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SHORT COMMUNICATION

Cytokinins and the CRE1 receptor influence endogenous gibberellin levels in *Medicago truncatula*

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ABSTRACT

Gibberellins (GAs) and cytokinins (CKs) are hormones that play antagonistic roles in several developmental processes in plants. However, there has been little exploration of their reciprocal interactions. Recent work in *Medicago truncatula* has revealed that GA signalling can regulate CK levels and response in roots. Here, we examine the reciprocal interaction, by assessing how CKs and the CRE1 (Cytokinin Response 1) CK receptor may influence endogenous GA levels. Real-Time RT-PCR analyses revealed that the expression of key GA biosynthesis genes is regulated in response to a short-term CK treatment and requires the CRE1 receptor. Similarly, GA quantifications indicated that a short-term CK treatment decreases the GA_1 pool in wild-type plants and that GA levels are increased in the *cre1* mutant compared to the wild-type. These data suggest that the *M. truncatula* CRE1-dependent CK signaling pathway negatively regulates bioactive GA levels.

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Gibberellin (GA) and cytokinin (CK) hormones are known to play antagonistic roles in several plant developmental processes, and some studies have reported development-dependent interactions between these two hormonal pathways.[1] In *Arabidopsis thaliana* seedlings, the up-regulation of GA level and/or GA responses was reported to inhibit CK signaling,[2] and similarly, GA signaling negatively regulates CK levels and/or signaling in *A. thaliana* and *Medicago truncatula* roots.[3,4] However, the reciprocal regulation of endogenous GA levels by CKs is less clear. In *A. thaliana* inflorescences, semi-quantitative RNA gel blot analyses suggested that CKs had no effect on the expression of a GA-responsive gene or a GA biosynthesis gene.[2] However, in the *Arabidopsis* shoot apical meristem, a CK-treatment induced the expression of *AtGA2oxidase* (*GA2ox*) gene, which encodes an enzyme that deactivates bioactive GA,[5]" and a genome-wide expression analysis reported the downregulation of several GA biosynthesis genes after a short-term CK treatment in whole *Arabidopsis* seedlings.[6] Similarly, transcriptomic studies performed in *M. truncatula* roots revealed a significant change in the expression of GA biosynthesis and response genes after short-term CK treatment,[7] suggesting that CKs may suppress endogenous GA levels. However, no study has examined the actual changes in endogenous GA levels in response to CKs. Here, we explored whether CKs and the CRE1 (Cytokinin Response 1) receptor that mediates CK responses[8] influenced endogenous GA levels in *M. truncatula*.

Based on transcriptomic data gained in *M. truncatula* roots,[7] we selected two genes rapidly regulated by a CK treatment; *GA20ox1* (GA_{20}-oxidase) and *GA2ox1* (GA_2-oxidase). The *GA20ox1* gene is predicted to encode an enzyme that catalyzes the formation of the inactive GA precursor GA_{20}, which can then be converted to bioactive GA_1 by a *GA3ox* enzyme.[9] The *GA2ox1* gene encodes a putative enzyme that catalyzes the deactivation of GA_1 into inactive GA_8[9]. The expression of these genes was analyzed by real time RT-PCR as previously described in Fonouni-Farde et al.,[4] in wild-type (WT) and *cre1* mutant roots in response to a short-term CK treatment (BenzylAminoPurine [BAP; Sigma]; 3h; 10^{-7}M) (Fig. 1A). In WT, the expression of *GA2ox1* was significantly induced by CKs, whereas the expression of *GA20ox1* was conversely repressed. These CK-dependent regulations were abolished in the *cre1* mutant (Fig. 1A), indicating that a subset of GA metabolic genes requires the CRE1 receptor to be transcriptionally regulated by CKs.

Based on the expression patterns observed in response to the BAP treatment (Fig. 1A), we hypothesized that there may be an increase of bioactive GAs in the *cre1* mutant compared to the WT. We therefore quantified GAs in WT and *cre1* mutant roots following a protocol derived from Boden *et al.*[10] (Supp. Methods). Like in the closely related legume pea,[11] GA_1 appears to be the predominant bioactive form in *M. truncatula*, as we could detect GA_1 but not GA_4. A small increase in GA_1 was detected in the roots of the *cre1* mutant compared to the WT, although this change was not significant (Fig. 1B).

As GA_1 levels were near the detection limit in *M. truncatula* roots, we also quantified GAs in shoots of WT plants after a short-term CK treatment (BAP; 3 h; 10^{-7}M) (Fig. 1C, D) and

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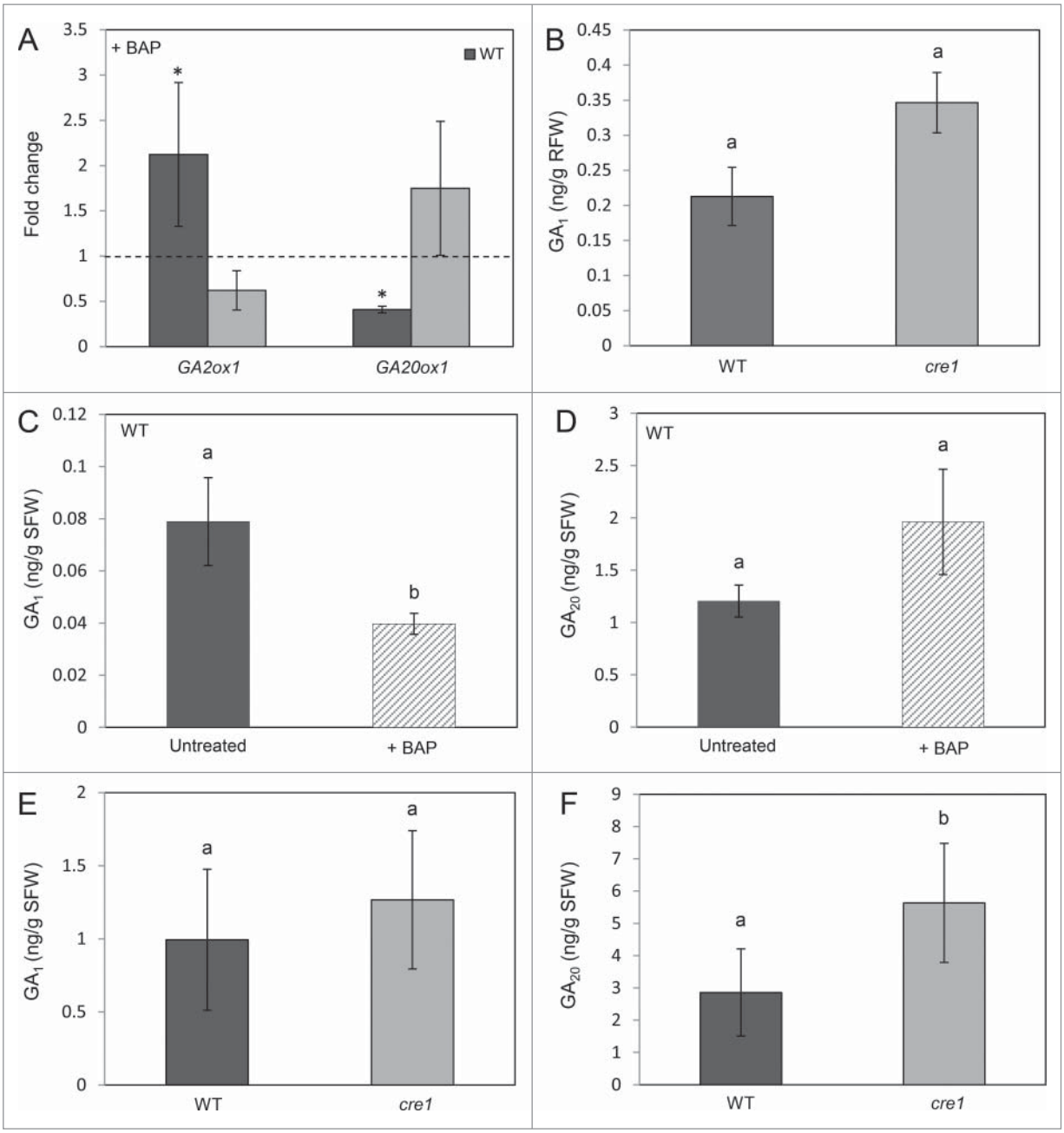

Figure 1. CKs and CRE1 regulate GA-metabolic genes and endogenous GA levels. (A) Quantification by RT-qPCR of the expression of *GA2ox1* and *GA20ox1* GA-metabolic genes, previously shown to be regulated by CKs (Ariel et al., 2012), in wild-type (WT) or in *cre1* mutant roots after a BenzylAminoPurine (BAP [Sigma]; 3 h; 10^{-7} M) treatment. (B) Quantification (in ng/g of Root Fresh Weigh [RFW]) of the bioactive GA1 in WT or *cre1* mutant roots. (C-D) Quantification (in ng/g of Shoot Fresh Weigh [SFW]) of the bioactive GA_1 (C) or the precursor GA_{20} (D) in WT shoots treated or not with BAP (3 h; 10^{-7} M). (E-F) Quantification (in ng/g of SFW) of GA_1 (E) or GA_{20} (F) in WT or *cre1* mutant shoots. In (A), transcript levels are normalized relatively to untreated control roots to show fold changes and the dotted line indicates a ratio of 1. Error bars represent standard deviations. Asterisks indicate significant differences compared to the untreated control, based on a Mann-Whitney test ($\alpha < 0.05$). Results are the mean of three biological replicates, each replicate being a pool of 25 plants. In (B-F), error bars represent standard errors of the mean. Letters indicate significant differences based on a Mann–Whitney test ($\alpha < 0.05$). In (B), the GA_1 quantification was based on four biological replicates, each replicate being a pool of at least two plants. In (C-F), GA quantifications were based on at least five biological replicates, each replicate being a pool of at least two plants.

in the *cre1* mutant (Fig. 1E, F). In the WT, the level of the bioactive GA_1 was significantly reduced in response to the CK treatment, whereas a small but not significant increase was observed for the immediate precursor to GA_1, GA_{20} (Fig. 1C, D). To determine if the CRE1 receptor was involved in the regulation of endogenous GAs level, we quantified GA_1 and GA_{20} in WT and *cre1* shoots (Fig. 1E, F). While the content of GA_1 showed no statistical difference between the WT and the *cre1* mutant, the level of GA_{20} was significantly higher in the *cre1* mutant (Fig. 1F). Taken together, these results indicate that CKs and the CRE1 receptor are able to regulate the level of a GA endogenous precursor and/or of a biologically active GA in *M. truncatula*.

In this study, we show that CKs through the action of the CRE1 receptor influence the levels of GAs in *M. truncatula*. Together with previous results reporting that GAs can regulate CK levels and response in *M. truncatula* roots,[4,7] this suggests that there is a bidirectional antagonistic regulation of CK and GA

levels that likely contribute to a dynamic equilibrium between these two hormones. As bioactive GAs suppress the accumulation of DELLA proteins,[12] we can speculate that in various developmental contexts the CRE1-dependent CK suppression of GA content may increase the DELLA protein accumulation and therefore modulate the expression of target genes.[13,14]

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