Since the early eighties, studies related to the effect of
479 light on AR formation in woody plant cuttings have been pursued (Druart *et al.*, 1982;
480 Hammerschlag, 1987; Klopotek *et al.*, 2010). Several studies have addressed the effect
481 of light quality and/or intensity on the rooting of cuttings (Jarvis & Shaheed, 1987;
482 Baraldi *et al.*, 1988; Fuernkranz *et al.*, 1990; Fett-Neto *et al.*, 2001; Iacona & Muleo,
483 2010; Daud *et al.*, 2013), but the exact molecular and genetic mechanisms underlying
484 the role of light as well as the downstream targets of light signals during ARI remain
485 poorly understood. In this study, we showed that under cWL conditions de-rooted
486 Norway spruce hypocotyls were unable to produce AR and that this was unlikely to be

487 due solely to an increase in endogenous CK levels, as suggested by (Strömquist &
488 Eliasson, 1979; Bollmark & Eliasson, 1990) since exogenously applied auxin was
489 unable to stimulate AR development. This result led us to hypothesize that specific
490 wavelengths within the white spectrum could be responsible for this strong inhibition,
491 and we therefore grew seedlings under white or monochromatic LEDs. In these
492 conditions, we showed that AR developed under cRL even in the absence of exogenous
493 auxin application, while they did not develop under cBL.
494 The cRL-mediated ARI is possibly due to a faster reduction in JA and JA-Ile contents
495 compared to cWL. cRL also repressed the accumulation of CKs and ABA which was
496 observed under cWL. Reduction in the amounts of the three negative regulators to
497 below a certain threshold led to a de-repression of ARI.
498 The reduced amount of JA and JA-Ile in the base of cuttings could be partially explained
499 by downregulation of *de novo* JA biosynthesis**,** since we observed a reduced amount of
500 the JA precursor *cis*-12-oxo-phytodienoic acid (*cis*-OPDA) under cRL compared to
501 cWL conditions 24, 48 and 72 h after cutting (Supplemental data set **2**). This result is
502 supported by the downregulation of expression of *PaAOC-like*, a key gene in the JA
503 biosynthesis pathway. The link between light and the JA signaling pathway has been
504 proposed in several contexts, but how it affects JA biosynthesis is not yet known.
505 Interestingly, several reports have revealed mechanistic insights into the roles of RL or
506 red/far-red light ratio in the control of plant development or in adjusting growth-defense
507 tradeoffs (Kazan & Manners, 2011). For example, (Campos *et al.*, 2016) found that
508 mutating the RL-receptor *Phytochrome B* (*PhyB*) suppressed the stunted rosette
509 phenotype of the *JazQ* mutant, which harbors five null mutations in JAZ family
510 members. These data suggest that *PhyB*-mediated light signaling interacts with JA
511 signaling to control growth in Arabidopsis. Under our conditions, we found that the
512 expression levels of *PaMYC2-like*, a master transcriptional regulator of JA signaling,
513 were slightly but significantly downregulated under cRL compared to cWL. This result
514 could be explained either by a direct effect of RL on transcriptional regulation or by the
515 reduced amount of the bioactive form JA-Ile present under these conditions. The latter
516 assumption is supported by the fact that exogenous application of JA strongly induced
517 the expression of *PaMYC2-like,* an observation consistent with what has been reported
518 in Arabidopsis. The reduced amounts of JA and JA-Ile and the downregulation of the
519 expression of *MYC2-like* under cRL conditions led to a reduced JA response**,** as

520 indicated by downregulation of the steady state level of expression of the well-known
521 JA responsive genes (*PaJAZ10-like* and *PaJAZ3-like*).
522 Although the negative role of JA on ARI has been demonstrated in the model plant
523 Arabidopsis, its exact role in other species previously remained unclear (reviewed in
524 (Lakehal *et al.*, 2020b). Our results showed that JA is a negative regulator of ARI in
525 de-rooted Norway spruce hypocotyl and that it acts during the very early stages of
526 initiation**,** as indicated by anatomical characterization. We showed that under cRL,
527 certain cells, most likely cambial cells, have the competence to form ARs in a similar
528 way to that described for other conifers such as *Pinus taeda*, *P. contorta* and *P. radiata*
529 (Diaz-Sala *et al.*, 1996; Lindroth *et al.*, 2001b,a; Ricci *et al.*, 2008). When we exposed
530 the de-rooted hypocotyls to exogenous auxin, these cells exhibited rapid cell division
531 and re-orientation of division planes to organize the AR meristem**;** in contrast when
532 exogenous JA was added**,** no cell divisions were observed. In addition, we showed that
533 JA repressed the positive effect of auxin when the compounds were applied together,
534 supporting the conclusion that, as in Arabidopsis, JA acts downstream of auxin in the
535 control of ARI in de-rooted Norway spruce hypocotyls. Moreover, we showed that, like
536 JA, CK inhibits ARI and counteracts the action of auxin, but when combined with JA
537 no additive or synergistic effect was observed, which is in agreement with our recent
538 results showing that cytokinins act downstream of JA to repress ARI in Arabidopsis
539 hypocotyls (Lakehal *et al.*, 2020a).
540 Our data also showed that total CKs accumulated at the base of cuttings when the de-
541 rooted seedlings were exposed to cWL, whereas their amount remained constant over
542 time under cRL conditions. Under cWL, the increasing amounts of the iP-type CKs
543 could be due to *de novo* biosynthesis since we also observed an increased level of the
544 precursors iPR and iPRMP over time. (Bollmark & Eliasson, 1990) showed that de-
545 rooted Norway spruce hypocotyls kept under high irradiance WL (270 $\mu E.m^{-2}.s^{-1}$) could
546 not develop ARs, probably because of the accumulation of CKs. Nevertheless, the
547 effect of WL may be more complex**,** since under our conditions, although the WL
548 irradiance was much lower (75 $\mu E.m^{-2}.s^{-1}$), CKs still accumulated. In our conditions, as
549 a response to wounding, JA and JA-Ile accumulated at the base of hypocotyls 6 h after
550 cutting, and their content started decreasing thereafter, whereas iP-type CKs began to
551 accumulate 48 hours after cutting. Recently we showed that MYC2-mediated JA
552 signaling promotes the accumulation of CKs through direct control of expression of the
553 *APETALA2/ETHYLENE RESPONSE FACTOR 115* transcription factor in Arabidopsis

554 hypocotyls (Lakehal *et al.*, 2020a). We therefore propose that in Norway spruce
555 hypocotyls, wound induced accumulation of JA and JA-Ile triggers *de novo* cytokinin
556 biosynthesis which results in inhibition of rooting. Interestingly, under constant cRL**,**
557 the more rapid reduction in JA and JA-Ile contents and subsequent downregulation of
558 MYC2-mediated JA signaling may prevent the induction of *de novo* CK biosynthesis
559 and this would allow ARI, although we also cannot rule out a direct effect of cRL on
560 the repression of CK accumulation.
561 In conclusion**,** we propose that de-rooted Norway spruce seedlings do not develop AR
562 under WL**,** on the one hand because they accumulate CKs and ABA which are
563 repressors of ARI and on the other because of the presence of light in the blue region
564 of the spectrum which has a negative effect independently of JA, CKs and ABA.
565 Understanding the role of blue light requires further investigation. In contrast**,** we
566 showed that red light has a positive effect on ARI**,** most likely because it represses the
567 JA signaling pathway and the accumulation of CKs.
568
569 **Acknowledgements:**
570 The authors sincerely thank Marta Derba Maceluch from the UPSC Microscopy
571 platform for her advice and help with histological characterization.
572
573 **Authors contributions :**
574 SA, AL and CB conceived and designed the experiments. SA, AR, FB, ON and AL
575 performed the experiments. SA wrote the first draft of the manuscript. SA, AL and CB
576 reviewed and edited the manuscript. All authors read and approved the final article for
577 publication. AL and CB supervised the work. SA, ON and CB acquired funding.
578
579 **Funding:**
580 This research was supported by the Ministry of Higher Education and Scientific
581 Research in Iraq (to SA), by grants from the Swedish research councils FORMAS, VR,
582 VINNOVA, Kempestiftelserna, and the Carl Tryggers Stiftelse (to CB), and by grants
583 from the Ministry of Education, Youth and Sports of the Czech Republic (European
584 Regional Development Fund-Project "Plants as a tool for sustainable global
585 development" No. CZ.02.1.01/0.0/0.0/16_019/0000827), and the Czech Science
586 Foundation (Project No. 19-00973S) (to ON)
587

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751 **Figure Legends:**

752

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

755 (**a**) Percentage of rooting in hypocotyl cuttings kept under constant white light (cWL).
756 Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in
757 hormone free (HF) distilled water, or in the presence of 1 or 5 μM indole-3-acetic acid
758 (IAA), indole-3-butyric acid (IBA) or 1-naphthalene acetic acid (NAA), under cWL.
759 (**b**) Percentage of rooting in hypocotyl cuttings kept under cWL, cWL with a yellow
760 filter (YF), constant red light (cRL) or constant blue light (cBL).
761 Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in
762 hormone free (HF) distilled water under the different light conditions.
763 (**c**) Average number of ARs on Norway spruce hypocotyls kept under cWL, cBL or
764 cRL. Three-week-old Norway spruce seedlings were de-rooted and kept for 30 days in
765 hormone free (HF) distilled water. The hypocotyl cuttings were observed every day to
766 count emerging AR primordia and emerged ARs as in (**d**). Fifteen seedlings were
767 analyzed for each condition.
768 (**d**) Different stages of AR development in de-rooted 3-week-old Norway spruce
769 seedling in distilled water, under cRL. DAC: days after cut; scale bar = 2 mm.

770

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

773 (**a**) to (**h**) Three-week-old Norway spruce seedlings were grown under long day
774 conditions as described in Materials and methods. De-rooted seedlings were then kept
775 in constant cWL, cBL, or cRL for 6, 24, 48 and 72 hours. For each time point in each
776 condition 5 mm hypocotyls were taken and pooled for hormone quantification. Five
777 independent biological replicates were collected for analysis of IAA, ABA, SA and JA
778 contents, and additional five biological replicates were collected for cytokinin
779 quantification.
780 (**a**) free IAA content; (**b**) free salicylic acid (SA) content; (**c**) free jasmonic acid (JA)
781 content; (**d**) jasmonate-isoleucine (JA-Ile) content; (**e**) content of total cytokinins
782 (CKs); (**f**) total isopentyl-adenine type (iP-type) CKs; (**g**) isopentyl-adenine (iP)
783 content; (**h**) Free abscisic acid (ABA) content.

784 Values are the means with standard deviation **(**SD**)** of 5 biological replicates. Asterisks
785 indicate statistically significant differences under light conditions cRL versus cWL and
786 cBL versus cWL in t-test; *, **, and *** correspond to P-values of (0.05 > p > 0.01,
787 0.01 > p > 0.001, and p < 0.001 respectively; *n = 5*); Dashes indicate statistically
788 significant difference 6 h versus 24 h, 48 h or 72 h in t-test; #, ##, ### correspond to P-
789 values of (0.05 > p > 0.01, 0.01 > p > 0.001, and p < 0.001, respectively; n = 5; FW,
790 fresh weight.
791
792 **Fig. 3 Auxin enhances RL-induced adventitious rooting, whereas CKs and JA**
793 **repress this process**
794 Three-week-old Norway spruce seedlings were de-rooted and kept under cRL for 30
795 days in hormone free (HF) distilled water, or in the presence of 1 or 5 μM IBA (**a**)*;* or
796 in the presence of 1 or 5 μM NAA (**b**); or in the presence of 1 or 5 μM IAA (**c**); or in
797 the presence of jasmonate (JA) (**d**); or in the presence of benzyl adenine (BAP) (**e**); or
798 in the presence of both JA and IBA (**f**); or in the presence of both BAP and IBA (**g**); or
799 in the presence of both JA and BAP (**h**).
800 (**a)** Both concentrations of exogenously applied IBA significantly increased the average
801 number of ARs over time (repeated measures ANOVA; P < 0.0001).
802 (**b**) Both concentration of exogenously applied NAA significantly increased the
803 average number of adventitious roots over time (repeated measures ANOVA; P <
804 0.0001).
805 (**c**) Exogenously applied IAA did not have any significant effect compared to HF
806 conditions (Repeated measures ANOVA; P = 0.073).
807 (**d**) Exogenously applied JA had a significant negative effect on the average number of
808 ARs over time (repeated measures ANOVA; P < 0.0001). This negative effect was
809 concentration dependent (repeated measures ANOVA; P < 0.0001).
810 (**e**) Exogenously applied BAP significantly reduced the number of ARs compared to
811 HF conditions (repeated measures ANOVA; P < 0.0001).
812 (**f**) When JA was applied together with IBA, it significantly repressed the positive effect
813 of IBA. The combination 1 μM IBA + 2 μM JA significantly reduced the number of
814 roots compared to those in HF conditions by 24 days after cut (DAC) and 30 DAC (t-
815 test, degree of freedom df = 14; p-value = 0.02 and 0.001 respectively). The

816 combination 1 μM IBA + 20 μM JA significantly reduced the average AR number
817 compared to HF conditions (t-test, df = 14; p-value < 0.01).
818 (**g**) When BAP was applied together with IBA, it repressed the positive effect of IBA.
819 The combination 1 μM IBA + 0.01 μM BAP significantly reduced the average number
820 of ARs compared to the 1 μM IBA treatment. (13, 15, 18, 24 and 30 DAC; t-test, df =
821 14; p-value < 0.009, 0.007, 0.003, 0.0001and 0.02 respectively). The combination 1
822 μM IBA + 0.1 μM BAP significantly inhibited rooting induced by 1μM IBA at
823 (13,15,18,24 and 30 DAC (t-test, df = 14; p-value < 0.008,0.007,0.0009, 0.000000009
824 and 0.0009 respectively);
825 (**h**) Both JA and BA repressed AR compared to the HF control, but no significant
826 additive or synergistic effect was observed when JA and BAP were applied together.
827 The combination 0.01 μM BAP + 2 μM JA significantly inhibited rooting compared to
828 the HF control at 24 and 30 DAC (t-test, df = 14; p-value < 0.0007 and 0.003
829 respectively)**,** but the result was not significantly different from JA or BAP alone; The
830 combination of 0.01 μM BAP + 5μM JA significantly inhibited rooting compared to
831 the HF controls at 24 and 30 DAC (t-test, df = 14; p-value < 0.00004 and 0.0000003
832 respectively), but with no significant difference from JA or BAP alone.
833 (**a** to **h**) Results are expressed as average number of ARs per cutting. Error bars indicate
834 standard error (SE). For each treatment 15 hypocotyls were used, and the experiment
835 was repeated twice.
836
837 **Fig. 4 JA signaling is downregulated in hypocotyl cuttings kept under constant red**
838 **light (cRL) compared to hypocotyl cuttings kept under constant white light (cWL)**
839 (**a**) Relative transcript amount of putative JA-responsive genes *PaMYC2-like*, *PaJAZ3-*
840 *like*, *PaJAZ10-like* and *PaAOC-like* in three-week-old Norway spruce seedling
841 hypocotyls treated for 3 h with 50 μM JA or with a mock treatment. Values are relative
842 (on a log10 scale) to the values for mock treated seedlings which were arbitrarily set to
843 1. Error bars indicate ±SE obtained from two independent biological replicates, with
844 three technical replicates of each. A *t*-test was carried out, and values indicated by
845 asterisks differed significantly from the mock values ($P < 0.001$, $n \geq 80$).
846 (**b**) Relative transcript amounts of putative JA-responsive genes *PaMYC2-like*,
847 *PaJAZ3-like*, *PaJAZ10-like* and *PaAOC-like*, and the putative JA receptor *PaCOI1-*
848 *like*, at the base of three-week-old de-rooted hypocotyls kept under cRL or cWL for 6,

849 24, 48 and 72 h. The values are relative (on a log2 scale) to the values obtained from
850 seedlings kept under cWL, which were arbitrarily set to 1. Error bars indicate ±SE
851 obtained from two independent biological replicates, with three technical replicates of
852 each. A *t*-test was carried out, and asterisks indicate values that differed significantly
853 from the control values ($P < 0.001$, $n \geq 80$).
854
855 **Fig. 5 Histological analysis of AR development in Norway spruce hypocotyl**
856 **cuttings under cRL**
857 (**a**) Cross section through the base of a 21-day-old hypocotyl cutting just before transfer
858 to rooting conditions.
859 (**b**) Longitudinal section through the base of a hypocotyl cutting kept in hormone free
860 (HF) conditions for 5 days. Arrowheads indicate localized cell divisions, probably at
861 the points of origin of future ARs.
862 (**c**) Longitudinal section through the base of a hypocotyl cutting kept in the presence of
863 1 μM IBA for 10 days. Arrowheads indicate the presence of meristemoids.
864 (**d**) Longitudinal section through the base of a hypocotyl cutting kept in the presence of
865 1 μM IBA for 13 days. Arrows indicate an adventitious root primordium (ARP) and an
866 emerged adventitious root (AR).
867 (**e**) Cross section through the base of a hypocotyl cutting kept in HF conditions for 15
868 days. Arrows indicate an ARP and an emerged AR.
869 e, epidermis; Co, cortex; Pa, parenchyma; Ph, phloem; en, endodermis; c, cambium
870 region; P, pith; x, xylem. In all panels scale bars = 20 μm.
871
872 **Fig. 6 Histological analysis of ARI in Norway spruce hypocotyl cuttings under cRL**
873 **in the presence of IBA, JA or HF.**
874 (**a, d, g, j**) Cross sections were prepared from the base of de-rooted Norway spruce
875 hypocotyl cuttings, kept under cRL in hormone free (HF) distilled water for 3, 5, 10 or
876 13 days. In (**a**) the arrows show early cell activation, *i.e.* small cells with dense
877 cytoplasm and large nuclei. In (**d**) arrows indicate anticlinal and periclinal division. In
878 (**g**) arrows show the presence of tracheal elements. In (**j**) arrows indicate meristemoids
879 developing at the periphery of the tracheal elements.
880 (**b, e, h, k**) Cross sections were taken from the base of de-rooted Norway spruce
881 hypocotyl cuttings, kept under cRL in the presence of 1 μM IBA for 3, 5, 10 or 13 days.

882 In (**b**) arrows indicate cell divisions; In (**h**) arrows indicate the presence of
883 meristemoids. In (**k**) arrows indicate emerged adventitious roots (ARs).
884 (**c, f, i, l**) Cross sections were prepared from the base of de-rooted Norway spruce
885 hypocotyl cuttings, kept under cRL in the presence of 20 μM JA for 3, 5, 10 or 13 days.
886 e, epidermis; Co, cortex; Ph, phloem; en, endodermis; c, cambium region; P, pith; x,
887 xylem. In all panels scale bars = 20 μm.

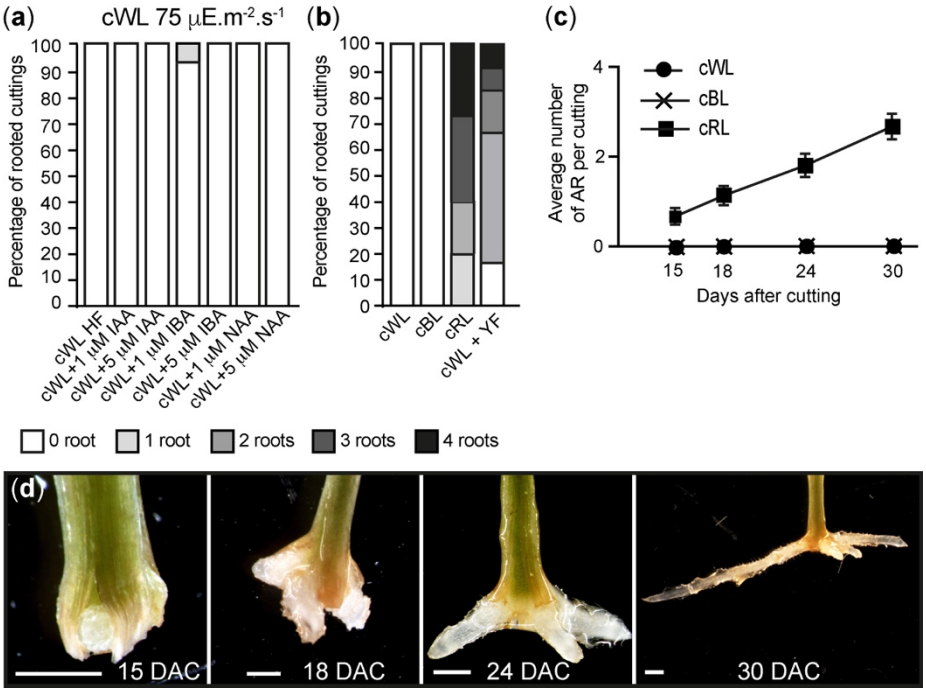

Fig. 1 Constant blue light inhibits, while constant red light promotes, ARI in de-rooted Norway spruce hypocotyls

(**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 cutting; scale bar = 2 mm.

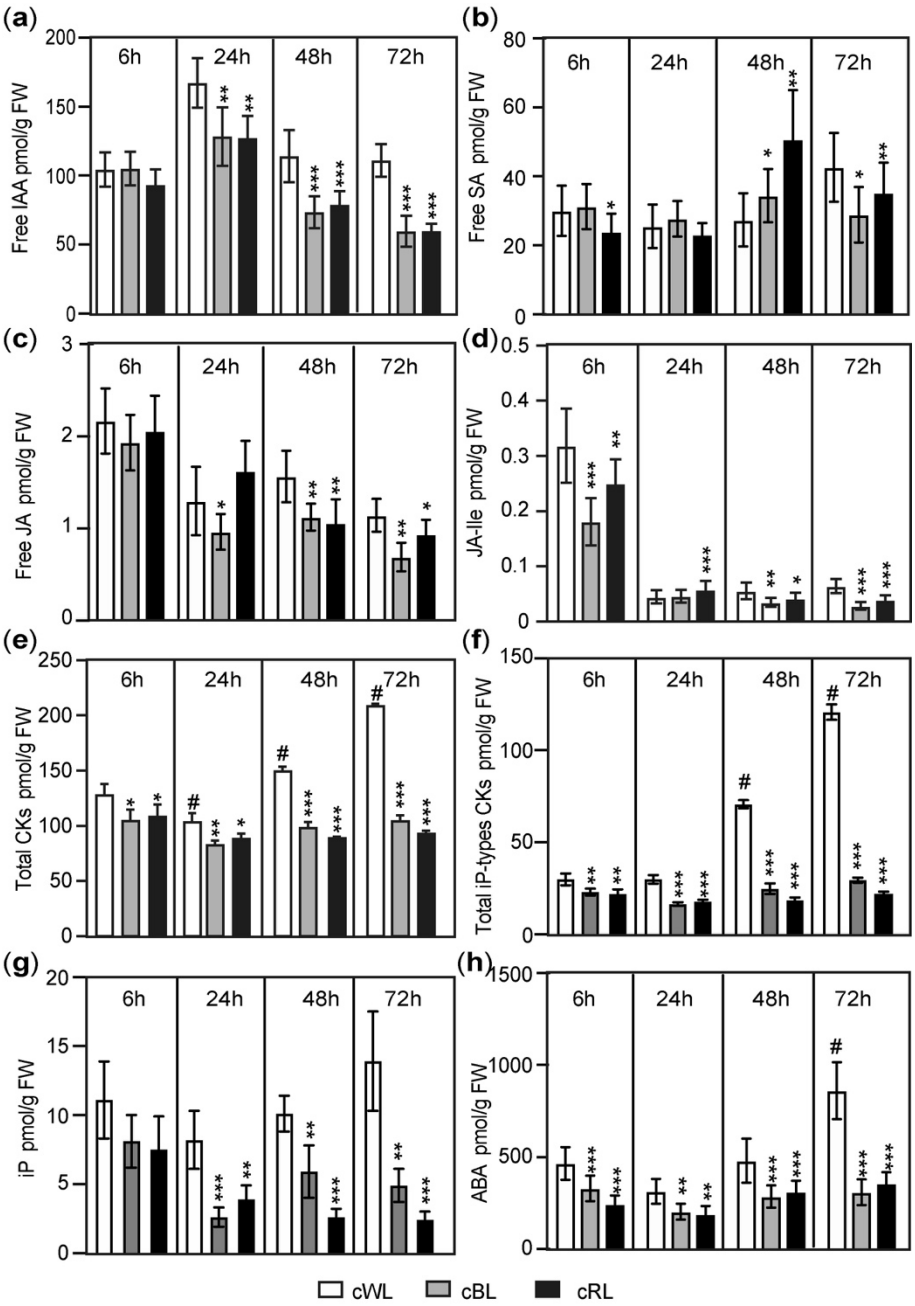

Fig. 2 Light spectral quality controls the homeostasis of endogenous contents of different hormones, which positively or negatively affect ARI

(**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.

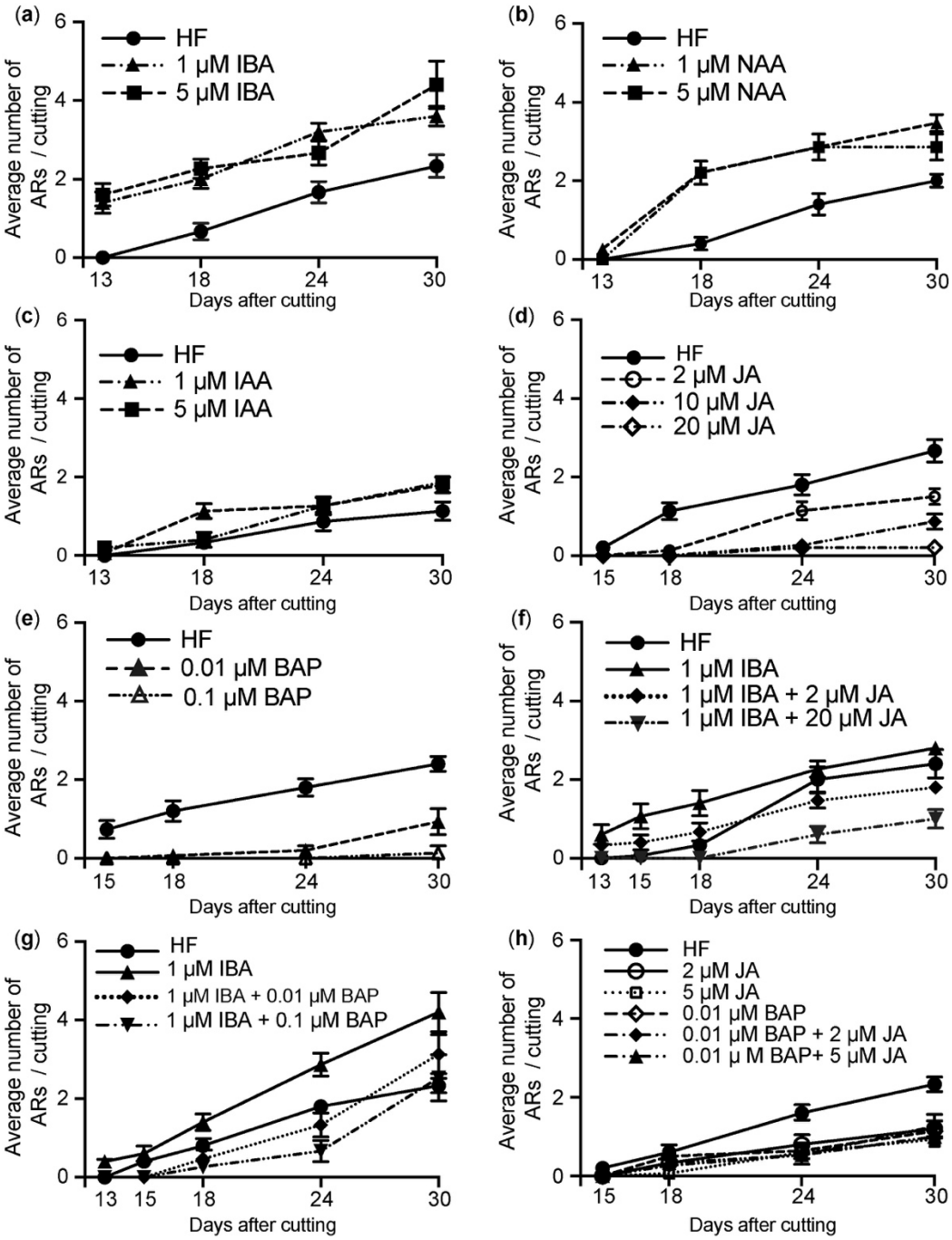

Fig. 3 Auxin enhances RL-induced adventitious rooting, whereas CKs and JA repress this process

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) (**e**); 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.

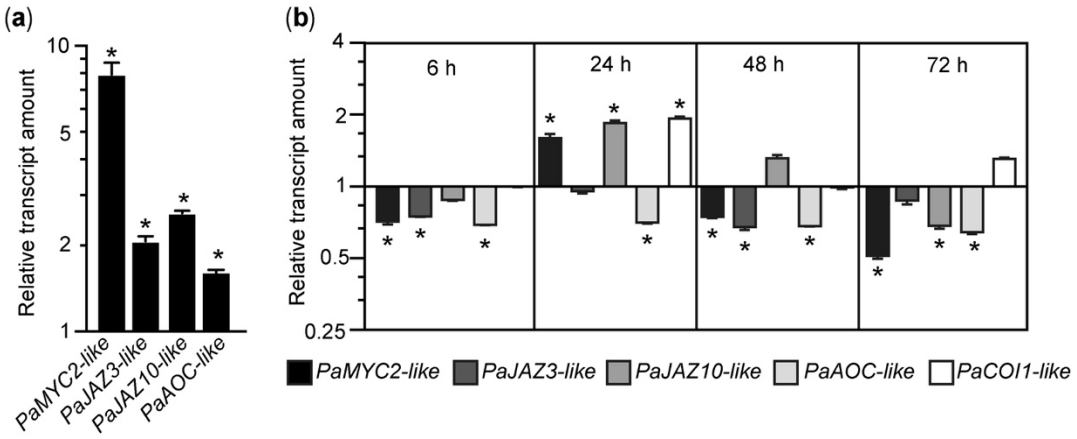

Fig. 4 JA signaling is downregulated in hypocotyl cuttings kept under constant red light (cRL) compared to hypocotyl cuttings kept under constant white light (cWL)

(**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 ($P < 0.001$, $n \geq 80$).

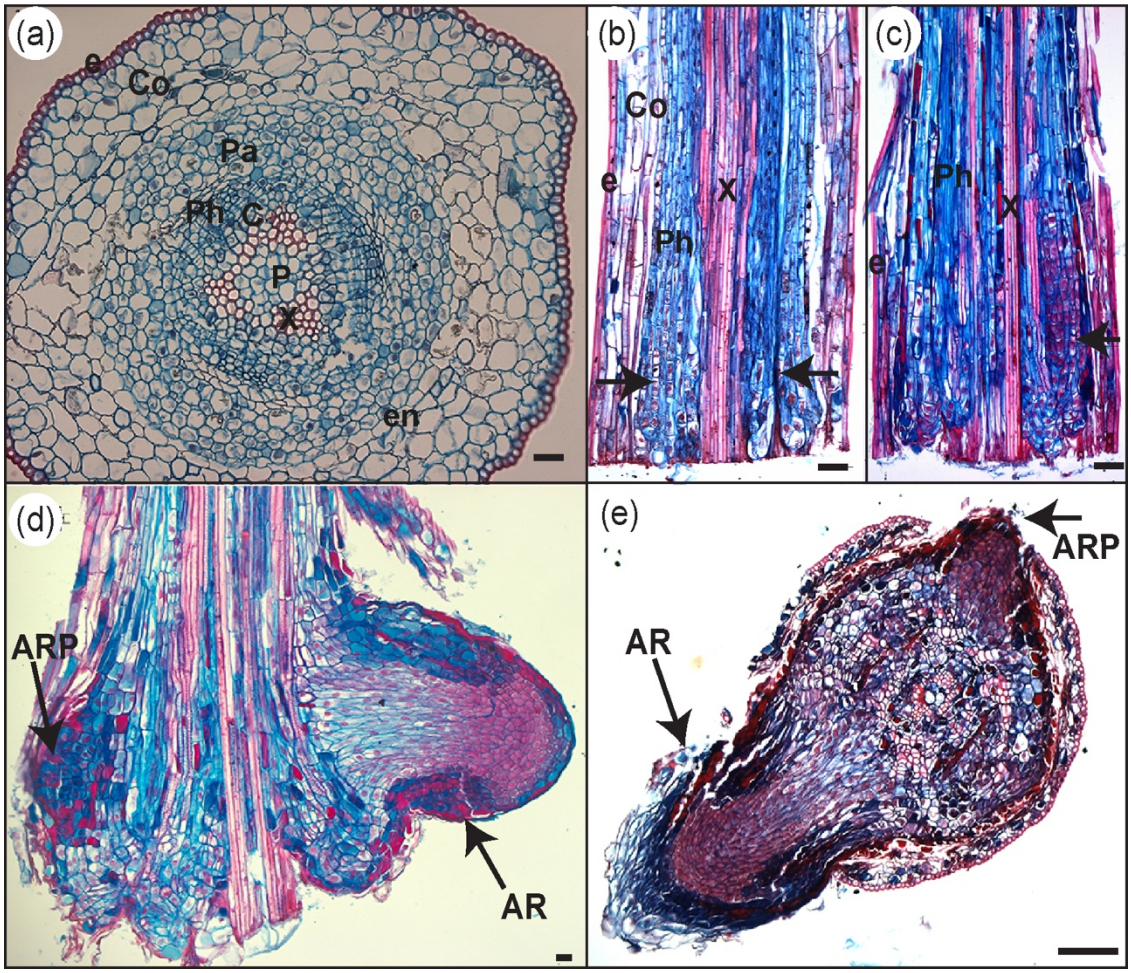

Fig. 5 Histological analysis of AR development in Norway spruce hypocotyl cuttings under cRL

(**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.

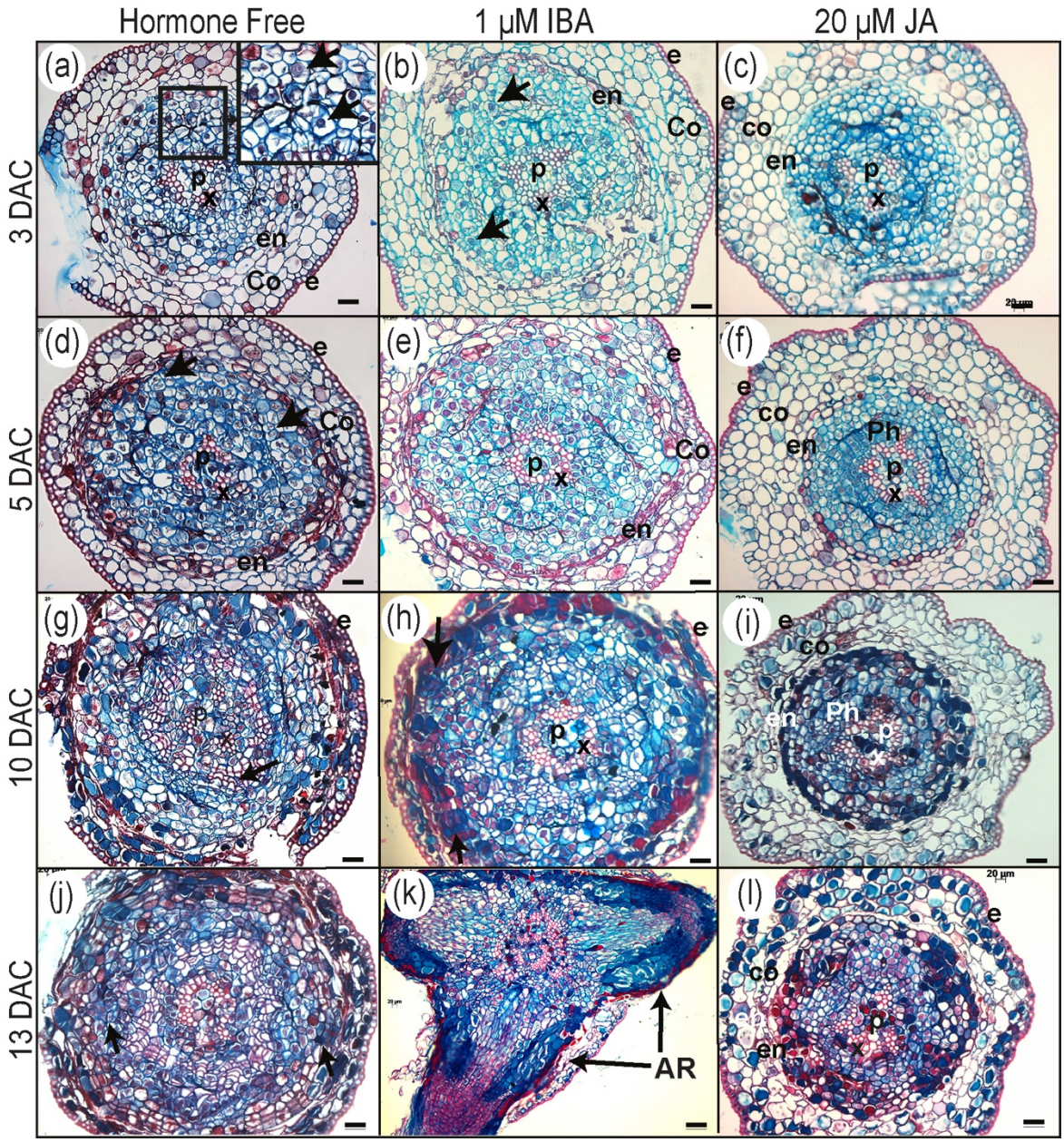

Fig. 6 Histological analysis of ARI in Norway spruce hypocotyl cuttings under cRL in the presence of IBA, JA or HF.

(**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.