Cytokinins are initial targets of light in the control of bud outgrowth

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Cytokinins Are Initial Targets of Light in the Control of Bud Outgrowth[1][OPEN]

Hanaé Roman, Tiffanie Girault, François Barbier[2], Thomas Péron, Nathalie Brouard, Aleš Pěnčík, Ondřej Novák, Alain Vian, Soulaiman Sakr, Jérémy Lothier, José Le Gourrierec, and Nathalie Leduc*

IRHS (Research Institute on Horticulture and Seeds), Université d'Angers, Agrocampus-Ouest, Institut National de la Recherche Agronomique, SFR 4207 QUASAV, 49070 Beaucouzé, France (H.R., T.G., F.B., T.P., N.B., A.V., S.S., J.L., J.L.G., N.L.); and Laboratory of Growth Regulators and Department of Chemical Biology and Genetics, Palacký University and Institute of Experimental Botany, Academy of Sciences of the Czech Republic, CZ-78371 Olomouc, Czech Republic (A.P., O.N.)

ORCID IDs: 0000-0002-8475-2976 (H.R.); 0000-0002-3248-0286 (N.B.); 0000-0003-3452-0154 (O.N.); 0000-0002-4785-7349 (A.V.); 0000-0002-6200-0116 (J.L.G.); 0000-0002-5323-0892 (N.L.).

Bud outgrowth is controlled by environmental and endogenous factors. Through the use of the photosynthesis inhibitor norflurazon and of masking experiments, evidence is given here that light acts mainly as a morphogenic signal in the triggering of bud outgrowth and that initial steps in the light signaling pathway involve cytokinins (CKs). Indeed, in rose (*Rosa hybrida*), inhibition of bud outgrowth by darkness is suppressed solely by the application of CKs. In contrast, application of sugars has a limited effect. Exposure of plants to white light (WL) induces a rapid (after 3–6 h of WL exposure) up-regulation of CK synthesis (*RhIPT3* and *RhIPT5*), of CK activation (*RhLOG8*), and of CK putative transporter *RhPUP5* genes and to the repression of the CK degradation *Rh*CK*X1* gene in the node. This leads to the accumulation of CKs in the node within 6 h and in the bud at 24 h and to the triggering of bud outgrowth. Molecular analysis of genes involved in major mechanisms of bud outgrowth (strigolactone signaling [*RwMAX2*], metabolism and transport of auxin [*RhPIN1*, *RhYUC1*, and *RhTAR1*], regulation of sugar sink strength [*RhVI*, *RhSUSY*, *RhSUC2*, and *RhSWEET10*], and cell division and expansion [*RhEXP* and *RhPCNA*]) reveal that, when supplied in darkness, CKs up-regulate their expression as rapidly and as intensely as WL. Additionally, up-regulation of CKs by WL promotes xylem flux toward the bud, as evidenced by Methylene Blue accumulation in the bud after CK treatment in the dark. Altogether, these results suggest that CKs are initial components of the light signaling pathway that controls the initiation of bud outgrowth.

Bud outgrowth is a process controlled by the intricate interactions between hormones, nutrients, and environmental cues (Ongaro and Leyser, 2008; Müller and Leyser, 2011; Leduc et al., 2014; Barbier et al., 2015b; Rameau et al., 2015). Among hormones, cytokinins (CKs) play a major promoting role that was revealed when exogenous CKs applied on axillary buds of intact pea (*Pisum sativum*) plants released these buds from dormancy (Sachs and Thimann, 1964, 1967; Kalousek et al., 2010; Dun et al., 2012). For years, it was thought that CKs synthesized in the roots were involved in the control of bud outgrowth (Bangerth, 1994; Turnbull et al., 1997; Nordström et al., 2004), but recent evidence has highlighted the implication of CK synthesized locally in the node bearing the bud (Tanaka et al., 2006; Liu et al., 2011; Xu et al., 2015). Indeed, following stem decapitation, auxin (indole-3-acetic acid [IAA]) repression of the expression of the CK synthesis *IPT* genes in the node terminates, and this allows the increase in CK levels in this same organ (Tanaka et al., 2006; Liu et al., 2011; Müller et al., 2015; Xu et al., 2015). Such an accumulation of CKs then antagonizes the promoting effect of strigolactones (SLs) on the negative branching integrator *BRANCHED1* (*BRC1*), and this leads to bud outgrowth (Dun et al., 2012; Rameau et al., 2015).

Environmental factors such as light modulate bud outgrowth and branching, allowing plants to better adapt their development to changing conditions (Leduc et al., 2014; Demotes-Mainard et al., 2016; Huché-Thélier et al., 2016). A low light intensity or a low ratio of

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[2] Present address: School of Biological Sciences and Queensland Alliance for Agriculture and Food Innovation, University of Queensland, St. Lucia, Queensland 4072, Australia.

* Address correspondence to nathalie.leduc@univ-angers.fr.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Nathalie Leduc (nathalie.leduc@univ-angers.fr).

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red/far-red (R/FR) light inhibits bud outgrowth and modulates the correlative inhibitions between buds in some species (Kebrom et al., 2006; Finlayson et al., 2010; Su et al., 2011; Demotes-Mainard et al., 2013; Furet et al., 2014; Drummond et al., 2015). In rose (*Rosa hybrida*) plants, darkness or far-red light (used as a single source of light) represses bud outgrowth (Girault et al., 2008). Even when buds are freed from apical dominance after decapitation, rose buds never grow out under such light conditions, and bud meristem organogenesis is inhibited (Girault et al., 2008).

In the control of branching, light acts both as a driver of photosynthesis for the supply of sugars and also as a photomorphogenic signal (Su et al., 2011). Such a signaling role for light is well described in the shade-avoidance syndrome, where perception by plant phytochromes (mainly phyB) of a low R/FR leads to bud inhibition even under a promoting photosynthetic photon flux density (Kebrom et al., 2006; Reddy et al., 2013). A signaling role for light in the triggering of bud outgrowth also was suggested in rose (Girault et al., 2008). In this species, bud outgrowth is inhibited within a few hours of dark exposure in rose plants first grown under white light (WL) and, thus, rich in sugars. Yet, as low an intensity of WL as 2 μmol m^{-2} s^{-1}, which is far below the light compensation point of rose plants (40–70 μmol m^{-2} s^{-1}, according to Zieslin and Tsujita, 1990; Ueda et al., 2000), is sufficient to trigger their outgrowth (Girault et al., 2008). This suggests that, in rose plants too, the triggering of bud outgrowth requires a light signal that is different from the energy supply involved in photosynthetic assimilation.

In accordance with this morphogenic role of light, several studies have shown that light acts through the modulation of hormones during bud outgrowth. In Arabidopsis (*Arabidopsis thaliana*), branching is promoted by the suppression of auxin signaling by phyB (Reddy and Finlayson, 2014) and by the negative transcriptional regulation of the SL signaling *AtMAX2* gene by high R/FR light (Finlayson et al., 2010; Su et al., 2011; González-Grandío et al., 2013). Evidence also was given that abscisic acid is involved in the bud response to R/FR light in this species (Reddy et al., 2013). In rose, when plants are exposed to darkness, inhibition of bud outgrowth correlates with the up-regulation of the SL signaling *RwMAX2* gene (Djennane et al., 2014) and of GA_3 degradation genes together with the down-regulation of GA_3 synthesis genes (Choubane et al., 2012). However, an exogenous supply of GA_3 is not able to circumvent dark inhibition (Choubane et al., 2012), showing that GA_3 is not the main actor in the light regulation of bud outgrowth.

Other important targets of the light regulation of bud outgrowth are sugars, which are an important source of energy and carbon and provide cell wall components for the achievement of outgrowth (Leduc et al., 2014; Barbier et al., 2015b). Recently, sugars were shown to act as an initial trigger of bud release in pea (Mason et al., 2014) and as an early modulator of the key hormonal mechanisms controlling bud outgrowth in rose (Barbier et al., 2015a) under a favorable light environment. In rose, the transcription and enzymatic activity of vacuolar invertase (*RhVI*) as well as transcription of the Suc transporter gene *RhSUC2* are under light control, and darkness leads to their repression concomitant with the inhibition of bud outgrowth (Girault et al., 2010; Henry et al., 2011; Rabot et al., 2012, 2014). However, in vitro culture of excised rose buds in the presence of sugars does not rescue dark inhibition (Henry et al., 2011; Rabot et al., 2012), suggesting that sugars are not the initial target of the light signal that controls outgrowth.

Despite the key regulatory role of CKs in bud outgrowth, very little information has been gained on the effect of light conditions on these hormones. Only recently, a transcriptomic analysis of sorghum (*Sorghum bicolor*) buds revealed differential regulation of some CK genes between wild-type and *phyB-1* buds, which suggests a correlation between the dormant state of *phyB-1* buds and the down-regulation of CKs (Kebrom and Mullet, 2016). In this study, we question whether CKs could be major targets of light in the control of bud outgrowth. We used quantifications of CK levels as well as of transcript accumulation of CK-related genes in buds and nodes of rose plants subjected to WL or darkness to address this question. Exogenous treatments of buds with CKs under darkness or with CK inhibitors under WL also were applied to further decipher the impact of light on CK metabolism and signaling during bud outgrowth. Finally, the expression of genes involved in the control of bud outgrowth (sugar-, SL-, auxin-, and cell cycle-related genes) was assessed after CK treatment in darkness to identify pathways acting downstream of the light-dependent CK signal.

RESULTS

Light Acts Mainly as a Morphogenic Signal in the Triggering of Bud Outgrowth

As shown previously (Girault et al., 2008), light is essential for triggering bud outgrowth in rose: when rose plants were exposed to WL, outgrowth of the bud just beneath the site of decapitation (Fig. 1A) always resumed and the bud produced an axis over 16 mm long with a mean of four new leaf primordia after 7 d (Fig. 1, B and D–F), but in the dark, the bud never grew out and leaf elongation and meristem organogenic activity were repressed (Fig. 1, C–F). Since photosynthesis and sugars promote bud outgrowth, we wondered whether they could be the initial targets of the light control over buds. Treatments with norflurazon, a photosynthesis inhibitor (Guseinova et al., 2005), together with masking experiments and sugar supplies were carried out to address this question.

When the axillary bud was placed in the dark using foil masking and leaving only the stem exposed to WL, no outgrowth occurred, while masking the bud with a transparent material allowed outgrowth (Fig. 2, A–C and F–H). This shows that light perception and

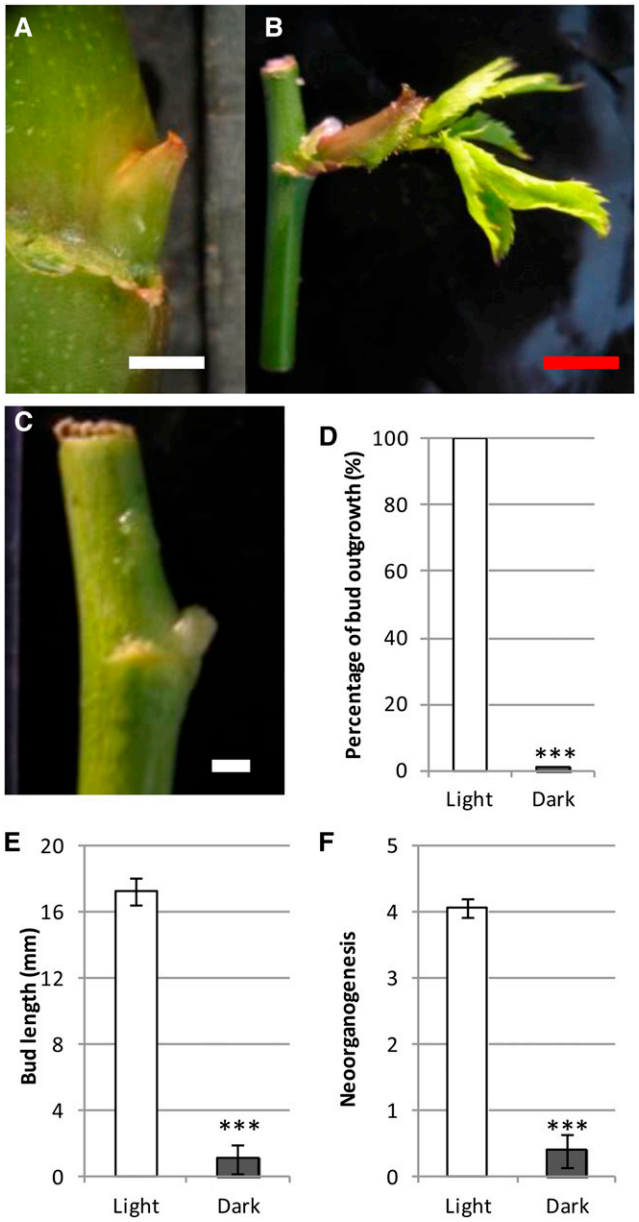

Figure 1. Effects of light environment on bud outgrowth in rose. A to C, Bud on the day of decapitation (Tdecap; A) and 7 d after WL (B) or dark (C) exposure. Red bar = 5 mm, and white bars = 1 mm. D, Percentage of bud outgrowth. E, Bud length. F, Neoorganogenesis after 7 d. Data are means ± SE; $n = 3$ biological replicates with at least 10 plants per replicate. Asterisks indicate significant differences between light and dark conditions ($P < 0.001$).

photosynthetic assimilation by the stem are not able to trigger bud outgrowth and confirms that light needs to be perceived by the bud itself to achieve outgrowth (Fig. 2, A–E; Girault et al., 2008). In another experiment, buds were treated with norflurazon (500 μM) 4 d before decapitation to ensure degradation of their chlorophyll prior to decapitation (Fig. 2, I and N). Norflurazon treatment was then continued for 7 d after decapitation. On the day of decapitation, axillary buds had turned white (Fig. 2N) and were deprived of chlorophyll, as shown by the absence of a chlorophyll signal in leaf tissues (Fig. 2, O–R) in contrast with buds exposed to WL (Fig. 2, J–M). Yet, when exposed to WL, these buds were able to grow out and to produce, within 7 d, an axis up to 4.6 mm long bearing, on average, 2.4 new leaf primordia (Fig. 2, J and O). This indicates that photosynthesis is not necessary to trigger bud outgrowth and that the light perceived by the bud acts mainly as a morphogenic signal in the initiation of this process. Photosynthesis would rather contribute to enhancing growth once the initiation of outgrowth has occurred.

To check whether the dark inhibition of bud outgrowth could be due to too-low sugar levels in the vicinity of the bud, Suc or Glc+Fru was applied on the cut end of the stem (Fig. 3). Concentrations of 100, 250, and 400 mM that were previously shown to support full outgrowth of excised rose buds under WL (Henry et al., 2011; Barbier et al., 2015a) were used as well as higher concentrations (600 and 800 mM). When applied under WL, sugar concentrations of 100, 250, and 400 mM allowed the same bud elongation and meristem organogenic activity as those supplied with water and supported outgrowth of 100% of buds (Fig. 3).

Higher concentrations had detrimental effects, with only 30% of the buds achieving outgrowth under 600 mM sugar supply and 0% at 800 mM (Fig. 3A). When sugars were furnished under the dark condition, no outgrowth was observed at low or highest sugar concentrations, and only some buds grew out with 600 mM Glc+Fru (10%) or 600 mM Suc (20%) supplied (Fig. 3A). Meristem organogenic activity resumed when Glc+Fru (600 and 800 mM) or Suc (400–800 mM) was provided, but a maximum of two new leaf primordia was produced (Fig. 3C). When bud elongation occurred, it was short and reached a maximum of 1 mm (Fig. 3B).

Thus, increasing sugar levels in the stem has a limited impact on the dark inhibition of bud outgrowth. This suggests that molecular actors other than sugars are the initial target(s) of this photomorphogenic control.

Darkness Affects CK Signal during Bud Outgrowth

We asked whether CK could be one of these initial targets. This question was addressed through the comparison of the relative expression of *RhARR3* and *RhARR5* genes in contrasting light environments. *RhARR3* and *RhARR5* code for proteins sharing high homologies with the type A Arabidopsis response regulators *AtARR3* and *AtARR5* (Supplemental Table S1), which are involved in the last step of CK perception (Fig. 9A). These genes are known to exhibit a strong and rapid transcriptional response to CK (Taniguchi et al., 1998; D'Agostino et al., 2000; Romanov et al., 2002; Hwang et al., 2012) and are involved in CK-mediated bud activation in Arabidopsis (Müller et al., 2015), potato (*Solanum tuberosum*; Hartmann et al., 2011), and sorghum (Kebrom and Mullet, 2016). Decapitation and

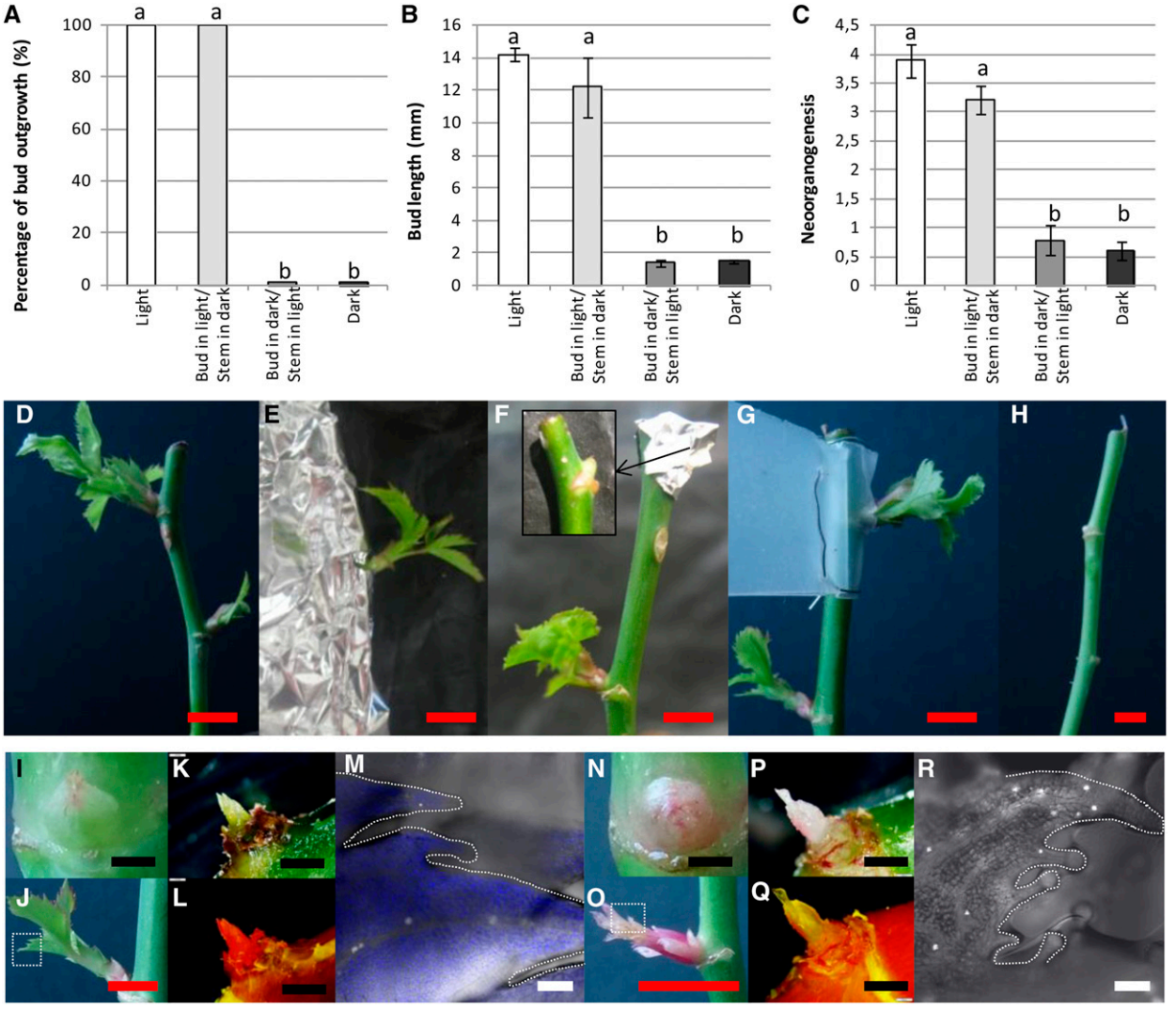

Figure 2. Effects of partial masking and of photosynthesis inhibition on bud outgrowth in rose. A to H, Percentage of bud outgrowth (A), bud length (B), and neoorganogenesis after 7 d (C) with bud and stem exposed to light (white columns, light treatment; D), or bud only exposed to light and stem placed under darkness using foil (light gray columns, bud in light/stem in dark; E), or bud placed in dark and the entire stem in light (dark gray columns, bud in dark/stem in light; F), or entire plant placed in dark (black columns, dark treatment, dark bag removed; H). Bud outgrowth occurred only when the bud itself was exposed to WL (D and E). Masking the bud with translucent material as a control allowed bud outgrowth as shown in G, where the strength of bud growth made even bud leaves protrude through the translucent material. Control plants for D and H with a translucent bag over the entire plant were published by Girault et al. (2008), showing that enclosing the plant in such a bag had no effect on the bud's capacity to grow out. I to R, Effect of norflurazon (500 μM) on bud outgrowth. In mock-treated plants (I–M), buds were green on the day of decapitation (I). Upon decapitation and exposure to WL, bud grew out and produced green leaves (J and K). Young green leaves are shown after the removal of older leaves and observation with a binocular microscope (K). Chlorophyll in these young leaves gives a red signal under UV light (L) and a blue signal by confocal microscopy (M). The square in J corresponds to M. Application of the photosynthesis inhibitor norflurazon 4 d before decapitation produced bleached bud due to the destruction of the chlorophyll on the day of decapitation (N). This bleached bud grew out even when norflurazon treatment was pursued for 7 d after decapitation (O). Leaves that expanded from the treated bud were white (P) and deprived of chlorophyll, as shown using UV light using a binocular microscope (Q) and by confocal microscopy (R; corresponding to the white square in O). Data are means ± SE; n = 3 biological replicates with at least 10 plants per replicate. Letters indicate significant differences by ANOVA between treatments. Red bars = 5 mm, black bars = 0.5 mm, and white bars = 100 μm.

exposure to darkness (Tdecap) for 24 h (T0) caused an increase in *RhARR3* and *RhARR5* expression in buds between these two time points (Fig. 4, A and C), while in nodes, no change (*RhARR3*) or even a decrease (*RhARR5*) in transcript accumulation occurred (Fig. 4, B and D). Transfer to WL at T0 triggered a significant further increase in *RhARR3* and *RhARR5* expression in buds and nodes. The level of these transcripts doubled

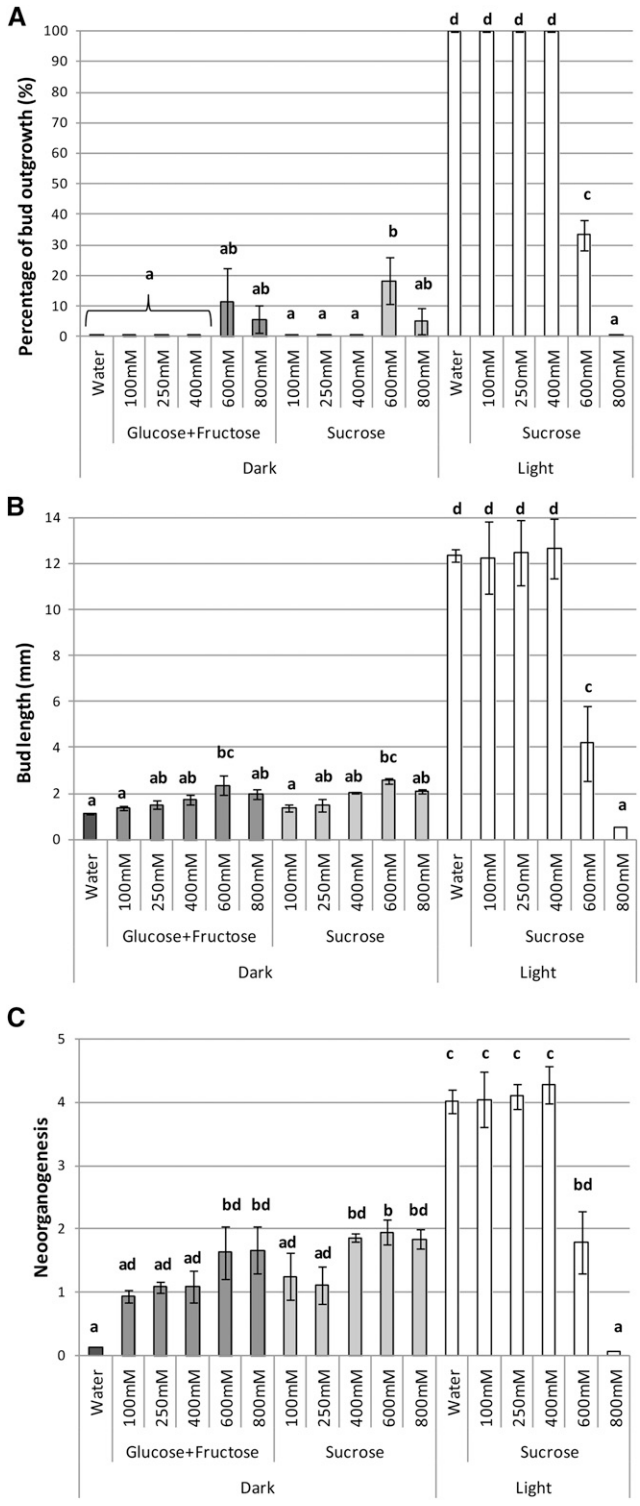

Figure 3. Effects of sugar feeding under darkness or WL on bud outgrowth. Sugars (Glc+Fru or Suc) were applied on the cut end of the stem in a lanoline drop at 100 mM or in aqueous solution for higher concentrations (250, 400, 600, and 800 mM) as in Supplemental Figure S1, C and D. Under WL, with water or sugar feeding up to 400 mM, all buds had grown out after 7 d, but sugar concentrations of 600 and 800 mM were detrimental in this condition, with reduction in percentage bud outgrowth (A), bud length (B), and neoorganogenesis (C). Under darkness, buds remained dormant when fed with water or with sugars up to 400 mM (A). When fed with Glc+Fru or with Suc at 600 mM, most buds remained dormant, but some (10% or 20%, respectively) grew out (A). Elongation of bud, however, was reduced (1 mm; B). Neoorganogenesis was promoted by sugar feeding at higher concentrations (C). Data are means ± SE; $n = 3$ biological replicates with at least 10 plants per replicate. Letters indicate significant differences by ANOVA.

within 3 h of WL exposure but remained unchanged in the dark (Fig. 4). The promoting effect of WL on *RhARR3* and *RhARR5* lasted for at least 48 h in both organs.

Together, these results indicate that CK signaling is not only modulated by decapitation, as reported before in other species (Tanaka et al., 2006; Foo et al., 2007), but also by the light environment and that the light effect on *RhARR3* and *RhARR5* is strong and long lasting.

Light Acts through the Control of CK Levels in Buds and Nodes during Outgrowth

A comparison of CK contents under dark and WL conditions showed that a 24-h exposure to WL led to an almost 10-fold increase in total CK in the bud and a more than 10-fold increase in active CKs as compared with T0 (Table I). In contrast, a very limited increase in total and active CKs was observed after the same period in darkness (Table I). In the node, total and active CK levels also nearly doubled after 24 h under WL, while in darkness, their levels dropped to less than half the amount measured at T0. This indicates that light controls CK levels in both bud and node. Promotion by WL of CK accumulation involved all CK forms (i.e. precursors, active, and storage forms; Table I; Supplemental Table S2). Interestingly, WL preferentially induced the accumulation of zeatin forms (tZ, tZR, and tZRMP; Fig. 5, A, C, E, and G) over isopentenyladenine forms (iP, iPR, and iPRMP; Fig. 5, B, D, F, and H) in the buds, while more iP forms accumulated in the node. In comparison, WL had a promoting effect on IAA accumulation only in the bud (Table I).

In order to assess whether the light effect on CK levels is a key mechanism in the photocontrol of bud outgrowth, we checked whether an exogenous supply of CK in the dark could release buds from the dark inhibition. When the synthetic CK 6-benzylaminopurine (BAP; 10 mM) was applied to the bud in darkness, a high and rapid (3 h) increase in the expression of *RhARR3* and *RhARR5* in both bud and node was observed, which was similar to the one triggered by WL (Fig. 4). Moreover, treatments with 0.05, 1, 10, or 30 mM BAP on the bud or on the cut end of the stem all led to bud outgrowth in the dark, while mock treatment (0 BAP) never did (Fig. 6, A–G). Up to 60% of the buds were released from dormancy and grew out with the resumption of the component mechanisms of bud outgrowth (i.e. leaf elongation and neoorganogenesis in darkness; Fig. 6, E–J). The effect of BAP treatment on bud outgrowth was specific to a control by CK. Indeed,

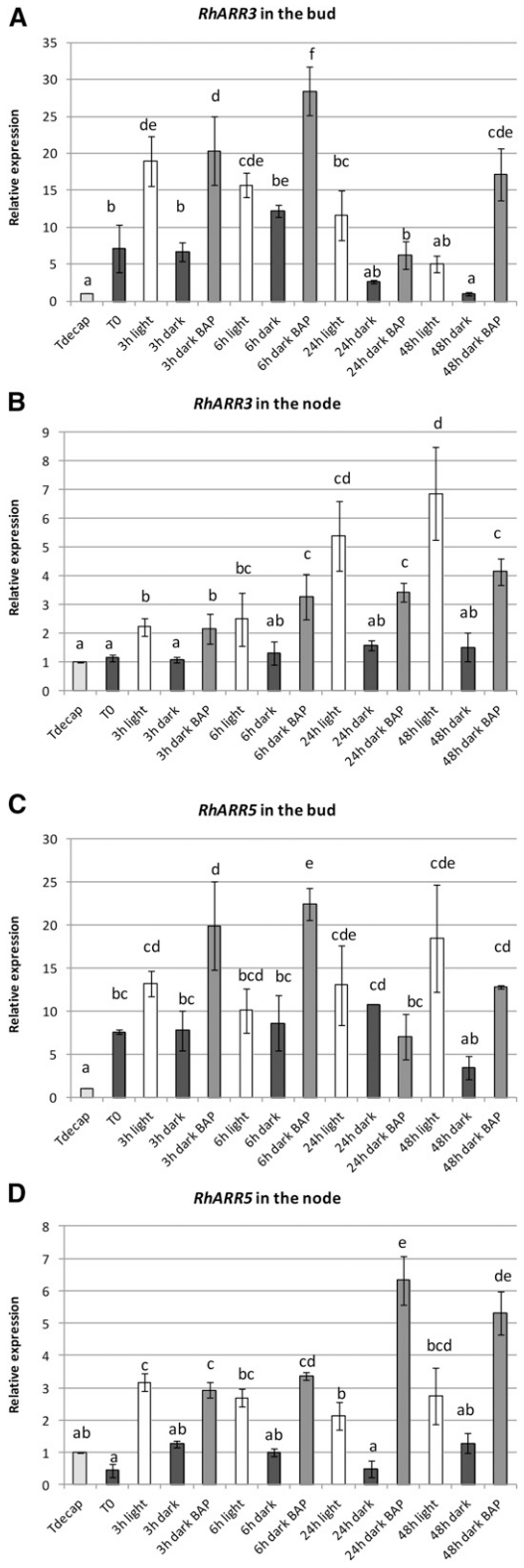

Figure 4. *RhARR3* (A and B) and *RhARR5* (C and D) expression in bud and corresponding node from decapitation to 48 h under WL or dark treatment. Relative expression is shown just prior to decapitation (Tdecap), after a 24-h dark treatment following decapitation (T0), and after 3, 6, 24, and 48 h of WL (white columns) or further dark (black columns) exposure after T0. Some of the dark-exposed plants also were treated with BAP (10 mM) on the bud at T0 (gray columns). Data are means ± SE of three independent batches with $50 < n < 80$ buds or $n = 20$ for node samples. Letters represent significant differences by ANOVA.

when two inhibitors of CK perception (LGR-991 and PI-55) were applied on the bud, this caused a repression of bud outgrowth that could not be prevented by BAP application (Fig. 7, B, C, and E–G). Also, strong inhibition of bud outgrowth in WL after stem treatment with lovastatin (LVS), a CK synthesis inhibitor, was restored by BAP application (Fig. 7, D and H–J). Finally, isopentenyladenine and zeatin, two natural CKs either applied alone (10 or 20 mM) or as a mix (20 mM), triggered the same response as BAP in the dark (Fig. 8).

Interestingly, none of the treatments modified the capacity of the buds below the treated bud to grow out, indicating a local effect of these molecules (Supplemental Fig. S1, D and E).

Light Regulates CK Metabolism and Transport at the Transcriptional Level

Stronger inhibition of bud outgrowth with LVS (Fig. 7, D and H–J; Supplemental Fig. S1, A–C) and more efficient promotion with CK applications when treatments were applied on the stem as compared with bud treatment (Figs. 6 and 8) suggest that the de novo synthesis of CK in the node may be involved in the photocontrol of bud outgrowth. Therefore, the effect of light on CK metabolism and transport was studied. Figure 9A gives a schematic overview of the CK biosynthesis, transport, and signaling pathway and of the main genes involved (Supplemental Tables S1 and S3). Transcript accumulation of these genes was followed from decapitation (Tdecap) to 48 h WL exposure. Supplemental Figure S2 gives the detailed transcript data.

Decapitation and exposure to darkness during the first 24 h after Tdecap promoted the expression of *RhARR* genes in the bud, as discussed above (Fig. 4), and also transcription of the CK synthesis *RhIPT3* gene in the node. Transcript levels of other CK genes, however, were not modified by this phase of the treatment (Fig. 9B).

When plants were then exposed to WL at T0, a strong and positive impact of WL on CK synthesis and activation genes in the node was observed compared with the dark condition (Fig. 9C; Supplemental Fig. S2). Up-regulation of *RhIPT3* and *RhLOG8* expression (4-fold) occurred as early as 6 h after light exposure, and transcript levels increased until 24 h. *RhIPT5* also showed a strong (8-fold) up-regulation by WL from 24 h. Rapid up-regulation of CK synthesis and activation genes by WL is consistent with the higher amounts of CK nucleotide precursor forms (752.4 pmol g^{-1} under WL and 154.2 pmol g^{-1} under darkness) and of

Table I. *Levels of CK metabolite groups, total CK, total active CK, and IAA in 1 g of extracted tissue*

Values shown are pmol g^{-1} (means ± SD). Asterisks indicate statistical differences between light and dark conditions for one time point (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

Tissue	Time Point	Light Conditions	Active CK		CK Nucleotides	Total CKs	IAA
			CK Bases	CK Ribosides			
Bud	T0		5± 0.7	18.2± 2.5	118.7± 21	144.7± 23	42.9± 9
	24 h	Light	12.4± 1.1***	256.7± 5***	1,129.1± 96***	1,419.9± 131***	72.4± 6*
	24 h	Dark	3.3± 0.3	29.9± 1.9	130.9± 15	170.4± 13	47.3± 7
Node	T0		4.9± 1.2	195.7± 40	391.1± 68	594.9± 105	30± 6
	6 h	Light	3.1± 0.8	143.3± 32	233.9± 41*	382.7± 55*	24.9± 5
	6 h	Dark	1.7± 0.4	93.4± 15	121.4± 19	219.1± 6.4	26.3± 3
	24 h	Light	10.6± 2.5**	330.3± 88**	752.4± 151**	1,102.3± 213**	34.4± 7
	24 h	Dark	1.4± 0.3	44.6± 6.2	154.2± 29	204± 31	31.8± 3

active forms (340.9 pmol g^{-1} under WL and 45.9 pmol g^{-1} under darkness) found in the node after 24 h of WL exposure compared with darkness (Table I; Supplemental Fig. S2; Supplemental Table S2). This indicates that WL acts through transcriptional up-regulation of CK synthesis genes to promote the accumulation of active CKs in the node, while darkness has a repressive action.

Interestingly, regulation by light of CK synthesis and activation genes was opposite in the bud compared with the node. For example, dark but not light stimulation was observed for *RhIPT3,5* and *RhLOG3,8* in the bud (Fig. 9C). This indicates that most of the newly synthesized and activated CKs produced in WL and found in the bud at 24 h (Table I) come from the node. Moreover, BAP application in the dark led to the repression of *RhIPT3,5* and *RhLOG3* in the bud (Supplemental Fig. S2B), suggesting that a negative feedback loop may take place in the bud to repress CK synthesis in response to incoming CK from the node. In darkness, the reduced de novo synthesis and activation of CK in the node would lead to a limited transfer of node CK to the bud. In this condition, no feedback regulation would occur, and the transcription of CK synthesis and activation genes would be promoted in the bud itself. It is worth noting that such transcriptional promotion by darkness of CK synthesis and activation genes in the bud is not strong enough to cause a sufficient increase in CK level in the bud (Table I) to trigger bud outgrowth in the absence of light (Fig. 1).

Transcriptional analysis also indicates that the increased accumulation of CK in the bud and in the node in WL (Table I) also resulted from a lower rate of CK degradation and possibly from an increased CK transport in WL. Transcription of *CYTOKININ OXIDASE* genes (*RhCKX1* and *RhCKX6* in the bud and *RhCKX1* in the node) was indeed rapidly (3 h) and strongly repressed in WL, and this repression lasted for at least 48 h (Fig. 9C). Concerning putative CK transporters, *RhPUP5* expression was highly correlated to the light control of bud outgrowth. In comparison with the dark condition, its expression in both bud and node was rapidly (3-fold at 3 h) promoted by WL exposure up to 48 h (Fig. 9C). In contrast, *RhENT1* seemed rather correlated to bud outgrowth inhibition, as its expression in the bud increased under darkness from 6 h (Fig. 9C).

Taken together, these results indicate that the initial up-regulation of the CK signaling gene *RhARR3/5* and of the CK synthesis gene *RhIPT3* (Fig. 9B) caused by decapitation (Fig. 4) is not sufficient to trigger bud outgrowth. The effect of decapitation has to be associated with a prolonged exposure to light that transcriptionally regulates a series of mechanisms (CK synthesis, degradation, and transport) leading to CK accumulation in the bud and allowing for its outgrowth.

Several Key Mechanisms of Bud Outgrowth Are Modulated through the Photocontrol of CKs

The above results have shown a rapid (3–6 h) control of light condition over CKs that tightly regulates the bud's capacity to grow out. This suggests that CK photocontrol acts upstream of many processes involved in bud outgrowth. We analyzed the expression of a set of genes involved in bud outgrowth to identify those regulated by CKs.

Figure 10 shows transcript levels in bud or node at 3 and 6 h up to 48 h after light and/or BAP treatment in darkness (for detailed expression data, see Supplemental Fig. S3). In buds, genes related to mechanisms promoting bud outgrowth (auxin synthesis [*RhTAR1* and *RhYUC1*], Suc degradation [*RhSUSY* and *RhVI*], Suc transport [*RhSWEET10*], cell cycle [*RhPCNA* and *RhCYCD3;1*], and cell wall expansion [*RhEXP*]) all had higher expression under WL (Fig. 10A, green color code) as compared with darkness, while genes involved in the repression of bud outgrowth (*RhBRC1* and *RwMAX2*) were promoted by darkness (Fig. 10A, red color code). As observed previously (Girault et al., 2010), the *NAD-DEPENDENT SORBITOL DEHYDROGENASE* gene (*NAD-SDH*) also was strongly induced in buds by dark treatment.

In nodes, up-regulation by light of *RhSWEET10*, *RhSUSY*, and *RhVI* also was observed as in buds, together with increased expression of the Suc transporter *RhSUC2* and of auxin influx *PIN1* genes. Also as in buds, repressor genes (*RhBRC1* and *RhMAX2*) were promoted by darkness in nodes, while only late (48 h)

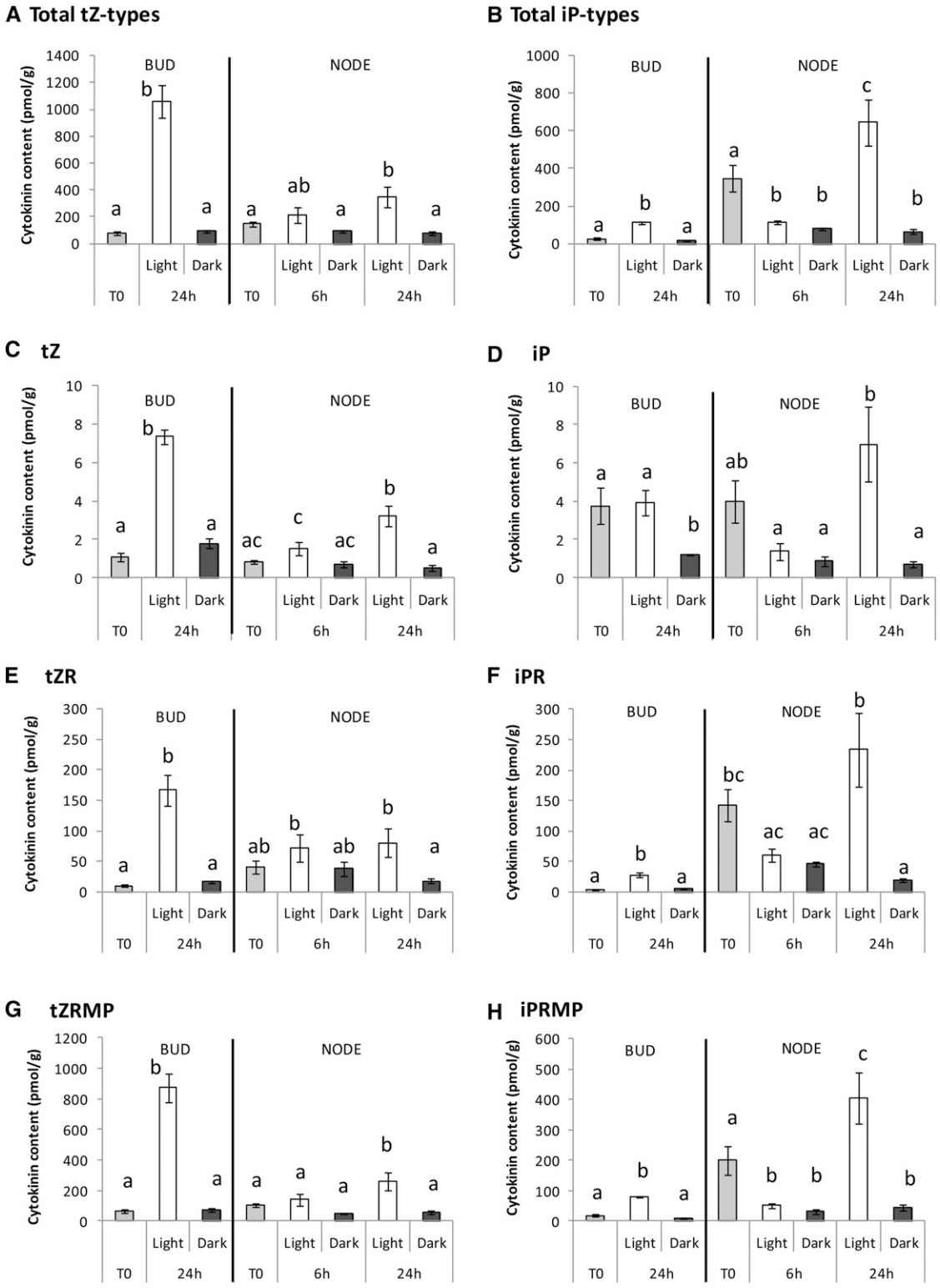

Figure 5. CK contents in bud and node under WL or dark treatment. Levels of trans-zeatin (tZ) type (A, C, E, and G) and of isopentenyladenine (iP) type (B, D, F, and H) CK are shown in bud and node tissues at T0 (gray bars) and following 6 and 24 h of WL (white bars) or of dark (black bars) treatment. Data show the total contents of tZ and iP types (A and B) and detailed levels of active (base) tZ (C) and iP (D) forms, of riboside tZR (E) and iPR (F) forms, and of nucleotide forms tZRMP (G) and iPRMP (H). Data are for 1 g of extracted tissue (pmol g^{-1}; means ± SD). Letters indicate significant differences by ANOVA between conditions for one given tissue.

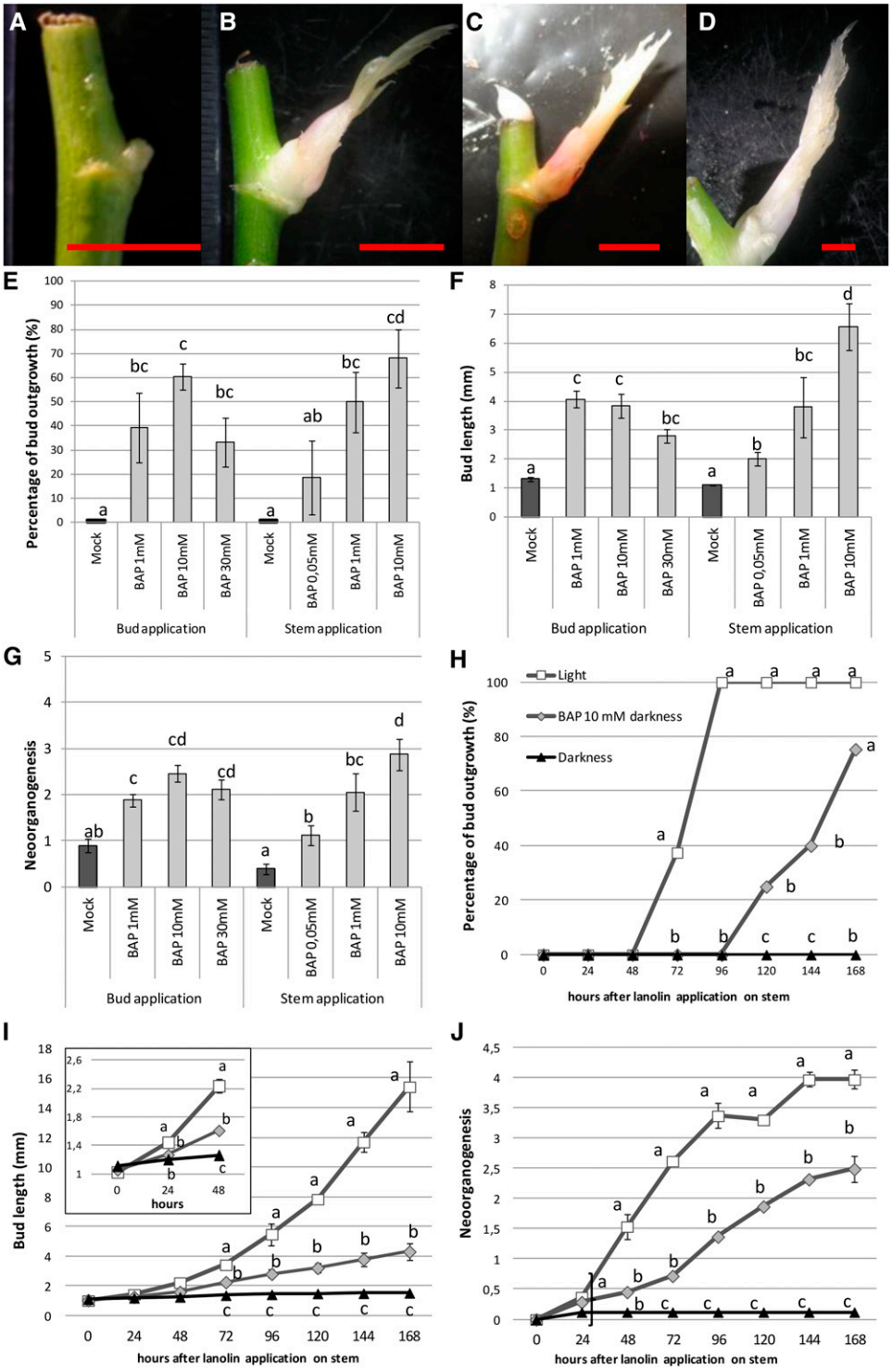

Figure 6. Effects of CK treatments under darkness on bud outgrowth. A to D, Buds 7 d (A–C) or 15 d (D) after treatment with a lanolin drop containing either only the solvent (mock; A) or a synthetic CK (BAP; 10 mM) applied on the bud (B) or on the stem (C and D). Red bars = 5 mm. E to G, Effects of BAP (1, 10, and 30 mM) applied on the bud and BAP (0.05, 1, and 10 mM) applied on the stem on the percentage of bud outgrowth (E), bud length (F), and neoorganogenesis (G) 7 d after BAP treatments. H to J, Kinetics of bud outgrowth (H), bud elongation (I), and neoorganogenesis (J) under WL and darkness with or without BAP (10 mM in lanolin) stem treatment under darkness. Data are means ± SE; $n = 3$ biological replicates with at least 15 plants per replicate. Letters indicate significant differences by ANOVA between the different concentrations in one tissue (E–G) and between the three conditions for the same time point (H–J).

promotion of *RhNAD-SDH* by darkness was observed in nodes compared with buds.

Altogether, these results show that light plays a major control in the regulation of bud outgrowth and that this light control takes place in both nodes and buds. Light control over many of these genes (*RwMAX2*, *RhVI*, *RhSUC2*, *RhSWEET10*, *RhPCNA*, *RhEXP*, *RhPIN1*, *RhNAD-SDH*, and *RhBRC1*) occurred very early (i.e. 3 or 6 h after light treatment in buds and/or nodes), indicating a possible direct control of these genes by light.

Application of BAP in darkness mimicked exposure to WL. For example, when BAP was applied in darkness, the expression of bud outgrowth repressor genes

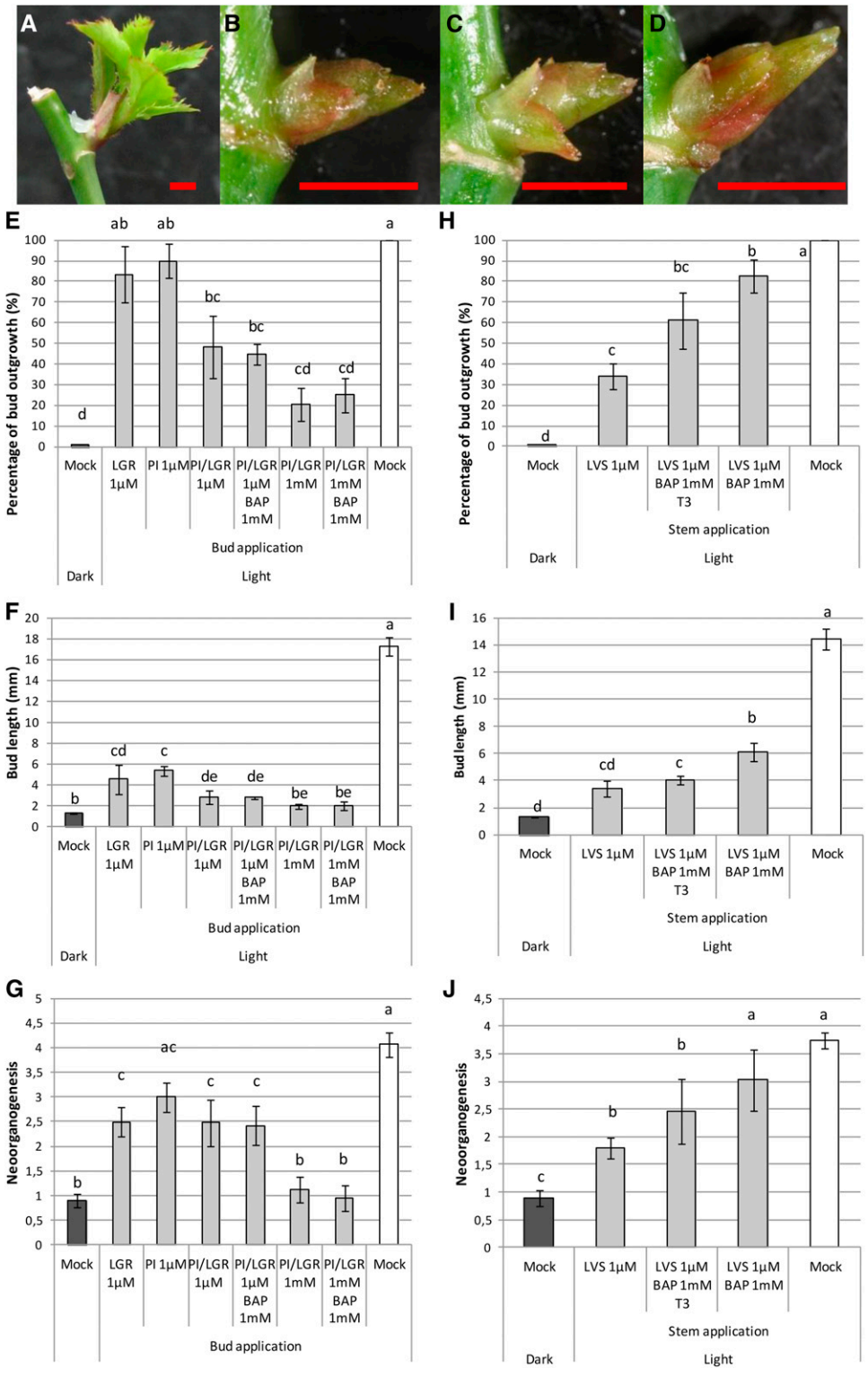

Figure 7. Effects of CK inhibitors (LGR-991 and PI-55 for perception and lovastatin [LVS] for synthesis) on bud outgrowth under WL. Inhibitors were applied alone or combined with BAP at T0. In one case, LVS treatment at T0 was followed by BAP treatment alone after 3 d. Perception inhibitors were applied on the bud, while the synthesis inhibitor was applied on the cut end of the stem, and plants were cultured for 7 d under WL. A to D, Bud treated by mock (A), LRG-991 (B), PI-55 (C), and LVS (D) at 1 μM each and after 7 d. Red bars = 3 mm. E and H, Percentage of bud outgrowth. F and I, Bud length. G and J, Neoorganogenesis after 7 d. Data are means ± SE; n = 3 biological replicates with at least 15 plants per replicate. Letters indicate significant differences by ANOVA. For stem application of PI-55 and LGR-991 and bud application of LVS, see Supplemental Figure S1, A to C.

(*RhBRC1* and *RwMAX2*) and of *RhNAD-SDH* was repressed within 3 or 6 h in both bud and node as in WL (Fig. 10B). On the contrary, the expression of all bud outgrowth promoter genes (*RhTAR1*, *RhYUC1*, *RhPIN1*, *RhSUSY*, *RhVI*, *RhSUC2*, *RhSWEET10*, *RhPCNA*, and *RhEXP*) except for *RhCYCD3;1* was increased by BAP treatment in darkness (Fig. 10B). These results indicate that the light control of CK acts upstream of the light regulation of all these different genes. While *RhCYCD3;1* is promoted by WL (Fig.

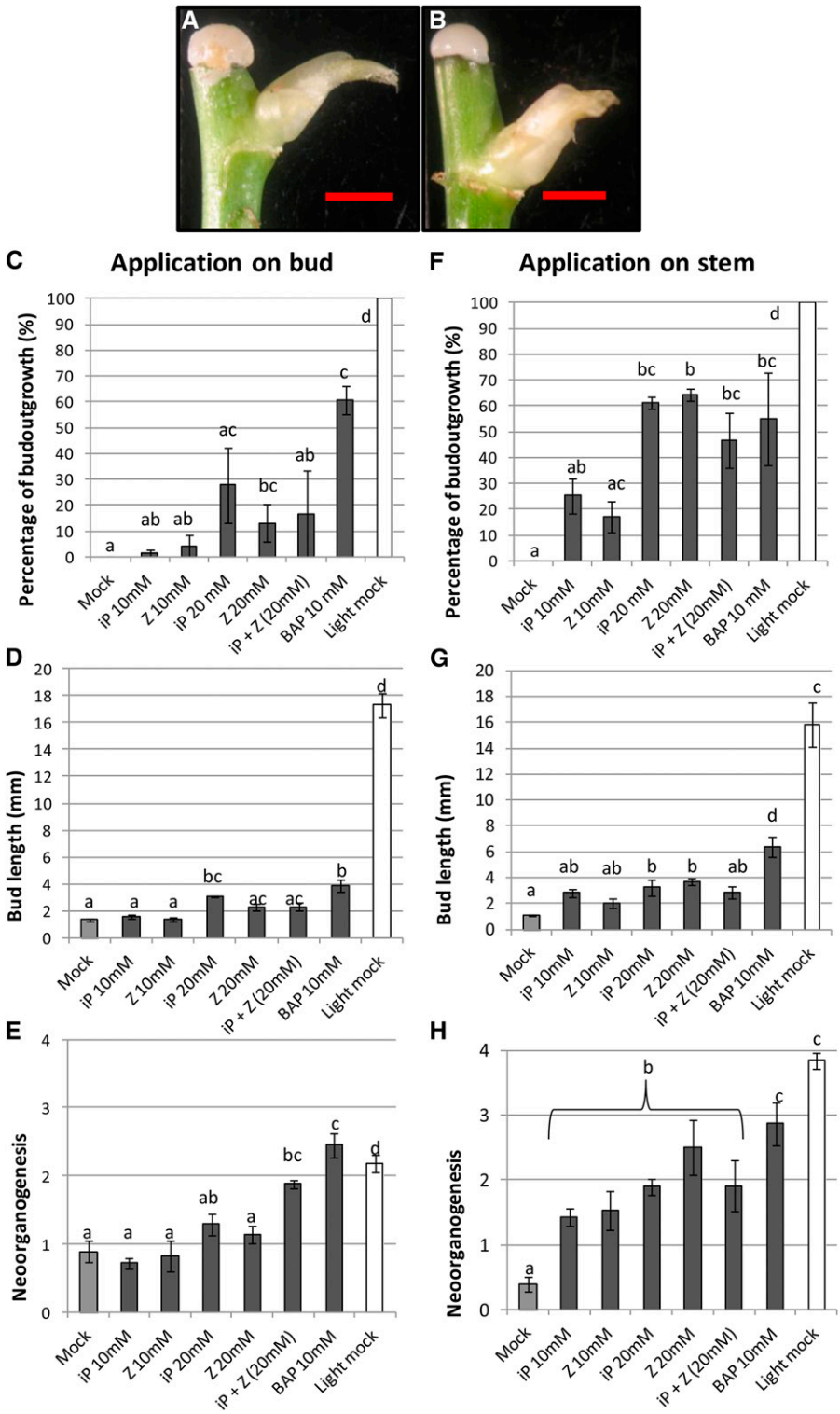

Figure 8. Effects of the natural CKs isopentenyladenine (iP) and zeatin (Z) on bud outgrowth in darkness. Plants were treated with a lanolin drop containing only the solvent (mock), iP (A), or Z (B) applied on the bud (C–E) or on the cut end of the stem (A, B, and F–H). Bud outgrowth was observed 7 d after treatments. A and B, Bud treated by iP (A) and Z (B). Red bars = 5 mm. C and F, Percentage of bud outgrowth. D and G, Bud length. E and H, Neoorganogenesis. Black and gray columns represent dark treatment, and white columns correspond to WL treatment. Data are means ± SE; $n = 3$ biological replicates with at least 10 plants per replicate. Letters indicate significant differences by ANOVA. Red bars = 5 mm.

10A), the repression of this same gene by CKs (Fig. 10B) suggests that another light signaling pathway independent of CKs may act in the control of bud outgrowth.

Since bud outgrowth is known to be associated with the formation of new functional vascular connections between the bud and the node (Ferguson and Beveridge, 2009), we also asked whether light control of CKs could play a role in this process. We used a histological approach involving soaking the plant roots in Methylene Blue solution to visualize xylem sieve flux: Methylene Blue is

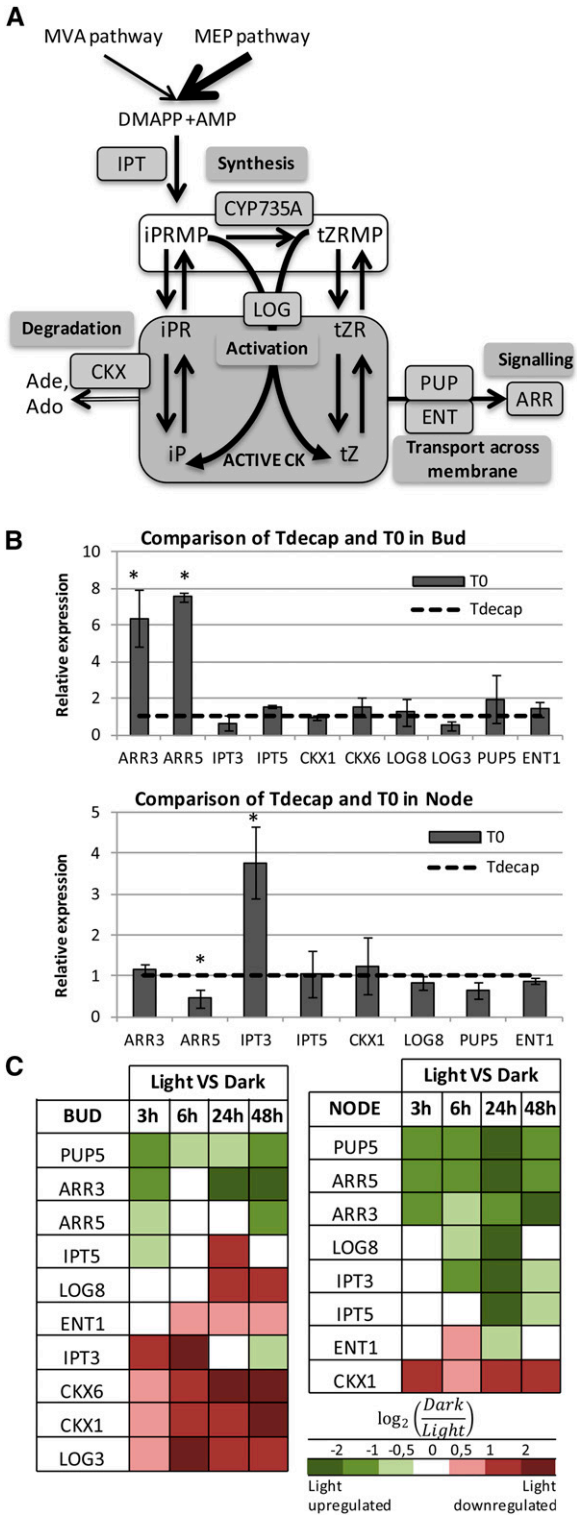

Figure 9. Effects of decapitation and light environment on CK-related genes. A, Simplified isoprenoid CK biosynthesis pathways. CK precursors originate from two pathways: the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways, both producing dimethylallyl-diphosphate (DMAPP). The *ADENOSINE PHOSPHATE-ISOPENTENYLTRANSFERASES* (*IPT*; Kakimoto 2001; Takei et al., 2001; Miyawaki et al., 2004) uses AMP, ATP, or ADP to form isopentenyladenosine-5′-monophosphate (iPRMP), IPRTP, and iPRDP, respectively. The cytochrome P450 *CYP735A* (Takei et al., 2004) catalyzes the next step where the iP nucleotides (iPRMP) are converted in zeatin nucleotides (tZRMP). The last step involves the LONELY GUY (LOG; Kurakawa et al., 2007) enzymes, which convert the nucleotide forms in active forms: isopentenyladenine (iP) and trans-zeatin (tZ). The iP and tZ ribosides and bases can be inactivated in an irreversible manner by the CYTOKININ OXIDASES (CKX; Whitty and Hall, 1974; Schmülling et al., 2003), which catabolized them into adenine (Ade) and adenosine (Ado). CK transport from cell to cell across the plasma membrane is achieved with the help of two families of transporters: the purine permease family (PUP), which transports free-base CKs (iP and tZ), and the equilibrative nucleoside transporters family (ENT), which transports CK ribosides (iPR and tZR; Kudo et al., 2010). CK perception at the plasma membrane involves a phosphorylation cascade from the receptor His kinase to His phosphotransferase, which finally activates Arabidopsis response regulators (ARR). This image is modified from Sakakibara (2006), Frébort et al. (2011), El-Showk et al. (2013), and Kieber and Schaller (2014). B, Effects of decapitation on the expression of CK-related genes in bud and node tissues: CK synthesis (*RhIPT3/5*), activation (*RhLOG3/8*), degradation (*RhCKX1/6*), and putative transport (*RhPUP5* and *RhENT1*) genes. Gene expression after decapitation and 24 h of dark treatment (T0; black bars) is expressed relative to expression on the day of decapitation (Tdecap; dotted lines) for each gene. Data are means ± SE of three batches with $50 < n < 80$ buds or $n = 20$ for node samples. Significant differences are represented by asterisks between T0 and Tdecap ($P < 0.05$). C, Evolution of the expression of CK-related genes from 3 to 48 h after T0 under WL or dark treatment. Changes in transcript levels are indicated by color codes: green indicating an increased expression under WL and red indicating an increased expression under darkness compared with T0. A color log scale is included at bottom right. All the qPCR data used to build these tables are shown in Supplemental Figure S3.

transported with the xylem sap and can be observed in hand sections of living stems and buds (Fig. 11, A and B). In dormant buds observed at T0, no accumulation of Methylene Blue was found in the bud (i.e. in the vascular tissues of the scales or of the preformed leaves), showing that no xylem sap from the node reaches the dormant bud in dark conditions (Fig. 11, A–C). However, 24 h after exposure to WL, Methylene Blue did accumulate in bud scales, indicating that an active vascular connection was established at this stage between the node and the bud (Fig. 11, D–F and J). Connection to bud preformed leaves was achieved at 72 h (Fig. 11, G and J), while no accumulation of Methylene Blue was observed in the youngest leaves and leaf primordia, in accordance with the absence of functional vascular tissues at this stage of their development (Fig. 11G). In contrast, in darkness, no accumulation of Methylene Blue in bud tissues was observed even after 7 d of culture (Fig. 11, H and J). This suggests that darkness impairs xylem sieve transport to the bud and that repression may likely constitute one of the causes of bud outgrowth inhibition in dark conditions. However, when BAP was applied on the cut end of the stem in darkness, an accumulation of Methylene Blue in bud scales and leaves occurred at 24 h (i.e. as early as that observed in WL; Fig. 11, I

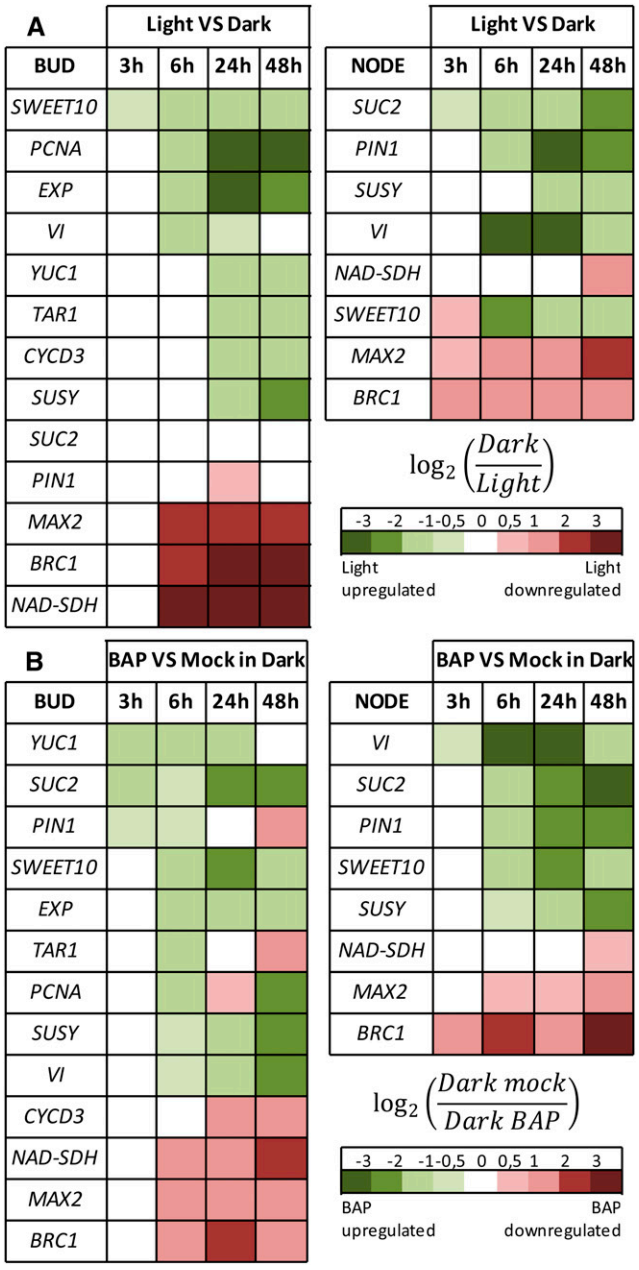

Figure 10. Evolution of the expression of genes involved in the control of bud outgrowth in the node and in the bud from 3 to 48 h after T0 under WL or dark treatment (A) or following bud treatment with BAP (10 mM) under darkness (B). Changes in transcript levels are indicated by color codes. In A, green indicates promotion by WL of gene expression, while red shows promotion by darkness compared with T0. In B, green indicates promotion by BAP treatment of gene expression, while red indicates repression of gene expression by BAP treatment compared with T0. Two color log scales are included. Transcript levels were obtained from three batches with $50 < n < 80$ buds or $n = 20$ for node samples. All qPCR data used to build these tables are shown in Supplemental Figure S4.

and J). This suggests that CKs actually promote xylem sieve transport to the bud and that light control over CKs also acts as a strong regulator of xylem sap flux to the bud.

DISCUSSION

Light Acts Mainly as a Morphogenic Signal in the Triggering of Bud Outgrowth

In rose, light is essential for bud outgrowth to resume, and the bud itself is the organ that needs to perceive this triggering light (Girault et al., 2008). Using norflurazon treatment that destroys chlorophyll, as described by Yoshida et al. (2011), and masking experiments, we bring evidence that photosynthesis in bud tissues is not essential for triggering the initial steps of outgrowth. Indeed, in the absence of chlorophyll in bud tissues, buds were able to achieve outgrowth (Fig. 2). These experiments thus further confirm that light acts as a morphogenic signal in the triggering of outgrowth (Girault et al., 2008). The photosynthetic component of the incident light may still contribute to enhance growth but is not essential to the initial steps of outgrowth.

CKs Trigger the Outgrowth of Darkened, Inhibited Buds

CKs have been known for a long time to promote bud release from dormancy in intact plants (Sachs and Thimann, 1964, 1967; Kalousek et al., 2010; Dun et al., 2012), and light has been shown in several species to strongly control bud outgrowth (Leduc et al., 2014). Yet, except for the transcriptomic study in sorghum buds carried out recently and showing the impact of *phyB* mutation on CK signaling (Kebrom and Mullet, 2016), no work has been carried out so far to decipher the relation between light and CK during bud outgrowth. Here, we give much evidence that the light signal that is perceived by the bud acts on CK and that CKs are initial targets and key control elements of the light response that control bud outgrowth. First, a limited increase in CK level in darkened buds was observed concomitant with the inhibition of bud outgrowth, while a massive increase in these hormones occurred upon the perception of WL and followed by outgrowth (Table I). Second, increasing CK levels in the vicinity of darkened and inhibited buds through the exogenous application of CKs was sufficient to release 60% of buds from dormancy and to trigger their outgrowth (Fig. 6). Third, both natural CKs (zeatin and isopentenyladenine) and synthetic CK (BAP) release buds from dark inhibition, showing that light indeed interacts with endogenous CKs (Fig. 8). Finally, light exposure rapidly (within 3–6 h) up-regulates CK genes, while darkness has a strong repressive effect (Fig. 9).

Light Controls CK Synthesis, Degradation, and Transport

When the interaction of light with CK was examined further, our experiments revealed that exogenous CK treatments could trigger a CK signal in darkness (as measured through increased *RhARR3/5* expression; Fig. 4) that was sufficient to cause bud outgrowth (Fig. 6). This implies that CK perception sensu stricto is little or not impaired by darkness and, in consequence, that light

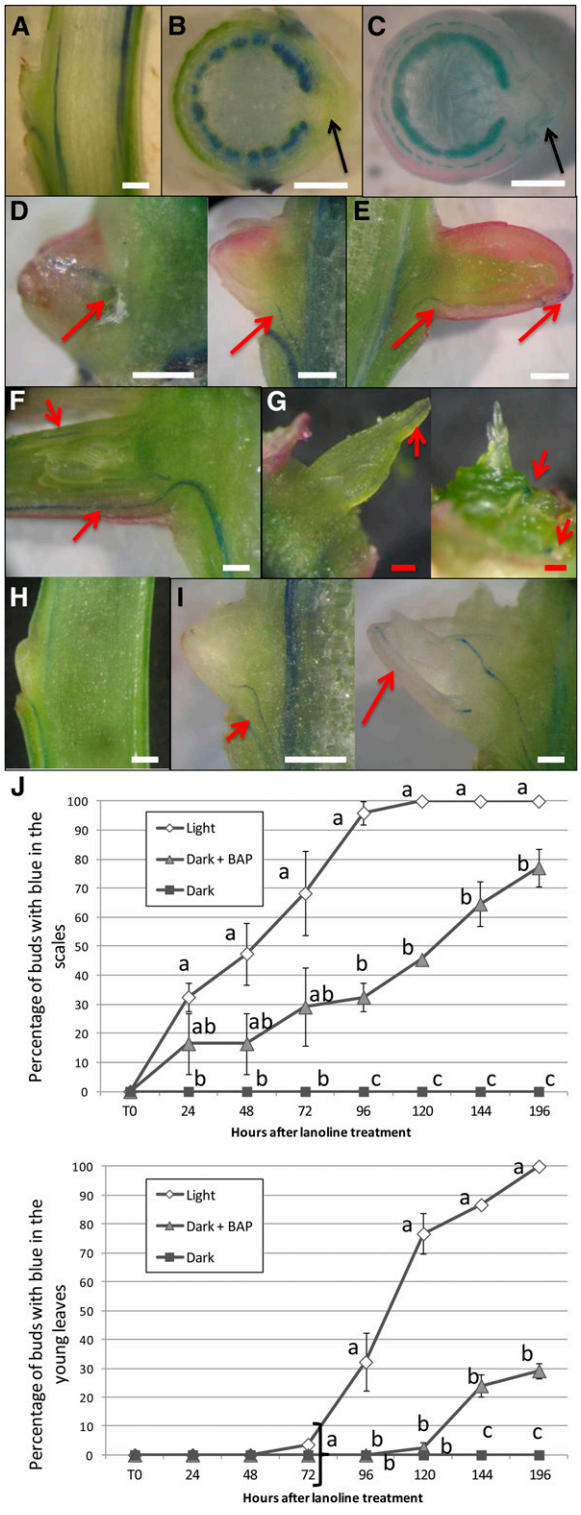

Figure 11. Activation by WL or BAP under darkness of vascular connections between bud and corresponding node during outgrowth. Plants treated or not with BAP (10 mM) on the cut end of the stem were placed under WL or darkness for 24 h up to 196 h. Roots were then soaked in Methylene Blue during 3 h under WL, and buds were observed. A to C, Bud and node at T0 showing no active vascular connection. A, Longitudinal section of the stem. B, Transverse section of the stem. No accumulation of Methylene Blue is observed at T0 in the bud, even though Methylene Blue is well transported by stem xylem. C, Same section after carmino-green staining showing xylem tissue in green. D to F, When the plant is exposed to WL from T0, the accumulation of Methylene Blue is already visible in bud scales at 24 h (D) and pursued at 72 h (E) and 120 h (F). G, From 96 h, the accumulation of Methylene Blue also is visible in the leaves but not in the leaf primordia. H, Under darkness, bud remains dormant and no accumulation of Methylene Blue is ever observed in the bud, even after 196 h. I, When BAP is applied under darkness, this leads as early as 24 h after treatment to active xylem connection between the node and the bud, as evidenced by Methylene Blue accumulation in the vascular tissues of bud scales. J, Percentages of buds showing Methylene Blue in scales and in leaves after light or CK treatment in darkness. Observations were made with a binocular microscope. Red arrows indicate the accumulation of Methylene Blue, and black arrows indicate bud. Data are means ± SE from three to five biological replicates, with n = 5 to 10 plants per replicate. Letters indicate significant differences by ANOVA between the three conditions for the same time point. White bars = 1 mm, and red bars = 0.1 mm.

rather acts on the upstream regulation of CK (i.e. homeostasis and transport). Some genes involved in CK synthesis (*LOG*) and degradation (*CKX*) were found to be under light control in other developmental processes and species (Carabelli et al., 2007; Schlüter et al., 2011; Bergougnoux et al., 2012). Here, we showed that the elevated amounts of active CKs in node and bud tissues under WL (Table I) were the result of the higher expression of CK synthesis genes in nodes (*RhIPT3* and *RhLOG8* from 6 h and *RhIPT5* from 24 h; Fig. 9C) and of the repression of CK degradation genes in both buds and nodes (*RhCKX1* and *RhCKX6* from 3 h). The rapid regulation of CK genes after WL exposure (3 or 6 h) indicates that CKs are one of the initial targets of the light transduction pathway during outgrowth.

Also, an increased accumulation of *RhPUP5* transcripts, a gene homologous to *AtPUP5*, was observed 3 h after WL exposure or 6 h after CK application in darkness (Supplemental Fig. S2C). *AtPUP5* is the closest isoform of *AtPUP4*, a putative CK transporter, whose expression is regulated by CK application in Arabidopsis (Gillissen et al., 2000; Brenner et al., 2005). Bearing in mind that the role of *PUP4* and *PUP5* in CK transport has not been demonstrated, this result may suggest that the accumulation of high levels of CKs in buds also could be driven by the promotion of CK transport by WL.

De Novo Synthesis of CKs in Node Is an Initial Step of the Light Signaling Pathway That Controls Bud Outgrowth

Application of CK on the cut end of the stem led to a stronger promotion of bud elongation and of meristem organogenesis compared with direct application onto the bud (Fig. 6). Also, de novo synthesis of CK (as measured by *RhIPT3/5* expression) in WL was promoted in the node while it was repressed in the bud. These observations are in agreement with previous observations suggesting that CKs provided by node

tissues are involved in the control of bud outgrowth (Tanaka et al., 2006; Liu et al., 2011; Xu et al., 2015). Since the accumulation of CKs in the node occurred as early as 6 h of WL exposure (Table I) and concomitant with the increased expression of *RhIPT* genes (Fig. 9C), and since exogenous application of the CK synthesis inhibitor LVS on the cut end of the stem led to a reduced outgrowth of buds under WL (Fig. 7), our results point to the primary role of newly synthesized CKs in the node as part of the initial trigger of bud outgrowth by WL.

Photocontrol of CKs Acts Upstream of SL Signaling *RwMAX2*, Auxin Transport *RhPIN1*, and Central Integrator *RhBRC1* Genes

Remarkably, when only exogenous CKs were applied on bud or stem in darkness, buds produced a significant outgrowth with well-developed leaves and the formation of new leaf primordia (Figs. 6 and 8). This indicates that, in the dark condition, exogenous CKs are able to trigger all the mechanisms needed to achieve outgrowth (i.e. node and leaf elongation and organogenic activity of the shoot apical meristem [SAM]). Taken together with the early promotion of CK synthesis, activation, and putative transport in the node-bud complex by WL (Fig. 9C; Table I), these observations suggest that light control over CK is an important and initial step of the light signaling pathway of bud outgrowth. Results from the analysis of gene expression during bud outgrowth after CK treatment in darkness bring support to this model and reveal a broad range of mechanisms and genes under the photocontrol of CK (Fig. 10).

Two models are currently proposed to explain the interactions between hormones during the control of bud outgrowth. According to the auxin canalization model, auxin export from the axillary bud is necessary for bud outgrowth and is blocked, during apical dominance, by polar auxin transport in the stem (Li and Bangerth, 1999; Bennett et al., 2006; Balla et al., 2011). In the second messenger model, auxin acts indirectly in the control of bud outgrowth (i.e. through inhibition of the CK signal and promotion of the SL signal; Tanaka et al., 2006; Brewer et al., 2009; Müller and Leyser, 2011). The antagonistic action of CK and SL leads to the modulation of the expression of the central integrator *BRC1* and to bud outgrowth (Dun et al., 2012; Rameau et al., 2015).

In this study, the response of *RhBRC1* to light and to CK treatment is of particular interest. Expression of *RhBRC1* in buds was rapidly (6 h) and highly (6-fold or greater) increased by dark treatment (Fig. 10A). This reveals a strong light control over this central regulator, in accordance with previous observations whereby the expression of *AtBRC1* in Arabidopsis buds is regulated by photosynthetic photon flux density (Su et al., 2011) and is increased in the presence of additional far-red light in WL (González-Grandío et al., 2013). BAP application in the dark led to a rapid inhibition of *RhBRC1* (i.e. as soon as 3 h in the node and 6 h in the bud), and inhibition even increased until 48 h (Fig. 10B). This suggests that *RhBRC1* is an initial target of CK photocontrol. Furthermore, the rapid modulation of *RhBRC1* expression by BAP suggests a direct control of its transcription by CK. Interestingly also, *RhBRC1* expression was found not only in the axillary bud but also in the node (Fig. 10). This is consistent with observations in chrysanthemum (*Dendranthema* × *grandiflora*), where expression of *DgBRC1* also was found in the stem (Chen et al., 2013), but not with previous reports in Arabidopsis and tomato (*Solanum lycopersicum*), where *BRC1* expression seemed restricted to the axillary bud (Aguilar-Martínez et al., 2007; Martín-Trillo et al., 2011). Further study should address the significance of *BRC1* expression in the node in the control of bud outgrowth.

A strong negative control by WL also was observed for the SL signaling gene *RwMAX2*, in accordance with our previous results in rose (Djennane et al., 2014). Expression of *RwMAX2* was promoted by darkness in both bud and node as early as 6 h (Fig. 10A). This spatial and temporal pattern of expression is consistent with the repressive effect of the SL signal on bud outgrowth and with the fact that local, and not long-distance, expression of *RwMAX2* drives the repression of bud outgrowth (Djennane et al., 2014). As highlighted for *RhBRC1*, the expression of *RwMAX2* also was strongly repressed by CK treatment in darkness and as early as 6 h (Fig. 10B). This result indicates that CK regulation by light rapidly affects SL signaling. So far, evidence was only given that SL could cause the inhibition of CK through the negative regulation of *PsIPT1* in pea (Dun et al., 2012), but no effect of CK on the SL signal has been reported. Further work should determine how CKs act on *RwMAX2* transcript levels and on other components of SL signaling genes. It should be pointed out that such negative effects of CK on *RwMAX2* transcripts also may contribute indirectly to the repression of *RhBRC1* that is promoted by the SL signal (Aguilar-Martínez et al., 2007; Braun et al., 2012).

The regulation of auxin homeostasis and transport by WL and CK appeared complex. Light promoted the expression of the auxin synthesis genes *RhTAR1* and *RhYUC1* in the bud and of the auxin efflux gene *RhPIN1* in the node (Fig. 10A). The light activation of *RhTAR1* and *RhYUC1* in the bud correlated well with the increased IAA content in the bud observed after 24 h of WL exposure (Table I). This auxin would then contribute to the ongoing outgrowth under WL. A promoting effect of CK treatment was observed on *RhYUC1* and *RhTAR1* in the bud and on *RhPIN1* in the node, indicative of a light control of these genes acting through CK (Fig. 10B). In the bud itself, no effect of light conditions was observed on *RhPIN1* transcript level up to 48 h, and exogenous application of BAP had little effect on the transcription of this gene in darkness (Fig. 10B). In pea, application of BAP on intact plants also led to an increase in *PsPIN1* expression followed by the relocalization of PIN proteins (Li and Bangerth, 2003; Kalousek et al., 2010). If a similar effect of CK on PIN distribution was demonstrated in rose, then our results

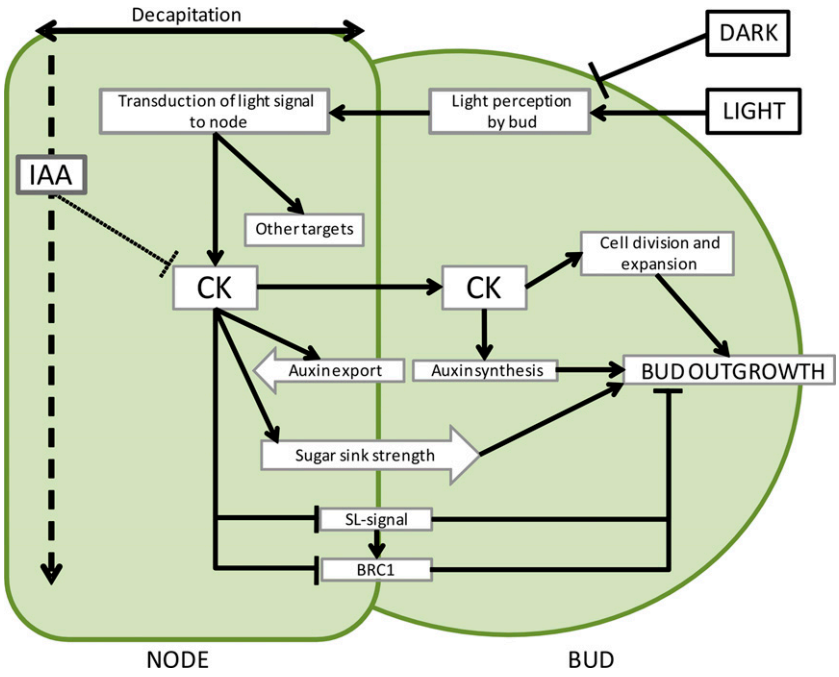

Figure 12. Model for the roles of CKs in the photocontrol of bud outgrowth in rose. After decapitation, perception by the axillary bud of favorable light conditions leads to the transduction of a photomorphogenic signal toward the corresponding node. This signal promotes the accumulation of CK in the node through rapid (3–6 h) stimulation of CK synthesis (*RhIPT3* and *RhIPT5*) and activation (*RhLOG8*) genes together with repression of the CK degradation gene *RhCKX1*. Transport of these neosynthesized CKs toward the bud may be promoted by the putative CK transporter *RhPUP5*, the transcription of which is enhanced by WL as soon as 3 h after light perception. In the bud, incoming CKs are preserved from degradation, since light inhibits expression of the CK degradation genes *RhCKX1* and *RhCKX6*. In both node and bud, neosynthesized CKs trigger a CK signal involving enhanced expression of the CK signaling genes *RhARR3* and *RhARR5* already at 3 h. The light-promoted CK signal then acts over several key actors of bud outgrowth: inhibition of the SL signaling gene *RwMAX2* and of the central negative regulator *RhBRC1* in both bud and node and increased sugar sink strength of bud through activation of the Suc transporters *RhSUC2* and *RhSWEET10*, of the Suc synthase *RhSUSY*, and of the vacuolar invertase *RhVI*. The simple sugars (Glc and Fru) provided to the bud contribute to bud outgrowth together with enhanced expression of the cell division gene *RhPCNA* and of the cell wall expansion gene *RhEXP* by CK signal. *RhCYCD3* also contributes to this growth; its expression is promoted by WL, independently of CK. The light-promoted CK signal also promotes auxin (IAA) accumulation in bud, as measured at 24 h through stimulation of the auxin synthesis genes *RhYUC1* and *RhTAR1* in the bud. The export of this neosynthesized auxin toward the node is permitted because auxin depletion in the node has occurred after decapitation, a process to which light-promoted CK signal may contribute to enhancing the expression of the auxin efflux gene *RhPIN1* in the node. The initial (3–6 h) impact of light over CK genes and CK accumulation as well as the rapid effects of the light-promoted CK signal over several mechanisms controlling bud outgrowth suggest that CKs are an initial and upstream component of the light signaling pathway. Other molecular targets of light independent of CK exist, as shown for *RhCYCD3* that takes part in the regulation of bud outgrowth. However, the capacity of CK-derived light signal to trigger a complete process leading to bud outgrowth (bud elongation and organogenic activity of the meristem) suggests that CKs drive a main part of the light regulation of bud outgrowth.

would suggest that dark inhibition of bud outgrowth is due, in part, to repressed auxin synthesis in the bud and to reduced polar auxin transport in the node, both light regulations being transmitted through CK.

Photocontrol of CK Acts on Sugar Sink Strength

Norflurazon and masking experiments in this study confirm our previous evidence that a bud relies on sugar already present in the plant rather than on instant sugar assimilation through photosynthesis to achieve outgrowth (Girault et al., 2010; Henry et al., 2011). An exogenous supply of sugars in darkness had little effect on the dark inhibition of bud outgrowth, as observed previously in vitro (Henry et al., 2011; Rabot et al., 2012). This confirms that the sugar level in the vicinity of the bud is not the initial limiting factor for bud outgrowth in the dark (Girault et al., 2010). Rather, light control of the vacuolar Suc invertase *RhVI*, the Suc synthase *RhSUSY*, and the two Suc transporters *RhSUC2* and *RhSWEET10*, as shown here (Fig. 10A), probably acts as a strong regulatory mechanism of bud sugar sink strength. Indeed, vacuolar invertases, Suc synthases, and Suc transporters are key components in the establishment of sink strength within sink organs (Zrenner et al., 1995; Sturm and Tang, 1999; Bihmidine et al., 2013). As a sink organ, a bud requires sugars from

the rest of the plant to grow out. Depending on the light environment, our results suggest that bud sugar sink strength would be promoted or repressed by the light signal perceived by the bud itself and that this would play a major role in the control of its outgrowth. In the bud, strong up-regulation of *RhVI* at 6 h by light would contribute to rapid sugar degradation required for the initiation of bud outgrowth (Fig. 10A; Girault et al., 2010; Mason et al., 2014; Barbier et al., 2015a). *RhSUSY* that is stimulated by WL at 24 h in the bud also would contribute to outgrowth, but in a later phase (Fig. 10A).

Interestingly, the expression of all the sugar-related genes studied here was regulated by exogenous CK applied under darkness. Promotion (or repression in the case of *RhNAD-SDH*) by CK was observed at least as rapidly (6 h) as the effect of WL, suggesting that the light regulation of sugar sink strength involves an upstream step through CK (Fig. 10B). Experiments using sugar supply in darkness also support this idea. Indeed, sugar supplies were less efficient (as measured by percentage bud outgrowth and bud elongation) than BAP supply. In other developmental systems and species, CKs were shown to adjust the sugar partitioning and sink strength of some organs through the regulation of sugar transporters and invertases (Thomas, 1986; Roitsch and Ehneß, 2000; Guivarc'h et al., 2002, Werner et al., 2008; Proels and Roitsch, 2009; Liao et al., 2013). Our results and those previous reports support the model whereby, in adverse light conditions, light through the down-regulation of CK would limit sugar sink strength in bud and thus repress outgrowth.

CKs Rescue Organogenesis in Darkened, Inhibited Meristems

Bud outgrowth requires the resumption of meristem organogenic activity and the elongation of the preformed organs, which involves promoting roles of cell cycle-related genes such as *PROLIFERATING CELL NUCLEAR ANTIGEN* (*PCNA*; Moldovan et al., 2007), cyclin (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998; de Jager et al., 2005), and cell wall expansion-related genes such as *EXPANSINS* (Fleming et al., 1997; Reinhardt et al., 1998; Zenoni et al., 2011). A strong photocontrol of *RhPCNA1*, *RhCYCD3;1*, and *RhEXP* was observed during bud outgrowth, as reported in Arabidopsis and sorghum (López-Juez et al., 2008; Finlayson et al., 2010; Kebrom et al., 2010). WL had an early (6 h) promoting effect on the expression of *RhPCNA1* and *RhEXP* and a later (24 h) effect on *RhCYCD3;1* (Fig. 10A). CK application allowed the resumption of SAM organogenesis in rose buds in darkness (Fig. 6, G and J), as reported previously for excised Arabidopsis and tomato meristems (Yoshida et al., 2011). This activation of SAM organogenic activity by CK appeared to be mediated by *RhEXP* and *RhPCNA1* (for which an increased expression was observed in darkness after CK treatment) but not by *RhCYCD3;1* (Fig. 10B).

CONCLUSION

This study has provided evidence for a positive photocontrol of CK during bud outgrowth, acting on both CK metabolism and possibly on CK transport and leading to a modulation of the CK signal. Triggering of bud outgrowth and the regulation of several key actors of bud outgrowth by the CK supply in darkness furnish evidence that CKs are initial and key actors of the light response in this process. A model based on our results is presented in Figure 12. Other pathways independent of CKs may coexist as evidence for *RhCYCD3;1*. Further work will contribute to their identification and to the study of their interactions with the CK pathway.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All the experiments were carried out using plants from a clone of a single genotype of *Rosa hybrida* 'Radrazz', as in our previous studies (Girault et al., 2008, 2010). Plants were grown in a greenhouse for 9 to 12 weeks after cutting in 0.5-L pots containing substrate (50% [v/v] Irish peat, 40% [v/v] perlite, and 10% [v/v] coconut fibers) and nutrients (1 kg m^{-3} PgMix 14:16:18-magnesium-trace elements; Yara). The method used to prepare plants for the experiments is described in Supplemental Figure S4. In intact plants of this rose cultivar, apical dominance is strong and axillary buds do not grow out until the apical bud flowers. Outgrowth of axillary buds then starts from buds just below the terminal flower. In order to trigger the outgrowth of basal buds, stems of plants with a terminal flower bud just protruding out of the young leaves (flower bud visible stage) were cut 0.5 cm above the third basal five-leaflet leaf, and all the remaining leaves were removed (Girault et al., 2008). Decapitation aimed to release the axillary bud just below the stem section from apical dominance. Defoliation aimed to remove any correlative interactions between the axillary bud and its corresponding leaf, which were shown to modulate the bud's capacity to grow out (Le Hir et al., 2006; Girault et al., 2008; Ferguson and Beveridge, 2009; Kebrom et al., 2010; Kebrom and Mullet, 2015). As shown previously, defoliation does not impair bud outgrowth (Girault et al., 2008).

Once decapitated and defoliated (at Tdecap), plants were all placed under darkness for 24 h after decapitation, using a dark bag. This first dark phase was used to synchronize the buds from all plants and to separate the effect of decapitation from the effect of the light treatment. The presence of a bag around the plant did not inhibit the bud's capacity to grow out due to a restricted air volume, since, when using a transparent bag, buds grew out normally (Girault et al., 2008). Some plants were grown in a large air volume in a darkened growth cabinet to further demonstrate that it is indeed the dark condition provided by the bag or the cabinet, and not the restricted air volume of the bag, that is responsible for bud outgrowth inhibition (Supplemental Fig. S5). After 24 h, plants were either transferred to WL (200 μmol m^{-2} s^{-1}, 16 h/24 h as described by Girault et al. [2008]) after removal of the bag or left in the bag for dark treatment. Darkness was used here as the null treatment for WL. The moment when the light treatment was applied, 24 h after decapitation, was called T0. The outgrowth of the distal bud (closest to the decapitation site and, thus, free from any apical dominance) was followed (Supplemental Fig. S4B).

Exogenous Applications of Chemicals and Growth Measurements

To study the effects of CKs on bud outgrowth, different CKs (BAP [Sigma], isopentenyladenine [Santa Cruz Biotechnologies], and zeatin [Sigma]) as well as the CK synthesis inhibitor LVS (Santa Cruz Biotechnologies) and the CK perception inhibitors LGR-991 and PI-55 (provided by the Department of Chemical Biology and Genetics, Palacký University and Institute of Experimental Botany) were applied either directly on the studied distal bud or on the cut end of the stem 24 h after decapitation (T0). LVS when used at 1 μM or at a lower concentration is known to act in blocking specifically the synthesis of the CK precursor from the mevalonate pathway (Alberts, 1988; Crowell and Salaz, 1992; Hartig and Beck, 2005). PI-55 and LGR-991 are inhibitors of CK

perception, acting, respectively, as competitors of the CK receptors Arabidopsis His kinase AHK4 and AHK3/4 (Spíchal et al., 2009; Nisler et al., 2010). PI-55 and LGR-991 up to a concentration of 5 μM were shown to be active in tomato (*Solanum lycopersicum*) seedlings with no toxic effect (Bergougnoux et al., 2012). In this work, the three inhibitors were used at a concentration of 1 μM or 1 mM except for LVS. Inhibitors and hormones were dissolved in 1% (v/v) dimethyl sulfoxide and then mixed in prewarmed (55°C) lanolin (Sigma). A 12-μL drop of the warmed paste was applied with a pipette tip. Preliminary studies showed that the warmed paste did not impair bud outgrowth. The drop was large enough to cover the bud or the section of the stem entirely. For the dark treatment, application of lanolin was achieved under green light. Plants were then placed under WL or back in darkness.

Sugars (Suc or a mix of Glc and Fru [Sigma]) were applied at T0 (24 h after decapitation) in lanolin at 100 mM as described above or as aqueous solutions for higher concentrations (250, 400, 600, and 800 mM) under darkness. For the supply of aqueous solutions, a 0.2-mL tube containing 100 μL of sugar solution was placed on the cut end of the stem after the tube base had been sectioned (Supplemental Fig. S4, C and D). Lanolin was used to seal the tube on the stem. Control plants bearing such a device were grown under WL to check that bud outgrowth was not impaired.

Bud outgrowth was observed 7 d after the above treatments. Bud length was measured using a numeric caliper, perpendicular to the stem. Buds were then dissected using a stereomicroscope: scales, young leaves, and leaf primordia were removed sequentially and counted until only the SAM remained (Supplemental Fig. S4E). The number of leaf primordia produced by the SAM during the 7 d, called neoorganogenesis, was calculated by subtracting the number of leafy structures (7.9 on average) found at T0. Buds were considered to have grown out when at least one visible leaf was protruding out of bud scales, as described previously (Girault et al., 2008). The mean bud lengths were calculated from the sizes of outgrowing and dormant buds from the same batch.

Detection of Chlorophyll and Masking Experiments

To study the role of photosynthesis in the triggering of bud outgrowth, foil masking and photosynthesis inhibitors were applied. Foil masking was carried out as described by Girault et al. (2008). Plants were either totally or partially covered by silver foil. All the corresponding controls were conducted with Parafilm or a translucent film and showed that the presence of such a film over the stem or the bud did not impair bud outgrowth. The photosynthesis inhibitor norflurazon (Sigma) was applied in a lanolin drop at 500 μM on the bud 4 d before the flower bud visible stage. At this stage, plants were decapitated and defoliated, and the lanolin drop containing norflurazon was removed. As for the sugar supply, norflurazon (100 μL) was then immediately supplied as a 500 μM aqueous solution (after dissolution in dimethyl sulfoxide) through the cut end of a 0.2-mL tube inserted on the cut end of the stem. Plants were then covered with an opaque bag for 24 h before exposure to WL for 7 d. The tube was filled every 2 d to ensure a constant supply of norflurazon.

The absence of chlorophyll after norflurazon treatment was checked using two methods. First, buds were observed using a stereomicroscope (Olympus SZX16, DP71, CellSens) equipped with a long path filter (ET500LP; emission spectra between 400 and 650 nm) and UV light (excitation wavelength of 460–480 nm). Exposure to UV light led to red fluorescence in the presence of chlorophyll or no fluorescence in the absence of chlorophyll. Some buds also were dissected and their young leaves observed using a confocal laser scanning microscope (Nikon EclipseTi) with a Plan Fluor 20× water-immersion objective (excitation wavelength of 638 nm, emission spectra between 662 and 737 nm). Laser power remained unchanged throughout the experiment. Composite images were produced with ImageJ1.43. Chlorophyll was detected as false-color blue dots inside the cells.

Measurements of Endogenous Phytohormones

Endogenous levels of CK metabolites and free IAA were detected by liquid chromatography-tandem mass spectrometry methods (Floková et al., 2014; Antoniadi et al., 2015). Samples (20 mg fresh weight) were homogenized and extracted in 1 mL of modified Bieleski buffer (60% methanol, 10% HCOOH and 30% water) together with a cocktail of stable isotope-labeled internal standards (0.25 pmol of CK bases, ribosides, and *N*-glucosides, 0.5 pmol of CK *O*-glucosides and nucleotides, and 5 pmol of [$^2H_5$]IAA per sample added). The extracts were purified using two solid-phase extraction columns, the octadecylsilica-based column (C18, 500 mg of sorbent; Applied Separations) and after that the Oasis MCX column (30 mg mL^{-1}; Waters). Analytes were eluted by a three-step elution using a 60% (v/v) methanol, 0.35 M NH_4OH aqueous solution and 0.35 M NH_4OH in 60% (v/v) methanol solution. CKs and free IAA levels were determined using ultra-HPLC-electrospray-tandem mass spectrometry using stable isotope-labeled internal standards as a reference (Rittenberg and Foster, 1940). Three independent biological replicates were performed.

Visualization of Xylem Flux Using Methylene Blue

Experiments with Methylene Blue (Sigma) were conducted on rose plants at the same stage as for the other experiments. After WL or dark treatment, the potting mix around the roots was removed and the roots were cleaned with distilled water. The plants were then placed in a solution of Methylene Blue (0.5% [v/v] in distilled water) for 3 h. Stems were hand cut longitudinally and observed as fresh tissues using a stereomicroscope.

Primer Design of Genes of Interest

Our previous work had identified a set of genes contributing to rose bud outgrowth (Girault et al., 2010; Henry et al., 2011; Djennane et al., 2014; Barbier et al., 2015a; Supplemental Table S3), and for some of them, we gave evidence for photocontrol during this process: a positive light control for *RhVI* (Girault et al., 2010; Rabot et al., 2012, 2014) and for *RhSUC2* (Henry et al., 2011) genes, and a negative light control for *RhNAD-SDH* (Girault et al., 2010) as well as for the SL signaling *RwMAX2* (Djennane et al., 2014) genes. Here, we further identified new homologous genes in rose that may be important in the control of bud outgrowth: *SWEET10* involved in Suc transport (Chen et al., 2012), *PCNA* and *CYCD3;1* involved in the cell cycle (Shimizu and Mori, 1998; de Jager et al., 2005; Moldovan et al., 2007), and *EXP* involved in cell wall expansion (Fleming et al., 1997; Dal Santo et al., 2011; Zenoni et al., 2011; Supplemental Table S1). New primers designed for this study are shown in Supplemental Table S1, and already published primer sequences are shown in Supplemental Table S3. The new primers were designed from sequences of *Rosa chinensis* or *Fragaria vesca* (https://www.rosaceae.org/species/fragaria/all), both species exhibiting high homologies with the rose genome (F. Foucher, personal communication), using the interface ROSAseq of the *R. chinensis* database (https://lipm-browsers.toulouse.inra.fr/plants/R.chinensis; Dubois et al., 2012). The chosen *Rosa* spp. clusters were BLASTed back in the NCBI database and in the *F. vesca* database for verification (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were designed using the National Center for Biotechnology Information primer BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and PCR products were sequenced for confirmation.

Concerning CK genes, nine *IPT*, seven *LOG*, seven *CKX*, 15 *PUP*, and eight *ENT* genes were identified in Arabidopsis (*Arabidopsis thaliana*), but not all their isoforms were found in the *R. chinensis* database. Among the *Rosa* spp. clusters, only those expressed in bud and/or stem were chosen: *RhIPT3/5*, *RhLOG3/8*, *RhCKX1/6*, *RhPUP4/5*, and *RhENT1/3*. However, no qPCR primers could be designed for *RhENT3* and *RhPUP4*.

Tissue Sampling for Molecular Analysis

Tissues were harvested 24 h after decapitation (T0) or 3/6/24/48 h after light and BAP treatments. Buds and nodes (0.5 mm above and below the harvested bud; see Supplemental Fig. S4) were collected, immediately frozen in liquid nitrogen, and then stored at −80°C until use. Three biological replicates were made for each condition, using buds and nodes from 40 to 100 plants for each biological replicate.

RNA Extraction, Reverse Transcription, and Quantitative Reverse Transcription-PCR

Frozen tissues (60–80 mg) were ground using a mortar and pestle in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) including the DNase treatment (Qiagen). RNA quantity was checked using NanoDrop technology, and RNA quality was checked by gel electrophoresis. The absence of contaminating DNA was checked by PCR on RNA using primers amplifying an intron-containing region of the *RhGAPDH* gene (Girault et al., 2010).

The synthesis of cDNA was achieved using the Reverse Transcription Kit (Invitrogen) on 1 μg of RNA. The cDNA was purified with the QIAquick PCR Purification Kit (Qiagen), diluted 100 times with milliQ water, and stored at −20°C. Gene expression was analyzed by real-time quantitative reverse

transcription-PCR. The qPCR was carried out with a mix of the cDNA as the template, the primer pairs, and iQ SYBR Green Supermix (Bio-Rad), in a final volume of 15 μL, and using the Chromo4 Real-Time PCR Detection System (Bio-Rad), with the following program: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 95°C and 60 s at 60°C. Then, the abundance of the transcripts was expressed relative to the control condition, as described by Pfaffl (2001). The reference gene used is the elongation factor *RhEF1-α*, as described previously (Girault et al., 2010). Three technical repetitions of each biological sample were made.

To compare the effect of light versus dark treatments or mock versus BAP treatments, relative expression in dark/relative expression in light and relative expression with BAP treatment/relative expression with mock treatment were calculated and transformed into $\log_2$ values. A value between −0.5 and +0.5 indicates no significant change in relative gene expression according to the treatment. A value greater than +0.5 or less than −0.5 is significant as shown by Student's *t* test. A color scale helps visualize the changes in transcript levels and is included in the figures. All qPCR data also are presented as graphs in Supplemental Figures S2 and S3.

Statistical Analysis

Three replicates of each experiment were conducted for the morphological analysis, with at least 10 plants per repetition. Standard statistical analyses (means and SE) and graphs were made using Microsoft Excel. Statistical differences were calculated by Student's *t* test when comparing two conditions or by multiple analyses ANOVA using R 2.13.2 software when comparing more than two conditions (0.05 significance level).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Impact of the CK perception inhibitors LGR-991 and PI-55 on bud outgrowth after application on the stem and of the CK synthesis inhibitor LVS after application on the bud and impact on basal buds.

Supplemental Figure S2. Graphs showing the relative expression of CK-related genes in the node or in the bud 3 h/6 h and 24 h/48 h after treatment, under WL and darkness, with or without CK applications.

Supplemental Figure S3. Graphs showing the relative expression of genes involved in the control of bud outgrowth in the node or in the treated bud 3 h/6 h and 24 h/48 h after treatment, under WL and darkness, with or without CK applications.

Supplemental Figure S4. Detailed description of the experimental procedures used.

Supplemental Figure S5. Effect of the opaque bag on bud outgrowth inhibition under darkness.

Supplemental Table S1. List of unpublished gene primers used in this study.

Supplemental Table S2. Detailed CK quantifications (bases, ribosides, nucleotides, and conjugated forms of trans-zeatin, cis-zeatin, dihydrozeatin, and isopentenyladenine) in nodes and buds after WL and dark treatments.

Supplemental Table S3. List of primer sequences published in previous work on roses.

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