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ORIGINAL RESEARCH

Overexpression of *Brassica rapa NGATHA1* Gene Confers De-Etiolation Phenotype and Cytokinin Resistance on *Arabidopsis thaliana*

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Abstract *Brassica rapa NGATHA1* (*BrNGA1*) encodes a B3-type transcription factor. By analyzing Arabidopsis overexpressors of *BrNGA1* (*BrNGA1ox*), we have previously demonstrated that *BrNGA1* may be involved in negative regulation of cell proliferation during lateral organ and root growth. In the present study, we have found that *BrNGA1ox* seedlings grown in the dark display de-etiolation phenotypes, such as short hypocotyls, open and elongated cotyledons, and developing true leaves. *BrNGA1ox* seedlings as well as adult plants and calli are also resistant specifically to exogenous cytokinins. These data raise the possibility that the de-etiolation phenotypes of *BrNGA1ox* seedlings may result from an alteration in cytokinin response. We set out to test whether the de-etiolation phenotype is due to cytokinin overproduction or constitutively activated cytokinin response. First, *BrNGA1ox* was crossed to the *CKX2ox* plant, an overexpression line of CYTOKIN OXIDASE 2, which is responsible for degradation of active cytokinins. We found, however, no difference in the de-etiolation and shoot growth phenotypes between *BrNGA1ox* and *BrNGA1ox CKX2ox* plants. Next, we measured the transcripts level of *ARR5* and *ARR7*, frequently employed as molecular markers for cytokinin signaling and yet found no difference in their transcripts levels of the wild-type and *BrNGA1ox* seedlings and shoots. These data indicate that biological role of *BrNGA1* involved in de-etiolation seems to be associated with neither cytokinin overproduction nor its altered signaling. Possible molecular mechanisms by which BrNGA1 may interfere with cytokinin responses and etiolation are discussed.

Keywords *Brassica rapa* · NGATHA transcription factor · De-etiolation · Cytokinin

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Introduction

The plant hormone cytokinins are important regulators of plant growth and development, including cell division and differentiation, photomorphogenesis, apical dominance, and leaf senescence (Sakakibara 2006; Riefler et al. 2006). Among natural cytokinins, N^6-prenylated adenine derivatives, such as N^6-(Δ^2isopentenyl)adenine (iP) and *trans*-zeatin (tZ), are major active forms in *Arabidopsis thaliana* (hereafter Arabidopsis; Sakakibara 2006). iP is synthesized via transfer of an isoprenyl group to the adenine base of adenosine phosphates and, then, consecutive removals of phosphates and ribosyl moieties; tZ synthesis involves hydroxylation of the isoprenyl group (Sakakibara 2006). Both iP and tZ are degraded by cytokinin oxidases (Werner et al. 2003; Sakakibara 2006).

Plants respond to cytokinins through a His-Asp phosphorelay system, consisting of receptor kinases, histidine phosphotransfer proteins, and response regulators (To and Kieber 2007; Kieber and Schaller 2010). In the Arabidopsis genome, there are three cytokinin receptor kinases (AHK2,

AHK3, AHK4/CRE1/WOL) that contain a cytokinin-binding domain, a histidine kinase domain, and a receiver domain. Five histidine-phosphotransfer proteins mediate the phosphotransfer from the receptor kinases to the response regulators (ARRs). There are 23 ARRs that function as either positive or negative elements in cytokinin signaling. Perturbations in cytokinin signaling by various combinatorial mutations in those components cause alteration in various cytokinin responses (Higuchi et al. 2004; Riefler et al. 2006; To and Kieber 2007).

Many studies have presented that dark-grown (etiolated) seedlings treated with exogenous cytokinins show photomorphogenetic phenotypes, such as shortening of hypocotyls, cotyledon expansion, and leaf formation (Chory et al. 1994; Chin-Atkins et al. 1996). Cytokinin-resistant mutants, such as *cin4* and *cnr1*, also show short hypocotyls, expanded cotyledons, and leaf development in the dark (Vogel et al. 1998; Laxmi et al. 2006). *altered meristem program1* (*amp1*), *high organogenic capacity* (*hoc*), and *pga22* that are cytokinin-overproducing mutants or transgenic plants display the characteristic de-etiolation phenotype as well (Chin-Atkins et al. 1996; Catterou et al. 2002; Sun et al. 2003). These studies suggest that cytokinins are involved in promotion of photomorphogenesis, even in the dark.

Recently, we have analyzed Arabidopsis overexpressors of a *Brassica rapa NGATHA* gene, *BrNGA1*, which encodes a B3-type transcription factor (Kwon et al. 2009). Our study demonstrated that the BrNGA1 transcription factor may be involved in negative regulation of cell proliferation during lateral organ and root growth. In the present study, we report that *BrNGA1* overexpressor seedlings, when grown in the dark, have short hypocotyls and expanded cotyledons and develop true leaves. We also show that the de-etiolated phenotypes are associated with altered response to cytokinins. However, *BrNGA1* action in the de-etiolation seems not to be involved in either cytokinin overproduction or an alteration in the canonical pathway of cytokinin signaling. We discuss possible molecular mechanisms by which BrNGA1 may interfere with cytokinin responses and etiolation.

Materials and Methods

Plant Materials

All the plants used in this study are in the Columbia-0 (Col) accession of *A. thaliana*. Arabidopsis overexpressors of *BrNGA1* (*BrNGA1ox*) are prepared as described previously (Kwon et al. 2009). The *CKX2ox* seeds were kindly provided by Dr. Thomas Schmülling. Seeds were stratified in water at 4°C for 3 days and then sown on wet soil and transferred to a growth room at 23°C under a photoperiod of 16-h light/8-h darkness.

Hormone Treatment and Measurement of Growth

Seeds were sterilized in 50% of a bleaching solution followed by 70% ethanol and stratified in water at 4°C for 3 days. Those seeds were sown on the half-strength MS medium plates containing 1% of sucrose with various addenda and vertically incubated in the dark at 23°C for 7 days. For hormone treatment, the MS plate contained various concentrations of individual hormones, all of which were purchased from Duchefa: 0.25 μM of abcisic acid (ABA), 1 μM of 2,4-dichloriphenoxyaceticacid (2,4-D), 1 μM of 1-aminocyclopropane-1-carboxylate (ACC), 1 μM of N^6-benzyladenine (BA), 0.5 μM of epibrassinolide (BR), and 10 μM of gibberellic acid 3 (GA). For ABA treatment, seeds were first allowed to germinate on a hormone-free MS plate for 24 h, then transferred to ABA-containing plates, and incubated for 6 days. For treatment of shoots with cytokinin, plants were incubated horizontally on half-strength MS plates containing various concentrations of *trans*-zeatin (tZ) under a photoperiod of 16-h light/8-h darkness for 26 days.

Callus Induction Assay

For the callus induction assay, hypocotyls were excised with sterilized razor blades and cultured for 10 days in dark condition and transferred to the light condition for 1 month on the MS medium supplemented with varying concentrations of 2,4-D and BA.

Reverse Transcription and Quantitative PCR Analysis

For determination of transcripts levels of type-A *ARR* genes, total RNAs were extracted with RNA extraction kit (GE Healthcare) from 17-day-old, light-grown shoots or 7-day-old dark-grown hypocotyl segments, and subjected to reverse transcription (SuperScriptII, Invitrogen). Quantitative PCR was performed using a mix containing SYBR premix Ex-Taq (TaKaRa Bio) on an ABI 7300 real-time PCR system (Applied Biosystems). PCR conditions are following: a 10-min initial denaturation at 95°C, 40 cycles of 95°C for 15 s, and 56°C for 1 min. The reaction was performed using primer pairs specific to the genes of interest: *ARR5* (5′-GCCTCGTATCGATAGATGTCTT GAAGG-3′ and 5′-TCTGATAAACTCAGACTTTGCGCGT-3′), *ARR7* (5′-AGAGT GGAACTAGGGCTTTGCAGT-3′ and 5′-CTCCTTCTTTGAGACATTCTTGTATAC GAGG-3′). *ACTIN8* was amplified as a control by using the following primer set: 5′-AGCAGAACGGGAAATTGTGAGAG-3′ and 5′-CAAATATGGCTGGAAAAGGAC T-3′. The average

threshold cycle (Ct) values were generated and analyzed by SDS software version 1.3 (Applied Biosystems), which used the comparative Ct method (Livak and Schmittgen 2001). Fold changes compared with the untreated wild-type control were calculated after normalization to the Ct values of *ACTIN8*.

Results

Phenotypes of Dark-Grown *BrNGA1ox* Seedlings

As mentioned above, we have previously shown that in the light condition Arabidopsis plants overexpressing *BrNGA1* (*BrNGA1ox*) develop small and narrow leaves as well as short roots, which turns out to be due to reduction in cell proliferation activities (Kwon et al. 2009). In the study, two transgenic lines were analyzed in detail: *BrNGA1ox-2* showed strong phenotypes, and *BrNGA1ox-4* weak ones. Here in this study, we examined the effect of *BrNGA1* overexpression on the growth and development of dark-grown seedlings on MS agar plates in the dark for 7 days. We found that while dark-grown wild-type seedlings developed typical etiolated morphology with long hypocotyls and robust apical hooks, *BrNGA1ox-2* seedlings developed short hypocotyls and open hooks with elongated cotyledons and true leaves developing (Fig. 1a, b). All the *BrNGA1ox-2* hooks opened fully, and their cotyledon length was longer, by 1.7-fold, than that of the wild type (Fig. 1c). *BrNGA1ox-4* also showed similar phenotypes, albeit weaker than those of *BrNGA1ox-2*, indicating that *BrNGA1* overexpression induces de-etiolation in the dark condition.

Determination of Hormone Responsiveness of *BrNGA1ox* Plants

De-etiolation phenotypes of dark-grown seedlings have been often associated with defective hormonal responses (Chory et al. 1994; Li et al. 1996; Tian and Reed 1999). In order to examine whether *BrNGA1ox* phenotype is related to hormonal responses, seedlings were grown on the MS medium containing various hormones in the dark. Wild-type hypocotyls responded well to the synthetic cytokinin, BA, being reduced in length to only about 40% over untreated wild-type control (Fig. 2a). In contrast, *BrNGA1ox-2* hypocotyls showed only a slight inhibition by BA, indicating that *BrNGA1ox-2* seedlings are resistant to exogenous BA. *BrNGA1ox-4* also exhibited a substantial resistance to BA. However, *BrNGA1ox* hypocotyls were only slightly affected by other hormones, although they showed a marginal resistance to brassinosterolide and ACC, the immediate precursor of ethylene (Fig. 2a). Growth of wild-type

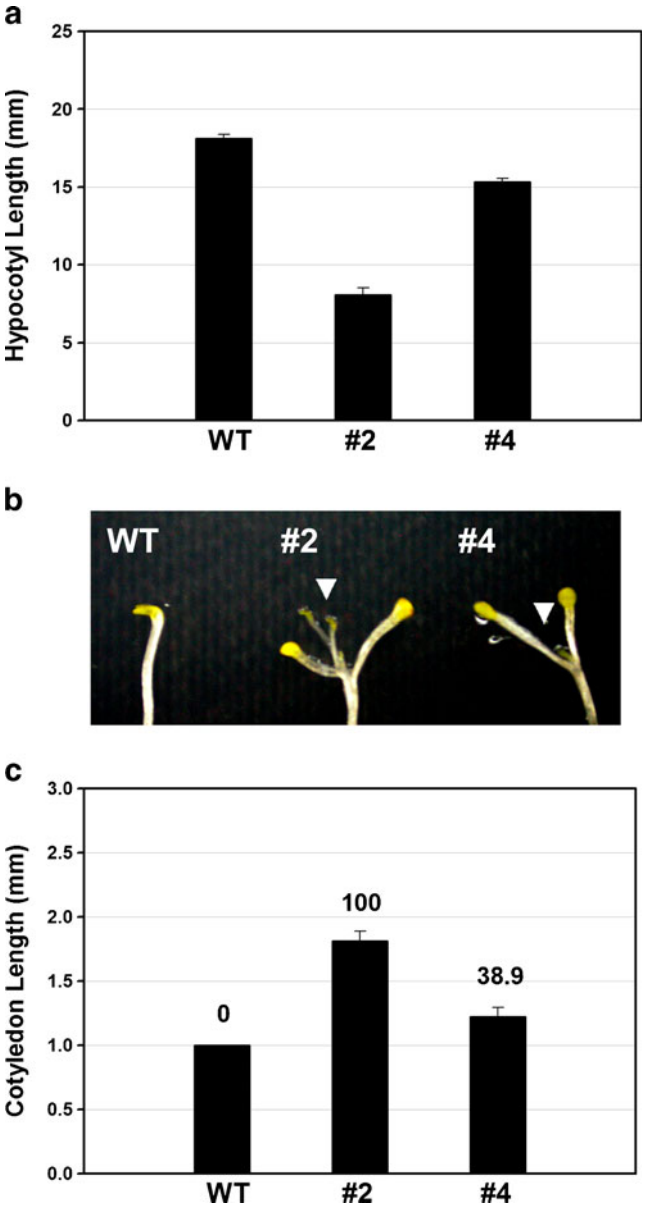

Fig. 1 Phenotype of etiolated seedlings. Seedlings were grown on vertical MS plates in the dark for 7 days. **a** Hypocotyl length. **b** De-etiolation phenotypes of seedlings. Note the developing true leaves (*arrowheads*) and elongated cotyledons in *BrNGA1* overexpressors. **c** Cotyledon length and percentage of cotyledon opening (the *numbers on the top of each column*) as de-etiolation indices

hypocotyls was inhibited by both the synthetic and natural cytokinins, BA and tZ, respectively, in a dose-dependent manner, whereas *BrNGA1ox-2* hypocotyls did not respond at all and *BrNGA1ox-4* did only moderately (Fig. 2b).

BrNGA1ox shoots were also resistant to exogenous cytokinins: wild-type plants did not survive on the MS medium plate containing high doses of tZ, whereas *BrNGA1ox* grew well, even forming the inflorescence stem, although their leaves tend to become smaller and narrower as cytokinin doses increase (Fig. 3a). In addition, wild-type

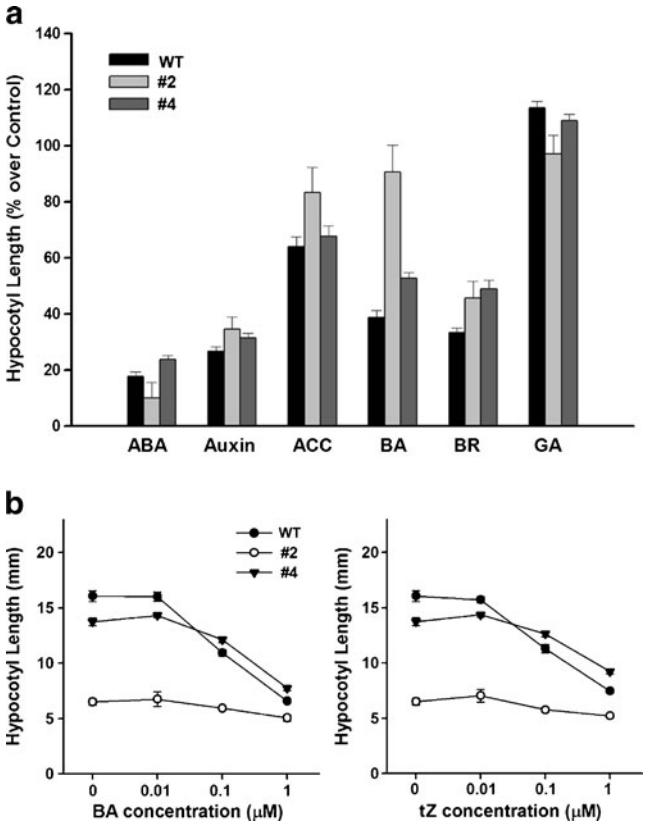

Fig. 2 Hypocotyl growth responses to exogenous hormones. **a** Changes in hypocotyl length of transgenic seedlings were compared with those of wild-type ones in response to application of various hormones. Final concentration of ABA, 0.5 μM; IAA, 1 μM; ACC, 0.5 μM; BA, 1 μM; BR, 0.5 μM, GA, 10 μM. **b** Hypocotyl length in response to various concentrations of exogenous cytokinins. Seedlings were grown on vertical MS plates containing hormones in the dark for 7 days

hypocotyl segments formed normal callus in the presence of auxin (1 or 10 μM) and cytokinin (0.01 μM); yet, callus growth was inhibited by high doses of tZ (Fig. 3b). In contrast, *BrNGA1ox* hypocotyls formed robust calli at those inhibitory concentrations of tZ (0.1 and 1 μM), except at an excessive concentration of tZ (10 μM; Fig. 3b).

Taken together, all these data are consistent with the notion that *BrNGA1* overexpression confers resistance to exogenous cytokinins on the Arabidopsis plant, as it induces a constitutive activation of cytokinin responses, resulting in de-etiolation in the dark.

Effects of *CKX2* Overexpression on Shoot and Hypocotyl Growth

A constitutive activation of cytokinin responses in *BrNGA1ox* plants may be caused by a high amount of endogenous cytokinins, in consequence nullifying the effect of exogenously applied cytokinins. To investigate the possibility, *BrNGA1ox-2* and a cytokinin oxidase-overexpressing plant, shortly *CKX2ox* (Werner et al. 2003), were crossed to each other or to wild-type plants. The F1 progeny of the crosses were used for analyses of shoot and hypocotyl phenotypes. *CKX2* gene encodes one of cytokinin oxidases that catalyze degradation of active cytokinins, such as iP and tZ. It has been demonstrated that overexpression of *CKX2* reduces endogenous level of cytokinins, resulting in cytokinin-deficient phenotypes, including small size of plants (Werner et al. 2003). *CKX2ox* plants heterozygous for the transgene were smaller than the wild-type plant (Fig. 4a). Similarly, heterozygous *BrNGA1ox-2* plants were also smaller than the wild-type plant (Fig. 4a; Kwon et al. 2009). Meanwhile, the F1 plant that harbors both heterozygous *BrNGA1ox* and *CKX2ox* did not differ in size from the heterozygous *BrNGA1ox* plant (Fig. 4a). The same pattern was also observed in hypocotyl growth in the dark: hypocotyl length of the F1 seedlings with both heterozygous *BrNGA1ox* and *CKX2ox* was not different from that of the heterozygous *BrNGA1ox* seedlings, although *CKX2ox* hypocotyl length was significantly longer than the wild-type one (Fig. 4b). These results indicate that downregulation of cytokinin level by *CKX2* overexpression did not affect growth phenotypes of *BrNGA1ox* plants, further suggesting that de-etiolation phenotypes and cytokinin resistance of *BrNGA1ox* plants are unlikely to be due to an excessive amount of endogenous cytokinins.

Expression Patterns of *Arabidopsis Response Regulator* Genes

Conclusion from the above experiments raises another possibility that de-etiolation phenotypes and cytokinin resistance of *BrNGA1ox* might be the result of a perturbation in cytokinin responsiveness, rather than a homeostatic alteration in cytokinin content. To test the hypothesis, we determined expression levels of the type-A *ARR* genes, *ARR5* and *ARR7*, by performing real-time PCRs after application of cytokinins to the shoot or hypocotyl tissues (Fig. 5a, b). It has been well documented that expression of *ARR5* and *ARR7* genes increases greatly in response to exogenous cytokinins (Argyros et al. 2008). However, we found no significant difference in either the basal or cytokinin-induced levels of *ARR* mRNAs between the wild-type and *BrNGA1ox* shoots and hypocotyl segments (Fig. 5a, b). These results indicate that *BrNGA1ox* plants show normal cytokinin signaling, at least, leading to the transcriptional activation of the type-A *ARR* genes, as wild-type plants do.

Discussion

Arabidopsis plants producing an excessive amount of endogenous cytokinin induce strong cytokinin responses.

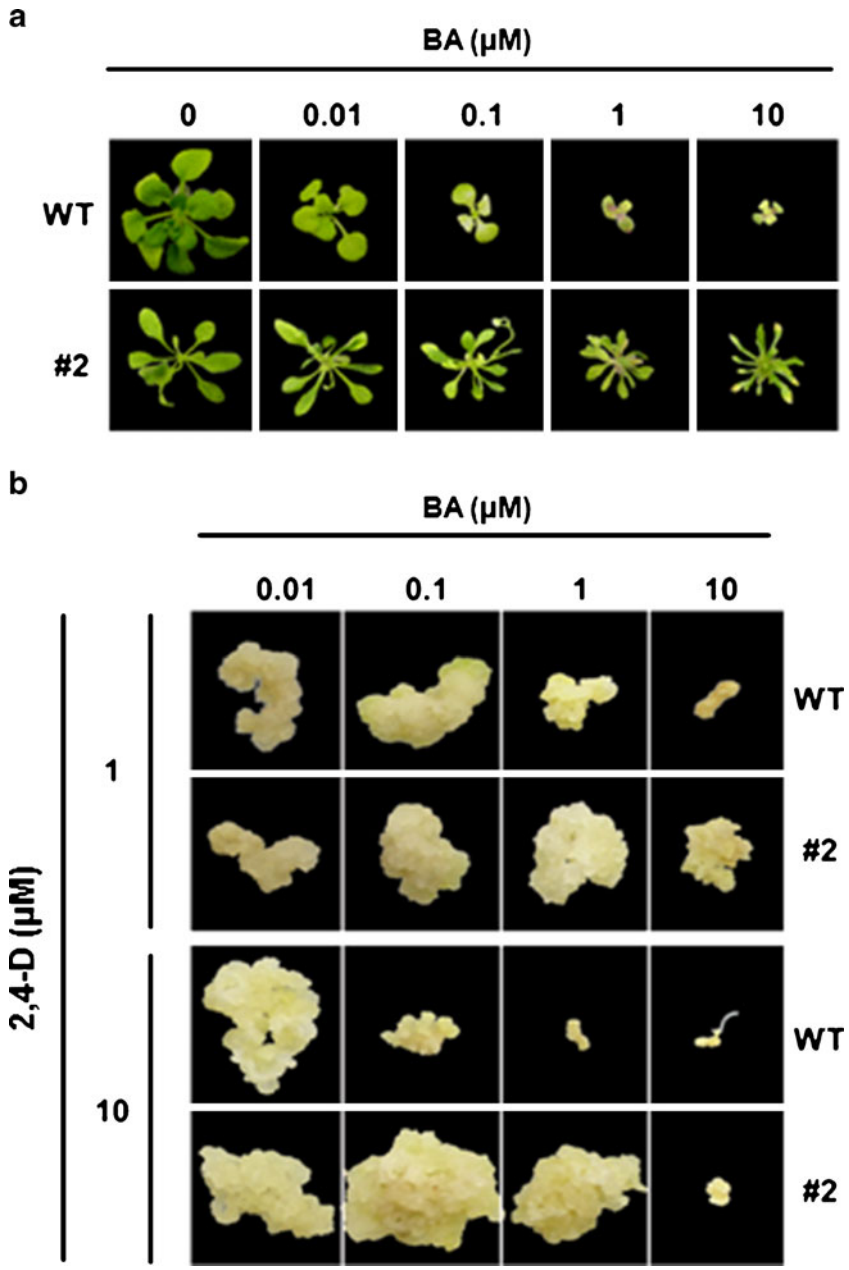

Fig. 3 Shoot and callus growth responses to various concentrations of exogenous cytokinins. **a** Shoot growth phenotypes in response to exogenous BA. Seedlings were grown on MS plates containing BA in the light for 26 days. **b** Callus growth in response to exogenous BA and 2,4-D

amp1 mutant overproducing cytokinins showed pleiotropic phenotypes, most of which can be phenocopied by exogenous treatment of cytokinins, including short hypocotyls and primary roots, de-etiolation in darkness, reduced apical dominance (Chaudhury et al. 1993). Another cytokinin-overproducing mutant *hoc* showed strong cytokinin responses similar to *amp1*, causing de-etiolation in the dark, such as short hypocotyls, cotyledon expansion, and true leaf development (Catterou et al. 2002). An increased level of cytokinins by conditional *AtIPT*-dependent biosynthesis also elicited typical de-etiolation responses, including short hypocotyl and roots, open cotyledons, the absence of apical hook, and true leaf initiation in darkness (Kuderová et al. 2008; Sun et al. 2003). Tobacco plants manipulated to express the *ipt* gene from *Agrobacterium tumefaciens* were short in height and have narrow and dark green leaves, underdeveloped roots, and increased growth of axillary buds (Hewelt et al. 1994; Medford et al. 1989; Smigocki 1991).

BrNGA1ox-2 and *BrNGA1ox-4* plants display strong and moderate phenotypes, respectively, that are reminiscent of cytokinin-overproducing responses in the light and dark. In other words, *BrNGA1ox* plants are resistant specifically to exogenous cytokinins (Figs. 2 and 3) and developed true leaves and open cotyledons in the dark as well as short hypocotyls (Fig. 1). They also produce more axillary leaves, which is indicative of reduced apical dominance, compared to wild type, and their leaf color is darker green

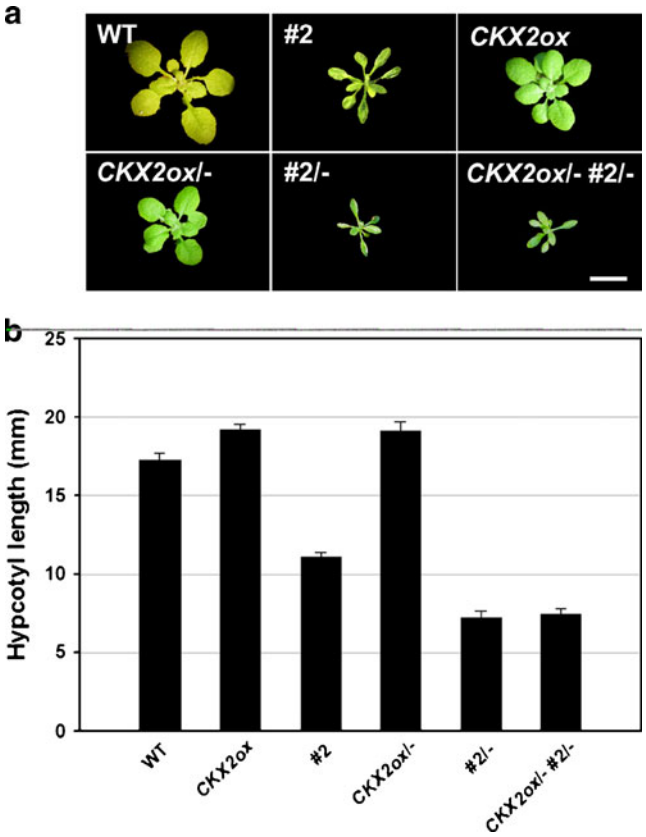

Fig. 4 Effect of *CKX2ox* on the shoot and hypocotyl growth. **a** Shoot phenotypes. Seedlings were grown on soil during 26 days. **b** Hypocotyl length. Seedlings were grown on vertical MS plates in the dark for 7 days. *Scale bar*=10 mm

than wild-type one (data not shown). These phenotypes are highly similar to those of cytokinin-overproducing plants. Therefore, it is conceivable that *BrNGA1* overexpression may induce constitutive cytokinin responses; thus, *BrNGA1* gene may play a role in regulating cytokinin responses.

When we prepared Arabidopsis plants overexpressing *A. thaliana NGA1* (*AtNGA1*) through *AtNGA4*, those overexpressors also developed shorter hypocotyls and cytokinin resistance (S.H.K and J.H.K, data not shown), suggesting that those phenotypes are a general feature of overexpressors of *NGA* family members. Furthermore, we also found that single or multiple loss-of-function mutants of *AtNGA1* through *AtNGA4* genes developed much longer hypocotyls than those of the wild type, although it remained to be determined whether or not those mutants displayed an altered responses to hormones, including cytokinins (S.H.K and J.H.K, data not shown).

How could *BrNGA1* overexpression induce constitutive cytokinin responses? First, we hypothesized that *BrNGA1* overexpression might induce overproduction of cytokinins. However, *BrNGA1ox* plants with *CKX2ox* were not different in shoot and hypocotyl phenotypes from *BrNGA1ox* plant lacking it, despite cytokinin-deficient phenotypes of the heterozygous *CKX2ox* plants (Fig. 4). These results suggest that phenotypes of *BrNGA1ox* may not be due to an increased amount of endogenous cytokinins. Next, it is possible that *BrNGA1* may constitutively activate the cytokinin phosphorelay signaling. However, we failed to detect any difference in the transcript level of the type A-ARR genes, *ARR5* and *ARR7*, indicating that overexpression of *BrNGA1* does not interfere with cytokinin phosphorelay signaling either.

Taken together, de-etiolation phenotypes of *BrNGA1ox* plants seem to be associated with neither cytokinin overproduction nor the phosphorelay signaling pathway. Nonetheless, we cannot rule out other possibilities in regard to cytokinin signaling pathway. First, BrNGA1 may be involved in other pathways in which induction of the type-A *ARR5* and *ARR7* is not required. For instance, type-C *ARR*s, *ARR22* and *ARR24*, are not transcriptionally regulated by cytokinins; yet, their overexpression inhibits cytokinin signaling, causing insensitivity to cytokinin (Kiba et al. 2004, 2005; Gattolin et al. 2006). Second, BrNGA1 may interfere with a later step of cytokinin responses following after the induction of type-A *ARR*s. Lastly, BrNGA1 may be involved in an alternative cytokinin

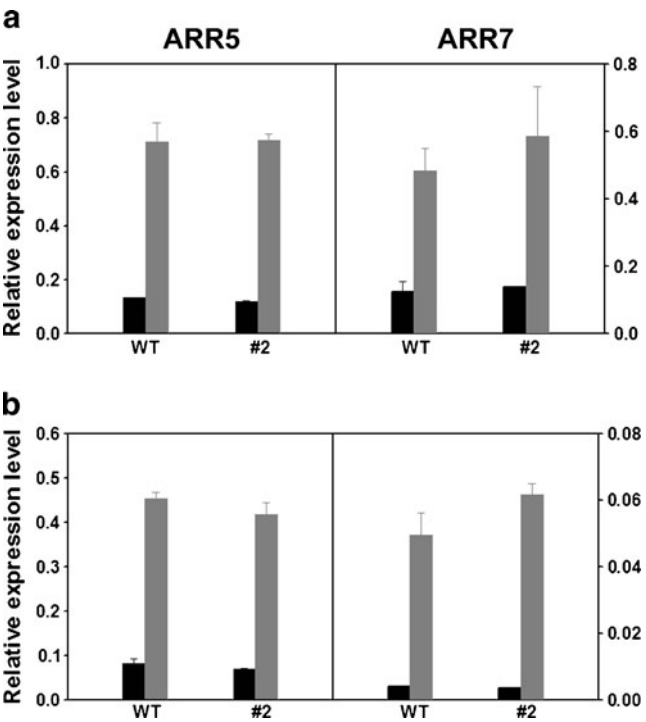

Fig. 5 Reverse transcription and quantitative PCR determination of mRNA levels of type-A *ARR* genes. Plants were grown on soil and treated with or without 10 μM BA for 1 h, and seedlings were grown on MS plates with or without 1 μM BA for 7 days. Total RNAs were isolated from the shoots and hypocotyls of wild-type and *BrNGA1ox-2*. **a** Expression level of *ARR5* and *ARR7* in the shoots. **b** Expression level of *ARR5* and *ARR7* in the hypocotyl. *Black bars* are control groups; *grays* are treated with BA

signaling. For instance, the CYTOKININ RESPONSE FACTORs do not require ARR pathway and, yet, mediate a part of cytokinin signaling (Rashotte et al. 2006). Furthermore, it still remains to be determined whether there are additional cytokinin perception systems or not (Kieber and Schaller 2010).

In conclusion, overexpression of *BrNGA1* gene confers de-etiolation and cytokinin resistance on dark-grown seedlings, although elucidation of the particular mechanism leading to those phenomena requires further studies in the future.

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