N-3-Oxo-Decanoyl-L-Homoserine-Lactone Activates Auxin-Induced Adventitious Root Formation via Hydrogen Peroxide- and Nitric Oxide-Dependent Cyclic GMP Signaling in Mung Bean

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N-Acyl-homoserine-lactones (AHLs) are bacterial quorum-sensing signaling molecules that regulate population density. Recent evidence demonstrates their roles in plant defense responses and root development. Hydrogen peroxide (H$_2$O$_2$), nitric oxide (NO), and cyclic GMP (cGMP) are essential messengers that participate in various plant physiological processes, but how these messengers modulate the plant response to N-acyl-homoserine-lactone signals remains poorly understood. Here, we show that the N-3-oxo-decanoyl-homoserine-lactone (3-O-C10-HL), in contrast to its analog with an unsubstituted branch chain at the C3 position, efficiently stimulated the formation of adventitious roots and the expression of auxin-response genes in explants of mung bean (Vigna radiata) seedlings. This response was mimicked by the exogenous application of auxin, H$_2$O$_2$, NO, or cGMP homologs but suppressed by treatment with scavengers or inhibitors of H$_2$O$_2$, NO, or cGMP metabolism. The 3-O-C10-HL treatment enhanced auxin basipetal transport; this effect could be reversed by treatment with H$_2$O$_2$ or NO scavengers but not by inhibitors of cGMP synthesis. Inhibiting 3-O-C10-HL-induced H$_2$O$_2$ or NO accumulation impaired auxin- or 3-O-C10-HL-induced cGMP synthesis; however, blocking cGMP synthesis did not affect auxin- or 3-O-C10-HL-induced H$_2$O$_2$ or NO generation. Additionally, cGMP partially rescued the inhibitory effect of H$_2$O$_2$ or NO scavengers on 3-O-C10-HL-induced adventitious root development and auxin-response gene expression. These results suggest that 3-O-C10-HL, unlike its analog with an unmodified branch chain at the C3 position, can accelerate auxin-dependent adventitious root formation, possibly via H$_2$O$_2$- and NO-dependent cGMP signaling in mung bean seedlings.

Many bacteria use small molecules as signals to communicate with each other and to coordinate their growth activities. The most common molecules are N-acyl-L-homoserine-lactones (AHLs), which facilitate quorum sensing to coordinate the behavior of individual cells in a population-dependent manner in gram-negative bacteria; such a phenomenon was first reported in *Vibrio fisheri* (Engebret and Silverman, 1984). In gram-negative bacteria, diverse AHLs were used to regulate gene transcription once perceived by a partner. The basic structure of the AHL family consists of a conserved homoserine-lactone ring and a four- to 18-carbon chain with an amide (N)-linked acyl side group. The acyl side group can be substituted with an oxo or hydroxyl group at position C3 (Waters and Bassler, 2005; Waters et al., 2008). In Arabidopsis (*Arabidopsis thaliana*), the branched carbonyl chain length influences the biological effects of AHLs, and N-decanoyl-homoserine-lactone (C10-HL) is the most active one for primary root growth and lateral root development (Ortíz-Castro et al., 2008). However, in *Agrobacterium tumefaciens*, only N-3-oxo-hexanoyl-homoserine-lactone (3-O-C10-HL) efficiently induces *tra* gene transcription (Zhang et al., 1993). These results demonstrate that the carbonyl chain structure of AHLs almost certainly plays an essential role in AHL biological functions, although the details are still unclear.

The importance of AHLs has been demonstrated in diverse fields, including agriculture, ecology, and medicine, with roles that range from the establishment of mutualistic or symbiotic relationships to their importance in virulent and pathogenic interactions (Daniels et al., 2002; Ortiz-Castro et al., 2008; Atkinson and
Williams, 2009; Williams and Cámara, 2009). Recent evidence indicates that plants are able to perceive AHLs; the application of AHLs to Arabidopsis or Medicago truncatula results in distinct transcriptional changes in the roots and shoots and affects primary root growth, lateral root formation, and root hair development (Mathesius et al., 2003; Ortiz-Castro et al., 2008; von Rad et al., 2008; Morquecho-Contreras et al., 2010). Similar to the structure of AHLs, the fatty acid amides, including N-acyl-ethanolamines (NAEs) and alkamides in plants, are also capable of regulating root and shoot architecture (Laxalt and Munnik, 2002; Campos-Cuevas et al., 2008; Morquecho-Contreras et al., 2010), and the Arabidopsis recessive mutant decanamide-resistant root1 (drr1) was isolated based on its continuous primary root and reduced lateral root formation in response to decanamide (an alkamide) or AHLs (Morquecho-Contreras et al., 2010). Further analysis demonstrated that DRR1 is required in the early stage of pericycle cell activation to form lateral root primordia, suggesting that plants may share a common pathway to perceive NAEs and AHLs for root development.

Accumulated evidence demonstrates that both nitric oxide (NO) and hydrogen peroxide (H2O2) are signals to participate in multiple physiological processes, including systemic acquired resistance, the hypersensitive response, leaf senescence, programmed cell death, stomatal closure, root gravitropism, cell wall development, pollen-stigma interaction, and development (Grant and Loake, 2000; Neill et al., 2002; Pagnussat et al., 2003; Hu et al., 2004; Mittler et al., 2004; Schuhegger et al., 2006; Xuan et al., 2008). Adventitious roots are derived primarily from plant stems and are essential for plant adaptation to environmental stress; studying the adventitious root formation is also significant for both fundamental and applied plant biology. The phytohormone auxin plays an essential role in stimulating adventitious root formation (Pagnussat et al., 2003, 2004). Significant progress has been made to elucidate the molecular mechanism of auxin action via the auxin/indole-3-acetic acid (Aux/IAA) and auxin-response factor (ARF) gene families. NO and cyclic GMP (cGMP) mediate auxin-induced root organogenesis (Pagnussat et al., 2003). H2O2 also acts as a signaling molecule in plant adventitious root formation (Li et al., 2007), but the interplay of H2O2, NO, and cGMP in AHL-mediated root development needs to be fully investigated.

Many Gram-negative bacteria strains such as Serratia plymuthica can produce AHLs as biocontrol agents to protect plants from damping-off disease and induce plant systemic resistance (Li et al., 2007). In this study, we investigated the effects of 3-O-C10-HL on adventitious

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**Figure 1.** Dose effects of AHL signal molecules on adventitious root formation in mung bean explants. A. Dose-response effects of a series of AHLs on adventitious root formation. The AHLs were applied to the mung bean explants at the indicated concentrations, and the adventitious root number was calculated after 1 week of treatment. The root number values are expressed as means ± se (n = 30 explants) from at least three independent experiments. Single asterisks indicate values significantly different at P < 0.05, and double asterisks indicate values significantly different at P < 0.01 (Student’s t test) compared with the control. B. Photographs were taken of mung bean explants after 4 d of treatment with the indicated AHLs at 100 nM. The chemical structures of the individual AHLs are shown below the photographs. [See online article for color version of this figure.]
root formation in hypocotyls-cutting explants of mung bean (*Vigna radiata*). Unlike the AHLs containing an unmodified branched chain such as C10-HL, 3-O-C10-HL treatment quickly induced increases in H$_2$O$_2$, NO, and cGMP and stimulated the basipetal transport of auxin and adventitious root formation. A decrease in H$_2$O$_2$ or NO generation suppressed the 3-O-C10-HL-induced effects. In addition, the application of exogenous cGMP partially rescued the inhibitory effect of H$_2$O$_2$ and NO scavengers on 3-O-C10-HL-induced adventitious root formation. These findings indicate that, unlike other AHLs that show an auxin-independent effect on adventitious root formation (Ortíz-Castro et al., 2008), 3-O-C10-HL regulates auxin-induced adventitious root formation via H$_2$O$_2$- and NO-dependent cGMP signals in mung bean seedlings.

RESULTS

3-O-C10-HL Induces Adventitious Root Formation in Mung Bean Seedlings

The culture filtrate from the *A. tumefaciens* C58 (with pTiC58, which constitutively overproduces AHL) strain induced adventitious root formation in the explants of mung bean seedlings, whereas the filtrate from mutant *A. tumefaciens* C58C1 (which is without the Ti plasmid and can not form AHL) did not exhibit the same ability. Adding 3-O-C10-HL to the *A. tumefaciens* C58C1 filtrate rescued the inability of *A. tumefaciens* C58C1 to stimulate adventitious root formation (Supplemental Fig. S1). Interestingly, adding C10-HL and N-octanoyl-homoserine-lactone (C8-HL) into such filtrate only partially increased the adventitious root number (Supplemental Fig. S1).

To investigate the possible role of 3-O-C10-HL in activating adventitious root formation, we compared it with other AHLs, including N-butyryl-homoserine-lactone (C4-HL), N-hexanoyl-homoserine-lactone (C6-HL), C8-HL, C10-HL, N-dodecanoyl-homoserine-lactone (C12-HL), N-tetradecanoyl-homoserine-lactone (C14-HL), N-3-oxo-octanoyl-homoserine-lactone (3-O-C8-HL), N-3-oxo-dodecanoyl-homoserine-lactone (3-O-C12-HL), and N-3-oxo-tetradecanoyl-homoserine-lactone (3-O-C14-HL), on adventitious root formation by adding each compound to the liquid culture medium of hypocotyl-cutting explants of mung bean seedlings. We found a positive dose-dependent relationship between AHLs, ranging in concentration from 10 to 100 nM, in the induction of adventitious root formation (Fig. 1A). Once AHL concentrations exceeded 100 nM, they exhibited an inhibitory effect on adventitious root formation (Fig. 1A). Comparing with other AHLs, the most effective compound inducing adventitious root formation was 3-O-C10-HL (Fig. 1). Time-dependent effects of 3-O-C10-HL on adventitious root formation were also observed, with a maximal root number seen at 7 d after treatment (Supplemental Fig. S2A). Previous reports showed that the ethylene, salicylic acid, or jasmonic acid signaling pathways are involved in plant responses to AHLs or plant growth-promoting rhizobacteria stimulation (Morquecho-Contreras et al., 2010; Shi et al., 2010). We used the ethylene action inhibitor AgNO$_3$, the salicylic acid synthesis inhibitor paclobutrazol, and the jasmonic acid synthesis inhibitor diethyldithiocarbamic acid to determine their roles in our system. Pretreatment with all these compounds reduced 3-O-C10-HL-induced adventitious root formation, although the effect of paclobutrazol was not as strong as that of AgNO$_3$ and diethyldithiocarbamic acid at the employed concentrations (Supplemental Fig. S2B).

3-O-C10-HL Stimulates Adventitious Root Formation by Inducing Endogenous H$_2$O$_2$ and NO Production

To assess the role of H$_2$O$_2$ in 3-O-C10-HL-induced adventitious root formation, we first detected H$_2$O$_2$ accumulation by the fluorescent molecular probe 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA; In-
vitrogen). As shown in Figure 2A, 3-O-C10-HL induced substantial \( \text{H}_2\text{O}_2 \) accumulation within 6 h in the root tips of mung bean seedlings (Fig. 2A; Supplemental Fig. S3B). Subcellular localization of \( \text{H}_2\text{O}_2 \) production by a cerium perhydroxide (CeCl\(_3\))-based cytochemical technique showed the presence of strong CeCl\(_3\) deposits, reflecting the accumulation of \( \text{H}_2\text{O}_2 \) around the plasma membrane, intercellular space, and primary cell wall after 3-O-C10-HL treatment (Supplemental Fig. S3B). Other AHLs without an acyl-modified branched chain, such as C8-HL and C12-HL, could not efficiently induce \( \text{H}_2\text{O}_2 \) generation (Supplemental Fig. S3C). Interestingly, the AHLs with an acyl-substituted branched chain, including 3-O-C8-HL and 3-O-C12-HL, could induce the generation of \( \text{H}_2\text{O}_2 \), although not as strongly as 3-O-C10-HL (Supplemental Fig. S3C). Butylated hydroxytoluene (BHT) and ascorbate acid (AsA) are efficient antioxidants (Beligni et al., 2002), and diphenylene iodonium (DPI) is an inhibitor of the plasma membrane NADPH oxidase that is one of the main sources of \( \text{H}_2\text{O}_2 \) generation after an environmental stress (Neill et al., 2002; Torres and Dangl, 2005). BHT, AsA, and DPI suppressed 3-O-C10-HL-induced \( \text{H}_2\text{O}_2 \) accumulation (Fig. 3; Supplemental Fig. S3B).

Similar to the \( \text{H}_2\text{O}_2 \) pattern, 3-O-C10-HL quickly and strongly induced NO generation detected by the NO fluorescent probe 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM DA; Fig. 2B). After 24 h of treatment, NO accumulation reached its peak (Supplemental Fig. S4A). Either 3-O-C8-HL or 3-O-C12-HL, but not C8-HL or C10-HL, also stimulated the generation of NO, although this stimulation was weaker than that of 3-O-C10-HL (Supplemental Fig. S4B). Pretreatment with BHT or DPI reduced the 3-O-C10-HL- or IAA-induced NO generation; treatment with the NO scavenger 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) reduced 3-O-C10-HL- or IAA-induced NO accumulation but did not obviously affect 3-O-C10-HL- or IAA-induced \( \text{H}_2\text{O}_2 \) generation (Fig. 3). Pretreatment with BHT, DPI, or cPTIO also significantly reduced 3-O-C10-HL- or IAA-induced adventitious root formation (\( P < 0.05 \); Fig. 4A).

Figure 3. The effects of different inhibitors or scavengers and donors on 3-O-C10-HL-induced accumulation of \( \text{H}_2\text{O}_2 \) and NO in mung bean explants. Three-day-old mung bean explants were subjected to 100 nm 3-O-C10-HL or a donor (100 nm C10-HL, 10 \( \mu \text{M} \) IAA, 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), 50 \( \mu \text{M} \) GSNO, or 1 \( \mu \text{M} \) 8-Br-cGMP) treatment for 3 d or were pretreated with different inhibitors or scavengers (10 \( \mu \text{M} \) NPA, 1 \( \mu \text{M} \) DPI, 1 \( \mu \text{M} \) BHT, 1 \( \mu \text{M} \) AsA, 50 \( \mu \text{M} \) cPTIO, 10 \( \mu \text{M} \) LY83583, or 75 \( \mu \text{M} \) ODQ) for 2 h. This treatment was followed by the addition of 100 nm 3-O-C10-HL for 24 h, and \( \text{H}_2\text{O}_2 \) (A) and NO (B) contents were determined. The experiments were performed in triplicate and repeated at least three times (\( n = 20 \)). The vertical bars represent means ± SE, and bars with different letters are significantly different at \( P < 0.05 \) (Tukey’s test). FW, Fresh weight.
Together, the pharmacological data reveal that 3-O-C10-HL induces H$_2$O$_2$ synthesis, leading to NO synthesis and adventitious root formation.

To investigate whether 3-O-C10-HL-induced adventitious root formation depends on IAA transport, we utilized decapitated explants to remove auxin synthesis in the apical portion of the shoot. We found that 3-O-C10-HL could partly induce adventitious root formation in the decapitated explants, although the magnitude of this effect was slightly less than that in the intact explants (Fig. 4). BHT, DPI, or CPTIO also blocked 3-O-C10-HL-induced adventitious root formation in decapitated explants (Fig. 4B). Interestingly, the absence of auxin transport did not alter the effect of exogenous H$_2$O$_2$ or NO on adventitious root numbers (Fig. 4).

Involvement of cGMP in 3-O-C10-HL-Induced Adventitious Root Formation

cGMP is an intracellular messenger downstream of NO action and is synthesized in response to auxin stimulation (Pagnussat et al., 2003). Consequently, we assessed the potential involvement of cGMP in 3-O-C10-HL-induced adventitious root formation. A rapid and substantial increase of cGMP was induced by 3-O-C10-HL, as measured by radioimmunoassay, and the maximum level was reached after 72 h of treatment (Supplemental Fig. S5A). Similar to the generation patterns of H$_2$O$_2$ and NO, 3-O-C8-HL and 3-O-C12-HL, but not C8-HL, C10-HL, or C12-HL, efficiently induced the synthesis of cGMP (Supplemental Fig. S5B). 1H-[1,2,4]-oxadiazole-[4,3-a]-quinazolin-1-one (ODQ) and 6-anilino-5,8-quinolinedione (LY83583) are inhibitors of guanylate cyclase (GC), the enzyme responsible for cGMP synthesis (Pagnussat et al., 2003; Hu et al., 2005). We found that pretreatment with ODQ or LY83583 did not substantially reduce 3-O-C10-HL- or IAA-induced H$_2$O$_2$ or NO generation (Fig. 3), whereas they efficiently suppressed 3-O-C10-HL- or IAA-induced cGMP generation (Fig. 5). Furthermore, both H$_2$O$_2$ and S-nitrosglutathione (GSNO) induced, whereas DPI, BHT, or CPTIO pretreatment decreased, 3-O-C10-HL- or IAA-induced cGMP synthesis (Fig. 5). ODQ or LY83583 also prevented 3-O-C10-HL- or IAA-induced adventitious root formation; such an inhibitory effect could be overcome by additional pretreatment with the cell-permeable cGMP derivative 8-bromo-cyclic guanosine monophosphate (8-Br-cGMP) (Fig. 6). Sildenafil citrate is a selective phosphodiesterase type 5 inhibitor and blocks the degradation of cGMP (Pagnussat et al., 2003). Pretreatment with sildenafil slightly increased 3-O-C10-HL- or IAA-induced cGMP accumulation (Fig. 5) and correspondingly enhanced 3-O-C10-HL- or IAA-induced adventitious root formation (Fig. 6).

Figure 4. The effects of different inhibitors or scavengers and donors on 3-O-C10-HL-induced adventitious root formation. A, Three-day-old mung bean explants (black bars, nondepleted; gray bars, IAA depleted) were subjected to 100 nM 3-O-C10-HL or donor (100 nM C10-HL, 10 μM IAA, 100 μM H$_2$O$_2$, or 50 μM GSNO) treatment for 6 d or were pretreated with different inhibitors or scavengers (concentrations are as shown in Fig. 2) for 2 h. This was followed by treatment with 100 nM 3-O-C10-HL for 7 d, and the adventitious root numbers were calculated. The experiments were performed in triplicate and repeated at least three times (n = 20). The vertical bars represent means ± se, and bars with different letters are significantly different at P < 0.05 (Tukey’s test). B, Photographs of mung bean seedlings following the treatments as in A were taken after 4 d of treatment. [See online article for color version of this figure.]
To understand whether cGMP-mediated rooting development depends on endogenous auxin synthesis in the plant apex, we treated the IAA-depleted explants (decapitated explants) with 8-Br-cGMP, 3-O-C10-HL, or IAA. The adventitious root number in the IAA-depleted explants was enhanced by 8-Br-cGMP or IAA, but the effect of 3-O-C10-HL was less dramatic (Fig. 6). Sildenafil treatment synergistically increased the effect of IAA or 3-O-C10-HL on the adventitious root formation in IAA-depleted mung bean explants (Fig. 6A). Similar to the intact explants, treatment with 8-Br-cGMP reversed the inhibitory effect of LY83583 and ODQ on the 3-O-C10-HL-induced increase of adventitious root formation. Interestingly, while the inhibitory effect of BHT and DPI on 3-O-C10-HL could be overcome with 8-Br-cGMP, application of exogenous H$_2$O$_2$ or NO donor GSNO could not compensate for this inhibitory effect (Supplemental Fig. S6A).

3-O-C10-HL Promotes Polar Auxin Transport

Polar auxin transport controls root development. To investigate whether 3-O-C10-HL stimulates adventitious root formation through auxin transport, we directly tested basipetal auxin transport in the treated explant. Figure 7 shows the net amount of basipetally transported IAA in a 3-cm apical segment after different treatments. Auxin transport was significantly increased by treatment with 3-O-C10-HL at 100 nM but was only slightly enhanced by the 3-O-C10-HL analogs C10-HL or C12-HL. Treatments with BHT, DPI, or cPTIO partly reduced polar auxin transport in the seedlings after 3-O-C10-HL treatment. Application of H$_2$O$_2$ or GSNO alone accelerated polar auxin transport; this effect was enhanced in combination with 3-O-C10-HL treatment. However, we found that 8-Br-cGMP alone did not increase polar auxin transport. In addition, the LY83583 and ODQ treatments alone did not interfere with 3-O-C10-HL-accelerated polar auxin transport (Fig. 7; Supplemental Fig. S7). As expected, naphthylphthalamic acid (NPA) efficiently blocked basipetal auxin transport in mung bean seedlings (Fig. 7) and ultimately decreased 3-O-C10-HL-accelerated adventitious root formation; the effect of NPA was partly reversed by the addition of 8-Br-cGMP, IAA, or sildenafil (Supplemental Fig. S6B).

H$_2$O$_2$ and NO May Function Upstream of the cGMP Signal to Stimulate 3-O-C10-HL-Induced Adventitious Root Formation and Corresponding Gene Expression

Adventitious root formation is regulated by a series of cell division-related or auxin-response genes, such as CDC2 (for cell division control protein 2), ARC (for auxin-regulated protein 2), and CDPK (for calcium-dependent protein kinase). The Aux/IAA gene family that is rapidly transcribed in response to auxin is also involved in adventitious root formation. Here, we found that treatment with IAA and 3-O-C10-HL increased the transcript levels of the mung bean genes *CDC2*, *ARC*, and *CDPK* as well as the Aux/IAA gene family members *AUX22c*, *AUX22d*, and *AUX22e* (Fig. 8). A similar effect was also observed for 3-O-C8-HL and 3-O-C12-HL, although not as strongly as 3-O-C10-HL, but other AHLs with unmodified chains, such as C4-HL or C8-HL, could not efficiently induce the transcription of these genes (Supplemental Fig. S8). Application of exogenous H$_2$O$_2$, the NO donor GSNO, or the cGMP analog 8-Br-cGMP alone also activated the transcription of these genes, whereas the H$_2$O$_2$ scavengers BHT and AsA, the H$_2$O$_2$ synthesis inhibitor DPI, the NO scavenger cPTIO, and the GC enzyme inhibitors LY83583 and ODQ all reduced 3-O-C10-HL induction of all of these genes to varying degrees, with *CDPK* being the least affected (Fig. 8). These data indicate that H$_2$O$_2$, NO, and cGMP synthesis are required for the 3-O-C10-HL-induced expression of the auxin and cell division-related genes.
DISCUSSION

AHLs Differentially Modulate Adventitious Root Formation in Mung Bean Explants

As a class of bacterial intracellular lipid signals, AHLs are used by gram-negative bacteria to monitor the bacterial population density or to coordinate bacterial social behaviors (Waters and Bassler, 2005; Waters et al., 2008). Recently, more evidence demonstrate that AHLs are able to modulate plant growth and development (Mathesius et al., 2003; Ortiz-Castro et al., 2008; von Rad et al., 2008; Méndez-Bravo et al., 2010; Morquecho-Contreras et al., 2010). In Arabidopsis, treatment with AHLs alters primary growth, lateral root formation, and root hair development; specifically, AHLs with different lengths of unmodified branched chains differentially affect the root architecture. Among them, the effect of C10-HL is the most obvious (Ortíz-Castro et al., 2008). In this work, we found that longer chains of AHLs show more biological activity than shorter ones in accelerating adventitious root formation in the hypocotyl explants of mung bean. Furthermore, AHLs with an acylated group at the C3 position of their branched chain, particularly 3-O-C10-HL, show the most obvious activity (Fig. 1). 3-O-C10-HL has a better capacity to induce the generation of H$_2$O$_2$, NO, and cGMP (Figs. 3 and 5), accelerate polar auxin transport (Fig. 7), and stimulate adventitious root formation (Figs. 4 and 6) than its analog C10-HL. Even though 3-O-C8-HL and 3-O-C12-HL displayed similar effects, in contrast to their analogs C8-HL and C12-HL, their effect is lower than that of 3-O-C10-HL. These data suggest that not only the branched chain length of AHLs, but also the substituted groups at their branched chain that form different spatial structures, may play a role in defining their biological activity. In agreement with our finding, Zhang et al. (1993) reported that the efficiency of Agrobacterium conjugation and the corresponding tra gene expression were both modulated by the length and nature of the lipophilic acyl chain of AHLs. Among the different AHLs, 3-O-C8-HL and 3-O-C10-HL showed a higher ability to induce tra gene expression than their analogs C8-HL and C12-HL. Ortiz-Castro et al. (2008) also reported that C10-HL was more effective in altering Arabidopsis root system architecture. It is possible that specific AHL structures are perceived by unique plant proteins to affect their signature phenotype, although this remains to be established (Bauer and Mathesius, 2004). Similarly, plant lipid signals, such as alkamides and NAEs, also show a structure-dependent biological activity. The degree of saturation, amino residue substitution, and acyl chain length all are important in determining the effect of
alkamides or NAEs on regulating primary root growth and lateral root development (López-Bucio et al., 2006, 2007).

**Branched Chain Structures of AHLs Affect Root Auxin Polar Transport**

Previous studies showed that modulating Arabidopsis primary and lateral root formation by some AHLs or alkamides, particularly those AHLs with an unmodified branched chain, is independent of auxin signaling (Ortiz-Castro et al., 2008; Méndez-Bravo et al., 2010; Morquecho-Contreras et al., 2010). For example, C10-HL treatment was unable to enhance GUS activity in Arabidopsis transgenic seedlings harboring the auxin-response **DR5:uidA** gene construct (Ortiz-Castro et al., 2008). However, Mathesius et al. (2003) reported that AHLs with an acyl-substituted branched chain, such as 3-O-C12-HL and 3-O-C16-HL, induce the tissue-specific activation of the auxin-response **GH3** and the chalcone synthase genes in the model legume *M. truncatula*; these findings imply that there is some level of variability in auxin signaling in response to AHLs. To confirm this, we measured the abilities of various AHLs to activate polar auxin transport. We found that the AHLs with an acyl-modified branched chain at the C3 position, such as 3-O-C10-HL, 3-O-C8-HL, and 3-O-C12-HL, could efficiently promote polar auxin transport, with 3-O-C10-HL showing the greatest response (Fig. 7). In contrast, AHLs without the acyl-modified branched chain at the C3 position, such as C10-HL or C12-HL, only slightly accelerated polar auxin transport. In agreement with the polar auxin transport data, 3-O-C10-HL strongly induced the accumulation of auxin-response transcripts, including **Aux22c**, **Aux22d**, and **Aux22e**, whereas AHLs lacking the modified branched

chain at the C3 position only weakly, or did not, induce the expression of these auxin-response genes. These results add further support to the observation that the structure of branched chains in the AHL family affects the auxin transport. These data also help explain why 3-O-C10-HL activated auxin-regulated gene expression in *Medicago* (Mathesius et al., 2003) and why C10-HL showed an auxin-independent effect on inducing lateral root development in Arabidopsis (Ortiz-Castro et al., 2008; von Rad et al., 2008; Shi et al., 2010), where it is likely that the presence or absence of the C3 branch chain differentiates between these two responses.

Alteration of root development by AHLs or plant growth-promoting rhizobacteria involves jasmonate, ethylene, and salicylic acid signaling pathways (Méndez-Bravo et al., 2010; Morquecho-Contreras et al., 2010; Shi et al., 2010). Here, we found that 3-O-C10-HL still retained the partial ability to promote adventitious root formation even in the presence of the auxin transport inhibitor NPA, indicating that auxin-independent signaling should not be excluded and may participate in 3-O-C10-HL-induced adventitious root formation. We found that treatment with an ethylene action inhibitor or jasmonic acid synthesis inhibitors indeed partially reduced 3-O-C10-HL-induced adventitious root formation (Supplemental Fig. S2), supporting the hypothesis that another signaling pathway in addition to, or interacting with, auxin signaling is also involved.

The Cross Talk of H$_2$O$_2$, NO, and cGMP May Mediate 3-O-C10-HL-Induced Adventitious Root Formation in Mung Bean Explants

Our data show clearly that 3-O-C10-HL treatment induced the rapid accumulation of H$_2$O$_2$ and NO (Figs. 2 and 3), which is confirmed by the result that the H$_2$O$_2$
scavenger BHT or the NO scavenger cPTIO efficiently abolished the generation of 3-O-C10-HL-induced H_2O_2 or NO. The plasma membrane NADPH oxidase inhibitor DPI efficiently suppressed 3-O-C10-HL-induced H_2O_2 production, indicating that NADPH oxidase might be primarily responsible for the generation of 3-O-C10-HL-induced H_2O_2. However, this goes with the caveat that DPI is a flavin analog and, therefore, other flavin-containing enzymes may also be responsible for the H_2O_2 generation seen here. Blocking polar auxin transport with NPA or IAA depletion through decapitation impaired 3-O-C10-HL-induced H_2O_2 and NO generation, and other AHLs without modified branched chains, such as C10-HL, only partially increased polar auxin transport and the generation of H_2O_2 or NO (Figs. 2 and 3). Together, these results suggest that 3-O-C10-HL-accelerated polar auxin transport likely contributes to the generation of H_2O_2 or NO. Auxin has previously been shown to stimulate H_2O_2 or NO generation in cucumber, tomato, and Arabidopsis (Joo et al., 2001; Pagnussat et al., 2003; Chen et al., 2010), but auxin did not stimulate NO generation in a tobacco (Nicotiana tabacum) suspension (Tun et al., 2001), suggesting that this is not a universal mechanism and may be context dependent. Plant alkamide as a lipid signal that acts as an AHL homolog can induce the accumulation of NO and adventitious root development in Arabidopsis (Campos-Cuevas et al., 2008), indicating that plants may have evolved a common mechanism to respond to exogenous AHLs, endogenous NAEs, and alkamide stimulation (Mendez-Bravo et al., 2010; Morquecho-Contreras et al., 2010). In our experiments, we noticed that suppression of 3-O-C10-HL-induced H_2O_2 generation with the H_2O_2 scavenger BHT or AsA or the H_2O_2 synthesis inhibitor DPI coordinately reduced the accumulation of NO, but blocking 3-O-C10-HL-induced NO accumulation with the NO scavenger cPTIO did not obviously affect 3-O-C10-HL-induced H_2O_2 generation (Figs. 2 and 3). We suggest that there is an interaction between H_2O_2 and NO, and H_2O_2 may modulate the NO signal during mung bean responses to 3-O-C10-HL stimulation. This conclusion is consistent with previous findings that H_2O_2 regulates NO metabolism in Arabidopsis (Wang et al., 2010).

cGMP is a secondary signal generated in response to NO or H_2O_2; it also mediates auxin-induced adventitious root formation and gravitropism bending in cucumber and soybean (Glycine max; Pagnussat et al., 2003; Hu et al., 2005). Here, we found that 3-O-10-HL induced the accumulation of cGMP that is similar to the increase of NO or H_2O_2; such an effect is partly reduced by pretreatment with the GC1 inhibitor ODQ or LY83583 (Fig. 5). In addition, direct treatment with H_2O_2, NO donor, or auxin stimulated 3-O-C10-HL-induced cGMP accumulation, whereas H_2O_2 and NO

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**Figure 8.** The effects of different inhibitors or scavengers and donors on the 3-O-C10-HL-induced transcript levels of auxin-response and cell division-related genes. Three-day-old mung bean explants were sub¬jected to 100 nM 3-O-C10-HL or different donor treatments for 24 h or pretreated with different inhibitors or scavengers (the concentrations are as shown in Fig. 2) for 2 h. This was followed by treatment with 100 nM 3-O-C10-HL for 24 h, and the stem basipetal auxin transport was measured immediately. RNA from the stem portion of the explants was extracted for use in the RT-PCR assay.

**Figure 9.** Schematic illustration of a proposed model for regulating adventitious root formation by 3-O-C10-HL in mung bean. In this model, IAA is synthesized in the apical bud and basipetally transported to the base of the hypocotyl. 3-O-C10-HL promotes the polar auxin transport to induce the generation of H_2O_2 and NO and subsequently induce the synthesis of cGMP to activate adventitious root formation. During this process, H_2O_2 and NO can provide positive feedback by regulating polar auxin transport to enhance H_2O_2 and NO accumulation, and H_2O_2 can also positively affect NO synthesis. [See online article for color version of this figure.]
signal pathway inhibitors or the polar auxin transport inhibitor NPA reduced 3-O-C10-HL-induced cGMP generation. By extension, auxin, \( \text{H}_2\text{O}_2 \), and NO signals may all be involved in activating 3-O-C10-HL-induced GC1 enzyme activity and cGMP synthesis. In support of this hypothesis, we found that the cGMP analog 8-Br-cGMP or sildenafil treatment simulated the accelerating effect of 3-O-C10-HL on both adventitious root formation and the transcript levels of the corresponding cell division-related and auxin-response genes. These results agree with the previous conclusion that cGMP is involved in auxin-mediated adventitious root formation (Pagnussat et al., 2003). However, the addition of 8-Br-cGMP or the GC1 enzyme inhibitor ODQ or LY83583 did not obviously impair 3-O-C10-HL-accelerated polar auxin transport, implying that auxin transport is required for cGMP generation, but cGMP does not appear to regulate auxin transport through a feedback mechanism (Fig. 9).

In summary, unlike most AHLs with unmodified branch chains, AHLs with acyl-modified branched chains, particularly 3-O-C10-HL, present special biological activities; they can accelerate auxin-mediated adventitious root formation through \( \text{H}_2\text{O}_2 \)- and NO-dependent cGMP signaling pathways. We propose one possible model in Figure 9. According to this model, treatment with 3-O-C10-HL at the root can rapidly induce the accumulation of \( \text{H}_2\text{O}_2 \), NO, and cGMP in an auxin-dependent manner; this then activates the transcription of genes involved in the cell cycle and cell division and leads to adventitious root formation. \( \text{H}_2\text{O}_2 \) and NO act downstream of the AHL signal but may enhance polar auxin transport through a positive feedback mechanism. Certainly, we must recognize that in the absence of available genetic information for mung bean, the model is drawn from biochemical, pharmacological, and physiological observations. However, to support our model, we treated Arabidopsis lines carrying an auxin-response \( \text{IAA}2: \text{uid}A \) report marker with different AHLs and found that 3-O-C10-HL treatment indeed intensified GUS activity in this transgenic Arabidopsis line, whereas C10-HL treatment had little effect. Furthermore, 3-O-C10-HL does not efficiently stimulate GUS activity in the \( \text{Attno}1/\text{IAA}2: \text{uid}A \) mutant deficient in NO generation or the \( \text{AtRboh}D/\text{IAA}2: \text{uid}A \) mutant deficient in \( \text{H}_2\text{O}_2 \) generation, providing the molecular genetic evidence for NO and \( \text{H}_2\text{O}_2 \) acting downstream of 3-O-C10-HL but upstream of auxin-induced gene expression (Supplemental Fig. S9). AUX1 and PIN2 have been clearly demonstrated to be functional as auxin influx and efflux carriers during auxin polar transport in Arabidopsis (Friml, 2003; Yang et al., 2006). We also found that 3-O-C10-HL, rather than C10-HL, increased the GFP fluorescence intensity in transgenic Arabidopsis with \( \text{Aux1:GFP} \) or \( \text{Pin2:GFP} \) lines, while such an effect was impaired in the mutants of \( \text{Attno}1 \) or \( \text{AtRboh}D \) lines, further supporting the role of \( \text{H}_2\text{O}_2 \) and NO in mediating 3-O-C10-HL-accelerated auxin polar transport (Supplemental Fig. S9). We recognize that the lack of complete inhibition in some of our pharmacological treatments may indicate that additional mechanisms may contribute to 3-O-C10-HL-induced adventitious root formation. In fact, our own data and other reports suggest that ethylene and jasmonic acid signals may be involved in plant responses to AHLs or plant growth-promoting rhizobacteria stimulation (Morquecho-Contreras et al., 2010; Shi et al., 2010). The roles of these, as well as other messengers, such as phosphatidic acid and carbon monoxide, have been reported to regulate adventitious root formation in cucumber (Lanteri et al., 2008; Xuan et al., 2008); however, their roles in AHL-induced plant adventitious root formation remain to be investigated in further experiments.

MATERIALS AND METHODS

Chemicals

All common chemicals were obtained from Sigma unless otherwise stated. A 10 mM stock solution of 3-O-C10-HL in ethanol was made by dilution, and it was adjusted to pH < 6. NPA obtained from Chem Service was used as the auxin transport inhibitor, BHT was used as the \( \text{H}_2\text{O}_2 \) scavenger, DPI was used as the plasma membrane NADPH oxidase inhibitor, and cPTIO was used as the NO scavenger (Toronto Research Chemicals, Inc.).

Plant Material and Growth Conditions

Mung bean (\( \text{V}i\text{gna radiata} \) ‘Baoshang’) seeds were sterilized with 1.0% NaClO for 15 min, washed with sterile water three times, and sown on enamel square trays containing sterile wetted filter paper. Germination and growth occurred under a 16-h photoperiod at a light intensity of 300 \( \mu\text{mol m}^{-2} \text{s}^{-1} \) and 25°C. The mung bean seedlings were either used intact or were decapitated by excising the apical bud immediately above the cotyledons and incubating the seedlings in quarter-strength Murashige and Skoog medium solution for 48 h before removing the primary root. For the explant seedling experiments, the intact seedling roots were removed, and the shoot and hypocotyl were placed in petri dishes containing water (as the control) or the different indicated chemicals.

\( \text{H}_2\text{O}_2 \), NO, and cGMP Content Assay

The samples after various treatments were harvested by flash freezing in liquid nitrogen and stored at −70°C until subsequent extraction. Frozen leaves (0.2 g) were ground to powder under liquid nitrogen and homogenized with 1 mL of 0.2 \( \times \) \( \text{HClO}_4 \) at 4°C. The extract was held on ice for 5 min and then centrifuged at 10,000 \( \text{g} \) for 10 min at 4°C. The supernatant was collected and either processed immediately or quick frozen at −70°C until further analysis. The concentration of \( \text{H}_2\text{O}_2 \) was measured as described previously (Hu et al., 2004). NO content was determined by hemoglobin assay following a reported method (Hu et al., 2005; Pasqualini et al., 2009). cGMP was assayed using a \( ^{[32\text{P}]} \)GMP radioimmunoassay kit (GE Biosciences) as described previously (Hu et al., 2005).

Fluorescence Microscopy

\( \text{H}_2\text{O}_2 \) or NO was imaged using H2DCF-DA or DAF-FM DA, respectively, using confocal microscopy as described previously (Hu et al., 2005). Briefly, the mung bean root, with or without the indicated treatments, was loaded with 10 \( \mu\text{M} \) H2DCF-DA or 5 \( \mu\text{M} \) DAF-FM DA in MES/KCl buffer for 30 min and washed (3 \( \times \) 3 min) in MES/KCl buffer. The treated root was viewed microscopically (excitation at 492 nm and emission at 520 nm for \( \text{H}_2\text{O}_2 \) fluorescence, excitation at 495 nm and emission at 515 nm for NO fluorescence; PCM2000 [Nikon]). For GFP observation, sample roots were excited with an argon laser at 488 nm, and the fluorescence emission was collected between 497 and 537 nm. The experiments were repeated at least five times, and similar results were obtained. All manipulations were performed under dim green light at 25°C.
Cytotoxic Chemical Detection of H$_2$O$_2$

CeCl$_3$ reacts with H$_2$O$_2$ to form cerium perhydroxides; this forms electron-dense deposits that can be visualized using transmission electron microscopy (Orozco-Cárdenas et al., 2001). The control and treated samples were rapidly sliced into approximately 10-mm strips, immediately vacuum infiltrated with 5 ms CeCl$_3$ in 30 ms MOPS buffer, pH 7.2, and incubated for 2 h. The leaf segments (3 × 3 mm) were then prefixed in 2.5% (v/v) glutaraldehyde buffered with 0.1 M cacodylate buffer, pH 7.2, at 4°C overnight, followed by three 10-min rinses in 0.1 M Na cacodylate buffer, pH 7.2. The leaves were then postfixed in 1% (v/v) buffered osmium tetroxide for 1 h at 4°C followed by three washes in distilled water. The leaves were stained in 1% (w/v) uranyl acetate for 1 h followed by dehydration in an ethanol series (30%, 50%, 70%, 90%, and 100% ethanol for 10 min each). They were treated with propylene oxide for 30 min prior to transfer to TAAB LV resin (TAAB Laboratories Equipment); propylene oxide (1:1) for 12 h in sealed vials. The leaves were then transferred to 100% TAAB LV for 2 h and moved to fresh TAAB LV for 24 h. Finally, the leaf segments were transferred to fit molds with TAAB LV and cured for 16 h at 60°C. The sections (90 nm) were cut with a diamond knife, stained with lead citrate, and viewed with a JEOL JEM-1400 electron microscope at an accelerating voltage of 80 kV.

Auxin Polar Basipetal Transport Assay

Auxin transport was assayed using [3H]IAA radiotracer assays as described previously (Lewis and Muday, 2009). In brief, the seedlings used for the transport assays were grown for 5 d at 300 μmol m$^{-2}$ s$^{-1}$ light intensity. After 1 week of growth, the primary root was removed from intact mung bean seedlings, and these seedlings were put into petri dishes containing half-strength liquid Murashige and Skoog medium (as the control) with or without additional different chemicals. For the auxin polar basipetal transport assay, 3-cm hypocotyl segments were cut 2 cm below the cotyledons, and the segments were placed on a damp paper towel (without inverting); for each experiment, 10 cut segments were prepared. After they were prepared, the individual segments were transferred from the paper towel to a microcentrifuge tube containing 0.1 mL of [3H]IAA. The cDNA was then isolated using an in vitro transcription kit (agricultural chemicals). After they were incubated for various times, the opposite ends of the segments from 2 cm were cut and assayed for the presence of radioactivity in a liquid scintillation counter. The nonpolar transport of auxin in the segments was determined by the addition of NPA (Chem Service) in the medium or the placement of the stem segments in a reverse orientation in the medium.

Gene Expression Analysis by RT-PCR

Total RNA was extracted as described previously (Hu et al., 2005; Xuan et al., 2008). After the total RNA was extracted, DNA-free total RNA (5 μg) from different treatments was used for first-strand cDNA synthesis in a 20-μL reaction volume containing 2.5 units of avian myeloblastosis virus reverse transcriptase XL (Takara) and 1 μM oligo(dT) primer. PCR was performed using 2 μL of a 2-fold dilution of the cDNA, 10 pmol of each oligonucleotide primer, and 1 unit of Taq polymerase (Takara) in a 25-μL reaction volume. The primer sequence information is listed in Supplemental Figure S10. To standardize the results, the relative abundance of tubulin was determined and used as the internal standard. The cycle number of the PCR was adjusted to obtain a clearly visible band for the sample with the highest transcript level. Each cDNA sample was run at least twice. Aliquots from the PCR were separated on 1.2% agarose gels and visualized using ethidium bromide. The specific amplification products of the expected sizes were observed, and their identities were confirmed by sequencing.

Histochemical Analysis

Transgenic plants that express the uidA reporter gene were stained in 0.1% 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide in phosphate buffer (NaH$_2$PO$_4$ and Na$_2$HPO$_4$, 0.1 μM [pH 7]) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 1 h at 37°C. The seedlings were cleared of chlorophyll by treatment with 70% ethanol at room temperature. Stained samples were observed and photographed as reported (Ortiz-Castro et al., 2008).

Statistics

Where indicated, the values are shown as means ± SD of at least three independent experiments. Data were analyzed by one-way ANOVA with Tukey’s test or Student’s t test; a value of P < 0.05 was considered to be significant for mean differences.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers S81594, AF129886, U08140, AB004931, AB004932, AB004933, and AY220546.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The effects of filtrates from the wild type and mutant strain A. tumefaciens C58 on adventitious root formation in mung bean explants.

Supplemental Figure S2. Time-course effects of 3-O-C10-HL on adventitious root formation, and the effects of different signaling pathway-related inhibitors on 3-O-C10-HL-induced adventitious root formation.

Supplemental Figure S3. The effects of different 3-O-C10-HL analogs on H$_2$O$_2$ accumulation in mung bean explants.

Supplemental Figure S4. The effects of different 3-O-C10-HL analogs on the generation of NO in mung bean explants.

Supplemental Figure S5. The effects of different 3-O-C10-HL analogs on the generation of cGMP in mung bean explants.

Supplemental Figure S6. The effects of different treatments on 3-O-C10-HL analog-induced adventitious root formation.

Supplemental Figure S7. The effects of different inhibitors or scavengers alone on polar auxin transport.

Supplemental Figure S8. The effects of the different structures of AHLs on the transcript levels of cell division-related and auxin-response genes in mung bean explants.

Supplemental Figure S9. The effects of 3-O-C10-HL and C10-HL on GUS staining and GFP fluorescence intensity in transgenic Arabidopsis lines with auxin-response marker genes.

Supplemental Figure S10. List of primer sequences used for RT-PCR in this study.

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