Effects of the removal of cotyledons on endogenous gibberellin levels in hypocotyls of young cucumber and tomato seedlings

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Abstract We have previously shown that gibberellin (GA) is required for tissue-reunion in the cortex of cut hypocotyls in cucumber (Cucumis sativus) and tomato (Solanum lycopersicum) seedlings, and that intact cotyledons are also necessary for this process. These results suggested that cotyledons might play an important role in controlling GA levels in the hypocotyl in these plant species. In this study, we found that a local application of a GA biosynthesis inhibitor uniconazole to cotyledons was effective to inhibit hypocotyl elongation, and that simultaneous application of GA canceled this inhibition. To study the role of cotyledons in GA content in the hypocotyl directly, cotyledons were removed from 7-days-old seedlings and endogenous GA levels in the hypocotyl were determined. Our results demonstrated that the cotyledon-removed seedlings contained lower levels of bioactive GAs and their precursors in the hypocotyls than did intact seedlings. Quantitative RT-PCR analysis indicated that transcript levels of LeGA20ox1 and LeGA3ox2 genes were elevated in the hypocotyl after the removal of cotyledons, suggesting that the reduced bioactive GA levels caused upregulation of these genes via the feedback regulation mechanism. Taken together, our results suggest that cotyledons are necessary for maintaining normal GA levels in young cucumber and tomato hypocotyls.

Key words: Cotyledon, GA20-oxidase and GA3-oxidase, gibberellin, hypocotyl.
Cotyledons are necessary for the GA maintenance in hypocotyls. The seeds were germinated and grown in artificial soil (Kurehakagaku, obtained from Sapporo-Nouen Co. (Sapporo, Japan)). The seeds of Cucumis sativus (Mil., cv. Moneymaker) were obtained from Sakata Seed Co. (Kanagawa, Japan), and tomato [Groot et al. 1987; Bensen and Zeevaart 1990; Rebers et al. 1999]) resulted in a failure in the tissue-reunion in cut hypocotyls. (3) Even after the removal of cotyledons, exogenous GA treatment can restore the ability of tissue-reunion in hypocotyls. GAs are a class of diterpenoids, some of which act as hormones that control many aspects of plant growth and development. In the GA biosynthesis pathways (Figure 1), GA₁ and GA₄ act as bioactive hormones, while others are precursors for bioactive GAs or inactivated forms. Recently, significant progress has been made in understanding GA biosynthesis and signaling pathways, including identifications of genes encoding GA metabolism enzymes and components in the GA response pathway in such model species as Arabidopsis, rice, pea and tomato (Ross et al. 1997; Rebers et al. 1999; Yamaguchi and Kamiya 2000; Olzewszi et al. 2002; Sun and Gubler 2004; Ueguchi-Tanaka et al. 2005). Nevertheless, our knowledge on transport of GA within the plant body is still limited. Previous studies using isotope labeled-GAs or grafting experiments with GA-deficient mutants suggested that GAs or GA precursors could move in the plant body (Rudich et al. 1976; Katsumi et al. 1983; Reid et al. 1983; Yang et al. 1995; Eriksson et al. 2006). However, the necessity and physiological significance of GA movement during plant development and in environmental responses still remain elusive. Also, it has not been well understood whether and how the GA status in one organ affects that in the others.

In this study, we have analyzed endogenous GA content and transcript levels of GA biosynthesis genes in order to address the possible contribution of cotyledons to altering GA levels in hypocotyls. Our results suggest that intact cotyledons are necessary for maintaining normal GA levels in young cucumber and tomato hypocotyls.

**Materials and methods**

**Plant materials and growth conditions**

Seeds of cucumber (Cucumis sativus L., cv. Shimoshirazu-jibai) were obtained from Sakata Seed Co. (Kanagawa, Japan), and those of tomato (Solanum lycopersicum; formerly Lycopersicon esculentum Mill., cv. Moneymaker) were obtained from Sapporo-Nouen Co. (Sapporo, Japan). The seeds were germinated and grown in artificial soil (Kurehakagaku, Tokyo, Japan) under continuous white fluorescent light (32 μmolm⁻²s⁻¹) at 26°C.

**Treatment with GA₄ and uniconazole**

GA₄ and uniconazole P (GA biosynthesis inhibitor: Izumi et al. 1985) were applied locally to cotyledons or hypocotyls as lanolin pastes. The lanolin pastes were prepared by adding anhydrous lanolin to an aqueous solution of the chemical or to distilled water (DW: as a control). Five days after the start of treatment, hypocotyl elongation was measured. Experiments were performed twice using independently grown seedlings with similar results.

**Quantitative Reverse Transcription (QRT)-PCR**

Total RNA was extracted from tomato seedlings using an RNasequeous RNA isolation kit with Plant RNA isolation aid (Ambion, Austin, TX). First-strand cDNA was synthesized from total RNA (1 μg) with Quantitect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). QRT-PCR with Taq-Man technology (Holland et al. 1991) or SYBR Green I regents (Qiagen) was performed using the first-strand cDNA as a template on a sequence detector system (ABI PRISM 7000, Applied Biosystems, Foster, CA) as described in Yamauchi et al. (2004). Results were normalized using an 18s rRNA as the internal control. Following nucleotide sequences of gene-specific primers and Taq-Man probes were used:

- for LeGA20ox 1, primer; 5' - CAA CTA CTA TCC ACC ATG CCA GAA-3' and 5' - TGA TGT TGG ATC ACA ATG AGG C-3', Taq-Man probe; 5'-FAM- CCG GAG CTC GCC TTA GGA ACG G-TAMRA-3', for LeGA3ox 2, primer; 5'- CCG GAC GAG ATT TTC CCT -3' and 5'-CCA GCC ATG CCA TCG G-3', Taq-Man probe; 5'-FAM- CCG GAC GTC GCC TTA GGA ACG G -TAMRA-3'.

SYBR Green primer for LeGA20ox 2, 5' -ACC AGA TCT TGC GTT AGG AAC TG -3' and 5' -CCT GAG ACG TTG A-3'; LeGA20ox 3, 5'-GGG CAG GGC CTC ATT GTG AT -3' and 5'-AAA CTT GAA GCC CAC CAA CAC T-3'; LeGA3ox 1, 5' -CAG ACC ACA TGG TCG AGA A-3' and 5'-CCT GAT GGT GTC ACT GGC TAT G-3'.

**Endogenous GA Measurements**

Cotyledons were eliminated from 7-days-old cucumber or tomato seedlings, and the plants were grown for an additional 1day. Intact seedlings were grown in an identical growth condition and were used as a control. About 30 g of fresh hypocotyls of cucumber seedlings were used for each GA measurement by gas chromatography-selected ion monitoring (GC-SIM). Quantitative analysis of GA was performed using ²H-labeled GAs as internal standards as described previously (Gawronska et al. 1995), using a mass spectrometer (Automass Sun, JEOL, Tokyo, Japan) equipped with a GC (6890N, Agilent Technologies, Palo Alto, CA, USA) and a capillary column (DB-1, Agilent Technologies). GA measurements were repeated three times using independently prepared plant materials. GA analysis in tomato hypocotyls was carried out using approximately 2 g (fresh weight) of plant materials. GA-containing fractions were subjected to LC-ESI-MS/MS analysis on a mass spectrometer (Q-Tof Premier, Waters) equipped with an Acquity Ultra Performance LC (Waters) using a reverse-phase column (Acquity UPLC BEH-C18, Waters). Experiments were repeated twice using independently prepared plant materials. Authentic GA samples and ²H-labeled internal standards were purchased from Professor Lewis Mander (Australian National University, Canberra).
Results
The effect of local application of uniconazole on hypocotyl elongation of cucumber seedlings

Our previous observations of the tissue-reunion process in cut hypocotyls suggested that normal GA biosynthesis in cotyledons is necessary for the maintenance of GA levels in the hypocotyl (Asahina et al. 2002). In this study, to further investigate this hypothesis, we measured elongation of hypocotyls after a local application of uniconazole (a GA biosynthesis inhibitor) to cotyledons, because hypocotyl elongation is known to be a GA-dependent process in cucumber (Katsumi et al. 1965b; Moore 1967; Sandhu et al. 1974; Taylor and Cosgrove 1989; Lopez-Juez et al. 1995). It was previously reported that radio labeled uniconazole applied to a cucumber leaf did not move to other organs significantly (Izumi et al. 1988). Therefore, a local application of this chemical to cotyledons would allow us to test the effect of local inhibition of GA biosynthesis in cotyledons.

Figure 2A shows that an application of uniconazole to cotyledons resulted in a decrease in the hypocotyl length, and a simultaneous application of GA4 canceled the inhibitory effect of uniconazole on hypocotyl elongation. Application of uniconazole to one of the cotyledons showed a weaker inhibitory effect on hypocotyl elongation (data not shown). Uniconazole-treatment on cotyledons was likely to cause GA-deficiency in the hypocotyl, because the inhibitory effect was canceled when GA4 was applied locally to the hypocotyls as well (Figure 2B).

GA levels in cucumber hypocotyls are reduced after the removal cotyledons

Our previous observations (Asahina et al. 2002) and the results in Figure 2 suggest that the maintenance of GA levels in hypocotyls is dependent on the presence of cotyledons in cucumber seedlings. To directly test this possibility, we determined endogenous levels of precursor and bioactive GAs in hypocotyls of intact and cotyledon-removed seedlings. In cucumber, GA4 in the non-13-hydroxylation pathway (Figure 1) acts as the major bioactive form (Smith et al. 1991). Figure 3 shows that hypocotyls after the removal of cotyledons contained a reduced amount of bioactive GA4 relative to those of intact seedlings (almost 50% reduction), and that a more drastic reduction in the level of GA12 was evident in the cotyledon-removed seedlings (almost 25% of the intact plants). These results indicate that cotyledons are necessary for maintaining the levels of both bioactive and precursor GAs in cucumber hypocotyls. These data are consistent with our notion that the removal of cotyledons causes a failure in the tissue reunion of cut hypocotyls (Asahina et al. 2002) and a reduction in the length of hypocotyls (Figure 2).

Transcript levels of GA biosynthesis genes in tomato seedlings

Our previous work using a GA-deficient mutant *gib-1* (Koornneef et al. 1990) has shown that, like in cucumber seedlings, GA is required for tissue-reunion in cut hypocotyls of tomato seedlings (Asahina et al. 2002). Also, the removal of cotyledons from wild-type tomato
seedlings resulted in inhibition of tissue-reunion in cut hypocotyls, as observed in cucumber seedlings (Asahina et al. 2002). To study the possible role of cotyledons in the maintenance of GA levels in hypocotyls in more detail, we took advantage of the tomato system, in which GA biosynthesis genes have previously been identified and characterized (Rebers et al. 1999). GA 20-oxidase and GA 3-oxidase catalyze conversions of GA_{12/53} to GA_{9/20} and of GA_{9/20} to GA_{4/1}, respectively (Figure 1), and are each encoded by a multi-gene family in many plant species (Lange et al. 1994; Chiang et al. 1995; Lester et al. 1997; Martin et al. 1997; Yamaguchi et al. 1998; Itoh et al. 1999; Rebers et al. 1999). In an attempt to assess the major family members expressed at the seedling stage, we carried out RT-PCR analysis using RNA samples prepared from aerial part of tomato seedlings and degenerate primers designed from conserved amino acid sequences of each enzyme in Arabidopsis, rice, and tobacco. Sequence analysis of the PCR products revealed that the majority of the clones isolated from the seedling sample corresponded to LeGA20ox1 for GA 20-oxidase and LeGA3ox2 for GA 3-oxidase, suggesting that these genes would represent the major GA 20-oxidase and GA 3-oxidase activities, respectively, at the seedling stage.

We determined transcript levels of LeGA20ox1 and LeGA3ox2 genes in each organ during seedling growth by QRT-PCR (Figure 4). Expression of both of these GA biosynthesis genes was detectable in every organ that we examined, but the LeGA3ox2 transcript was relatively more abundant in cotyledons than in hypocotyls at all time points (Figure 4). At stage 1 (before the visible expansion of true leaves, Figure 4), the level of LeGA20ox1 transcript was higher in cotyledons than in hypocotyls. These observations support the idea that GAs are produced in all organs including cotyledons in tomato seedlings.

**GA levels in tomato hypocotyls are reduced after the removal cotyledons**

To examine whether the removal of cotyledons affects GA levels in the hypocotyl of tomato seedlings as observed in cucumber seedlings (Figure 3), we measured endogenous levels of precursor, bioactive and inactive GAs in the hypocotyls of intact and cotyledon-removed plants. Unlike in cucumber seedlings, both the non-13-hydroxylation and the early-13-hydroxylation pathways (Figure 1) appear to operate in tomato seedlings (Koornneef et al. 1990; Rebers et al. 1999). Figure 5 shows that levels of all GAs that could be reliably quantified were reduced in the cotyledon-removed plants relative to those in intact seedlings reproducibly in duplicated experiments. The amounts of bioactive GA_{4} and GA_{12} after the removal of cotyledons were about 10% and 50% of those in intact seedlings, respectively.
Some GA biosynthesis genes are upregulated in the hypocotyl of cotyledon-removed tomato seedlings

To better understand the status of the GA biosynthesis pathway in tomato hypocotyls after the elimination of cotyledons, we determined relative transcript levels of GA biosynthesis genes by QRT-PCR (Figure 6). We found that the level of LeGA20ox2 transcript was highly elevated in hypocotyls after the removal of cotyledons, while expression of the other two LeGA20ox genes was not drastically altered under the same conditions. The LeGA3ox1 and LeGA3ox2 genes, both of which encode GA 3-oxidase, were expressed at higher levels in hypocotyls of the cotyledon-removed seedlings than in those of the intact ones. It has been shown in many plant species that some GA 20-oxidase and GA 3-oxidase genes are negatively regulated by GA activity (Martin et al. 1996; Hedden and Kamiya 1997; Elliot et al. 2001), which would function as part of the homeostasis mechanism in the GA biosynthesis and response pathways. Therefore, the observed upregulation of LeGA20ox and LeGA3ox genes in hypocotyls of the cotyledon-removed seedlings might be a consequence of reduced levels of bioactive GAs in this organ.

Discussion

Several lines of evidence presented in this study suggest that cotyledons play an important role in maintaining GA levels in hypocotyls of cucumber and tomato seedlings. We have demonstrated that GA levels in hypocotyls are decreased significantly when cotyledons are mechanically removed (Figures 3, 5). This was observed commonly in cucumber and tomato seedlings. These results are consistent with our previous findings that tissue-reunion of cut hypocotyls did not occur after the removal of cotyledons, while exogenous GA treatment to the cotyledon-removed seedlings was effective to restore the tissue-reunion (Asahina et al. 2002). Therefore, our current data strongly support the hypothesis that GA-deficiency in hypocotyls is responsible for the failure in tissue-reunion in the cotyledon-eliminated seedlings.

There is some evidence that suggests the capability of GA precursors and/or bioactive GAs to move across different cell types and between organs (Rudich et al. 1976; Katsumi et al. 1983; Reid et al. 1983; Yang et al. 1995). Therefore, the reduced GA levels in hypocotyls after the removal of cotyledons (Figures 3, 5) might be explained by the movement of GA(s) from cotyledons to the hypocotyl in intact seedlings. This hypothesis is supported by the fact that the effect of local inhibition of GA biosynthesis in cotyledons (based on the immobility of uniconazole applied to cucumber leaves, Izumi et al, 1988) on hypocotyl elongation was canceled by a local application of GA4 to cotyledons (Figure 2). A similar observation was reported using a local application of AMO1618 to cucumber cotyledons (Katsumi et al. 1965b), although the mobility of this inhibitor in plant is unknown. The hypothetical movement of GAs from cotyledons to the hypocotyl in cucumber and tomato seedlings should be demonstrated experimentally to confirm this model, because it is still possible that non-GA factor(s) produced in cotyledons is involved in the maintenance of hypocotyl GA levels. Recently, auxin...
indole-3-acetic acid) has been shown to play an essential role in maintaining normal GA levels in pea internodes (Ross and O’Neill 2001; O’Neill and Ross 2002). Application of indole-3-acetic acid alone, however, could not restore the inhibition of tissue-reunion in the cotyledon-eliminated cucumber seedlings (Asahina et al. 2002).

It is interesting to note that the removal cotyledons resulted in reductions in the levels of not only bioactive GAs but also their precursors (Figures 3, 5). At the same time, upregulation of some GA 20-oxidase and GA 3-oxidase genes was observed after the elimination of cotyledons in tomato seedlings (Figure 6). There are at least two possible mechanisms to explain these changes in GA levels and expression of GA biosynthesis genes after the removal of cotyledons. As the first model, we hypothesize that bioactive GA(s) synthesized in cotyledons move to the hypocotyl in intact seedlings. In this model, after the removal of cotyledons, the amount of bioactive GAs would be decreased in the hypocotyl due to their reduced supply from cotyledons, which in turn causes upregulation of GA 20-oxidase and GA 3-oxidase genes. Precursor GAs would not accumulate at high levels in the hypocotyl because they should be quickly metabolized by elevated GA 20-oxidase and GA 3-oxidase activities. The second hypothesis involves a movement of precursor(s) for bioactive GAs (such as GA12 or an earlier intermediate) from cotyledons to the hypocotyl. Once the supply of this GA precursor to the hypocotyl is blocked by the removal of cotyledons, the levels of both precursor and bioactive GAs would be decreased, which should then cause elevated expression of GA 20-oxidase and GA 3-oxidase genes via the feedback regulation. These two models are not mutually exclusive, and it is possible that the movement of both GA precursor(s) and bioactive GA(s) play a role in the maintenance of GA levels in the hypocotyl in intact cucumber and tomato seedlings.

In conclusion, we have investigated the status of the GA biosynthesis pathway in hypocotyls of cucumber and tomato seedlings after the removal cotyledons. Our results indicate that cotyledons play an essential role in the maintenance of GA levels in hypocotyl in these species.

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