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Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides

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Summary

The expression of the auxin responsive reporter construct, *GH3*:*gusA*, was examined in transgenic white clover plants to assess changes in the auxin balance during the earliest stages of root nodule formation. Reporter gene expression was monitored at marked locations after the application of bacteria or signal molecules using two precise inoculation techniques: spot-inoculation and a novel method for ballistic microtargeting. Changes in *GH3*:*gusA* expression were monitored after the inoculation of *Rhizobium leguminosarum* biovar *trifolii*, non-host rhizobia, lipo-chitin oligosaccharides (LCOs), chitin oligosaccharides, a synthetic auxin transport inhibitor (naphthylphthalamic acid; NPA), auxin, the ENOD40–1 peptide or different flavonoids. The results show that clover-nodulating rhizobia induce a rapid, transient and local downregulation of *GH3*:*gusA* expression during nodule initiation followed by an upregulation of reporter gene expression at the site of nodule initiation. Microtargeting of auxin caused a local and acropetal upregulation of *GH3*:*gusA* expression, whereas NPA caused local and acropetal downregulation of expression. Both spot-inoculation and microtargeting of *R. l.* bv. *trifolii* LCOs or flavonoid aglycones induced similar changes to *GH3*:*gusA* expression as NPA. *O*-acetylated chitin oligosaccharides caused similar changes to *GH3*:*gusA* expression as *R. l.* bv. *trifolii* spot-inoculation, but only after delivery by microtargeting. Non-*O*-acetylated chitin oligosaccharides, flavonoid glucosides or the ENOD40–1 peptide failed to induce any detectable changes in *GH3*:*gusA* expression. *GH3*:*gusA* expression patterns during the later stages of nodule and lateral root development were similar. These results support the hypothesis that LCOs and chitin oligosaccharides act by perturbing the auxin flow in the root during the earliest stages of nodule formation, and that endogenous flavonoids could mediate this response.

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Introduction

Legumes can engage in a symbiosis with soil bacteria of the genera *Rhizobium*, *Azorhizobium*, *Sinorhizobium* and *Bradyrhizobium*, resulting in the formation of nitrogen-fixing root nodules. These 'rhizobia' trigger the nodule developmental programme via the secretion of mitogenic lipo-chitin oligosaccharides, called LCOs, as reviewed by Dénarié *et al.* (1996), Schultze *et al.* (1994) and Spaink (1996). In plant roots, LCOs also trigger hair deformation, preinfection thread formation, cortical cell division, flavonoid induction and secretion, and induction of nodulin and cell cycle genes.

In temperate legumes, such as clover and alfalfa, nodules originate from inner cortical cell divisions that mostly occur opposite protoxylem poles (Hirsch, 1992). All cortical cells between a particular protoxylem pole and the epidermis are triggered into division, but only the inner cortical cells complete division, eventually forming a nodule primordium (Yang *et al.*, 1994). Although externally applied LCOs trigger cortical cell divisions, the nature of the endogenous plant signals induced by LCOs prior to the first cell divisions remain unknown.

Several studies suggest that LCOs work by affecting phytohormone regulation (Hirsch, 1992). Early work suggested that transverse gradients of signal molecules from the root stele and from infecting bacteria overlap to induce cortical cell divisions (Libbenga *et al.*, 1973). This model proposed that the nodule progenitor cells of the inner cortex are initially exposed to an optimal ratio and concentration of signal molecules before forming a nodule primordium. Nodules can also be induced by manipulating plant hormone levels (Bauer *et al.*, 1996; Cooper and Long, 1994) or by the addition of synthetic auxin transport inhibitors (Hirsch *et al.*, 1989). Flavonoids, which can act as endogenous auxin transport regulators (Jacobs and Rubery, 1988), are possible candidates to mediate the effect of LCOs on the plant hormone balance (Hirsch, 1992; Hirsch *et al.*, 1989). Recent studies suggest that the ENOD40–1 peptide,

which is induced from a very early stage in nodule development, affects the response to auxin in tobacco protoplasts during division (van de Sande *et al.*, 1996). The phytohormones cytokinins and auxins are most likely to be involved in nodule initiation, since they are necessary for activation and completion of the cell cycle (John *et al.*, 1993; Zhang *et al.*, 1996).

To investigate the role of endogenous signal molecules

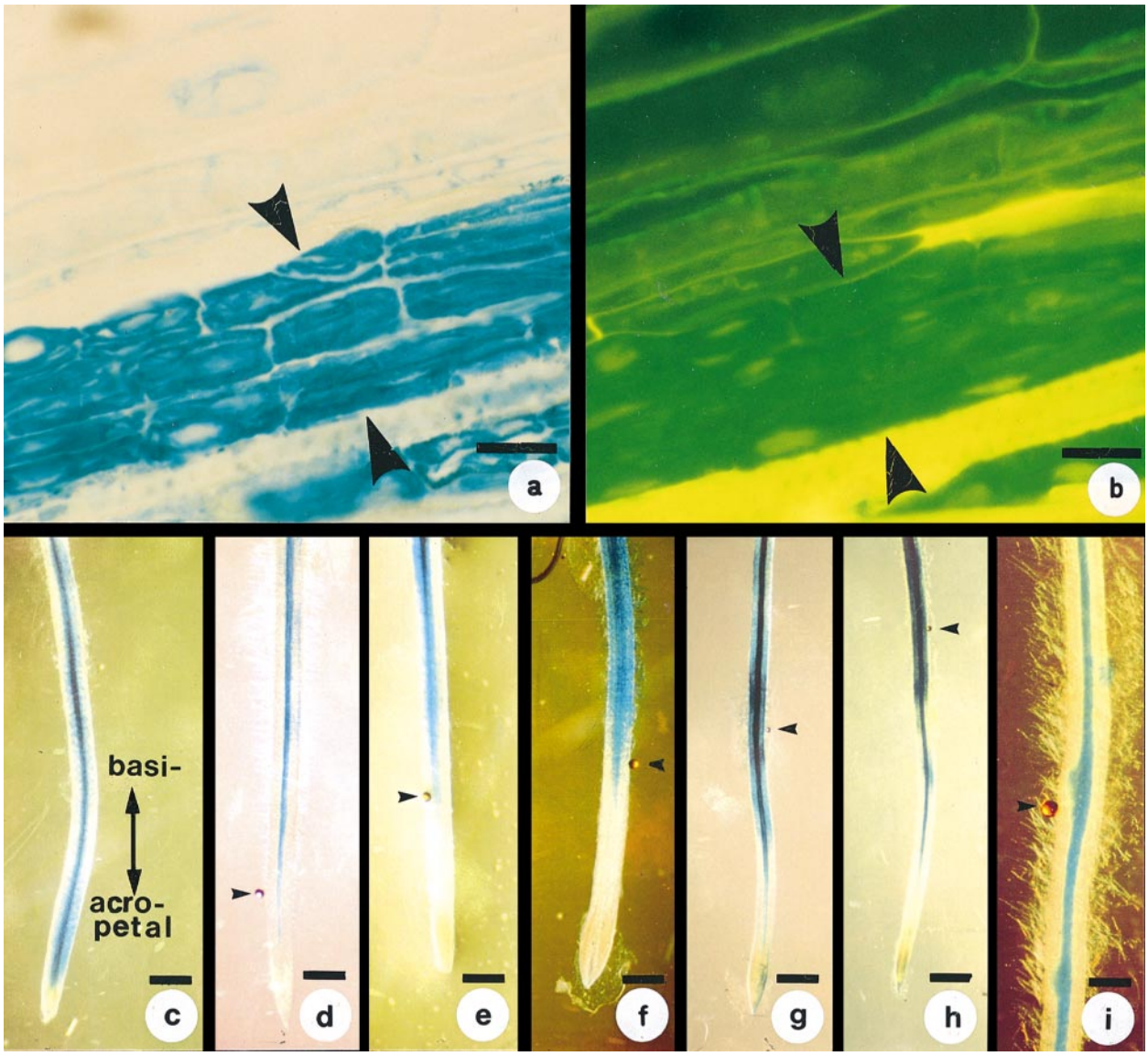

Figure 1. Localization of *GH3*:*gusA* activity in roots of uninoculated and inoculated transgenic white clover plants.
(a) and (b) show the distribution of GUS in subsequent longitudinal sections of an untreated root, located in the vascular bundle (between arrowheads). In (a) the distribution of GUS activity was detected by histochemical staining, whereas in (b) the GUS protein was detected with fluorescently (FITC) labelled anti-GUS antibodies (green). Non-specific yellow autofluorescence is seen in xylem elements.
(c–i) Whole roots showing the effect of spot-inoculated *R. l.* bv. *trifolii* (ANU843) upon *GH3*:*gusA* expression during the first 50 h (stages 1–5) of nodule development. The inoculation site is marked with an ion exchange bead (arrowhead).
(c) *GH3*:*gusA* expression was seen in the vascular bundle and, at lower levels, also in cortex cells in an untreated root. (d) One hour p.i. no changes in *GH3*:*gusA* expression were observed. (e) At stage 1 (5 h p.i.), strong reduction of *GH3*:*gusA* expression occurred in the cortex and vascular bundle acropetal from the inoculation site. (f) At stage 2 (10 h p.i.), the reduction of *GH3*:*gusA* expression acropetal from the inoculation site persisted, and an upregulation of *GH3*:*gusA* expression occurred basipetal from the inoculation site. (g) At stage 3 (20 h p.i.), *GH3*:*gusA* expression was enhanced in the cortex in a localized zone around the inoculation site and expression reappeared in the vascular bundle acropetal from the inoculation site. (h) At stage 4 (30 h p.i.), an extended zone of high *GH3*:*gusA* expression occurred around the inoculation site (transverse section shown in Figure 2e). Compared to 20 h p.i., the zone of enhanced *GH3*:*gusA* expression was located further basipetal and stretched further acropetal from the inoculation site. In about 50% of cases, *GH3*:*gusA* expression just behind the root tip was still reduced. (i) At stage 5 (50 h p.i.), a nodule primordium developed at the site of inoculation. A transverse section of this primordium is shown in Figure 2(f). The dividing cells expressed *GH3*:*gusA*, whereas the expression in flanking regions was not affected.
Bars = 50 μm in (a) and (b); 2 mm in (c), (d), (e), and (f); 3 mm in (g) and (h); 1 mm in (i).

in root nodulation, we monitored the inferred changes in the auxin balance occurring during nodulation in white clover using transgenic plants carrying the promoter of the auxin responsive gene, *GH3*, fused to *gusA* (Larkin *et al.*, 1996). *GH3* is a very early auxin responsive gene from soybean (Hagen *et al.*, 1984; Napier and Venis, 1995),

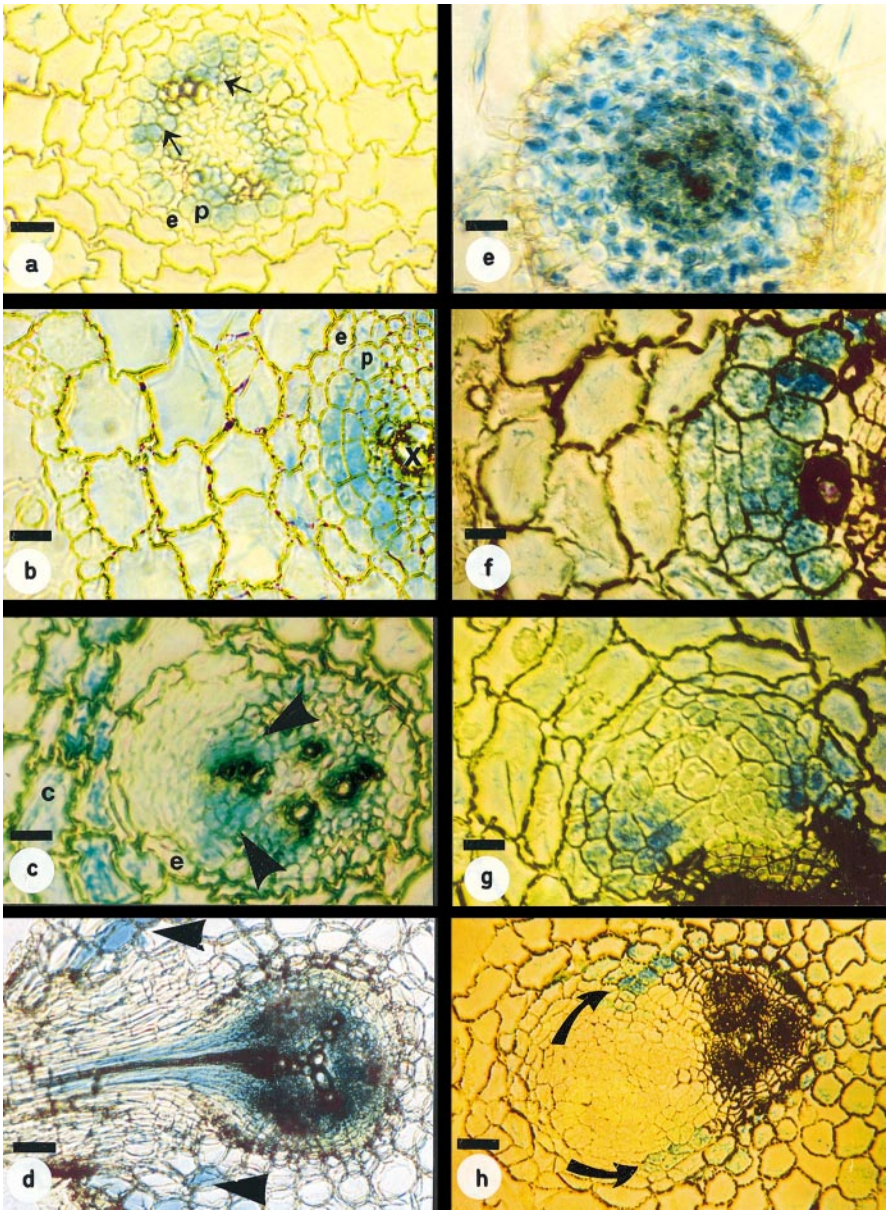

Figure 2. Expression of *GH3*:*gusA* during parallel stages of lateral root and nodule development.
(a–d) Stages of lateral root development. p = pericycle; e = endodermis; c = cortex
(a) In the root zone in which lateral root initiation had not yet started, *GH3*:*gusA* expression was localized in the vascular bundle, including undivided pericycle cells and parenchyma around the xylem poles (arrows). (b) The first dividing pericycle cells showed *GH3*:*gusA* expression, but no *GH3*:*gusA* expression was detected in the endodermis or the cortex ahead of the dividing cells. (c) After several rounds of cell divisions, the growing primordium showed no *GH3*:*gusA* expression. Dividing cells in the xylem parenchyma of the respective xylem pole (arrowheads) and cells in the cortex overlying the growing primordium expressed *GH3*:*gusA*. (d) In the emerged lateral root, cells of the newly formed central vascular cylinder expressed *GH3*:*gusA*. Some expression was present in the cortex of the main root at the site of breakthrough of the lateral root (arrowheads).
(e–h) Stages of nodule development after spot-inoculation with ANU843, shown as transverse sections at the site of inoculation.
(e) At stage 4 (30 h p.i.), enhanced *GH3*:*gusA* expression occurred in all cortical and vascular cells at the position of inoculation prior to cell divisions in the inner cortex. (f) At stage 5 (50 h p.i.), the first dividing inner cortex cells strongly expressed *GH3*:*gusA*, whereas adjacent cortex cells only showed very weak *GH3*:*gusA* expression. (g) At stage 6 (70–90 h p.i.), the growing nodule primordium showed *GH3*:*gusA* expression occurring at the side and base, but not in the centre of the primordium. (h) At stage 7 (4–5 days p.i.), *GH3*:*gusA* expression occurred in a bifurcated pattern (arrows) with no detectable *GH3*:*gusA* expression occurring in the centre of the young primordium.
Bars = 50 μm in (a) and (c); 20 μm in (b); 80 μm in (d); 100 μm in (h); 120 μm in (e); 40 μm in (f) and (g).

and the reporter gene, *gusA* encodes β-glucuronidase (GUS) (Jefferson *et al.*, 1987). Our earlier work indicated that endogenous *GH3*:*gusA* expression occurred in patterns that were consistent with the known role of auxin (Larkin *et al.*, 1996), thereby constituting a useful marker to study hormone-mediated differentiation processes. For lateral root formation, the requirement for auxin has already been established (Sussex *et al.*, 1995; Wightman

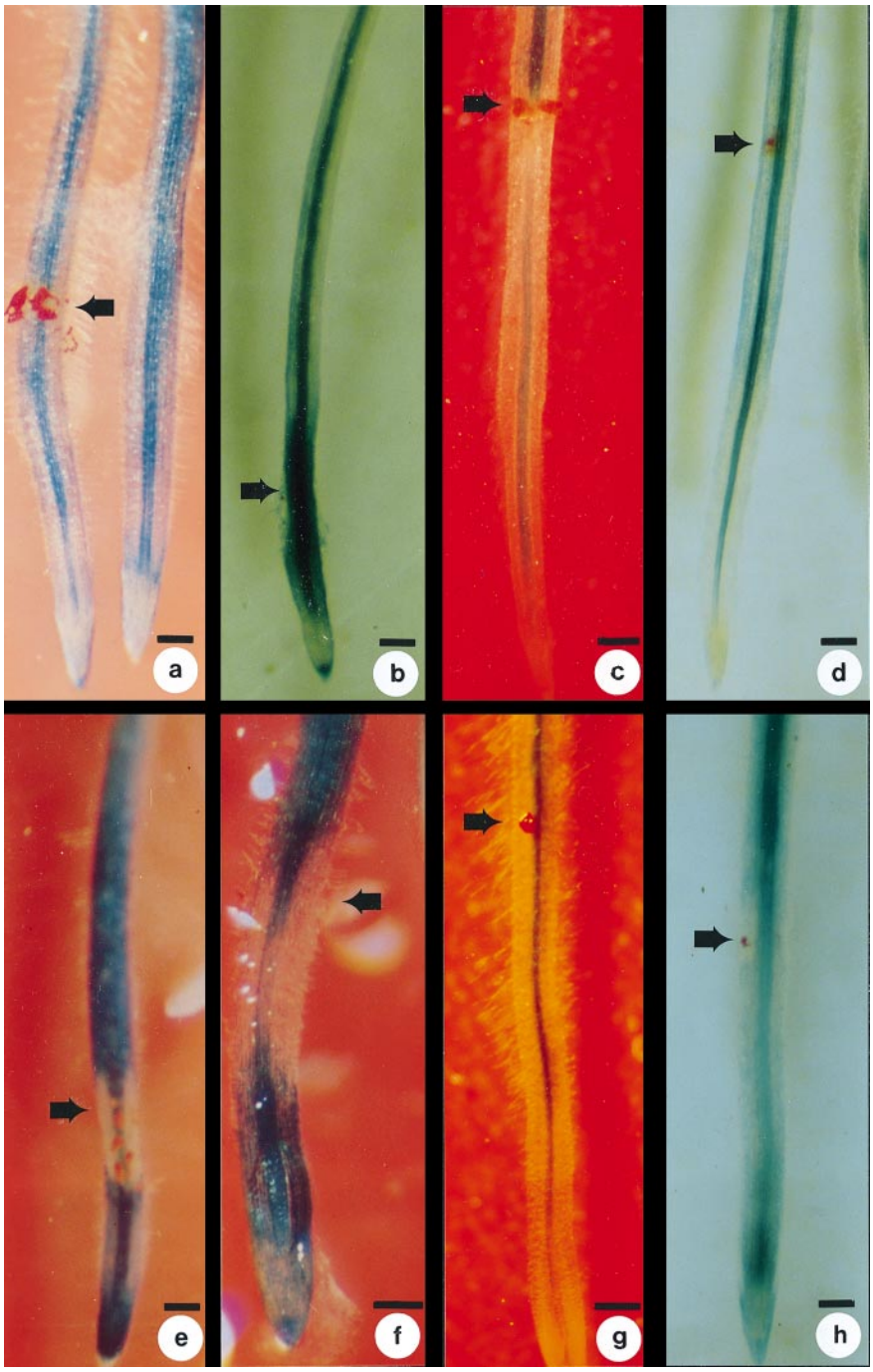

Figure 3. Alteration of *GH3*:*gusA* expression by microtargeted compounds after 24 h incubation.
The target site is marked with red ink and indicated by an arrow. Roots were microtargeted with: (a) sterile water (left) (for comparison, an untreated root is shown on the right); (b) the auxin IAA (10^{-6} M); (c) the synthetic auxin transport inhibitor, NPA (10^{-6} M); (d) the *Ms*ENOD40–1 peptide (10^{-10} M); (e) purified LCOs of strain ANU843 (10^{-8} M) (these roots were incubated in a higher substrate concentration which resulted in a higher than normal expression, this was done to enhance the effect of local downregulation of *GH3*:*gusA*); (f) an *O*-acetylated chitin pentamer (10^{-6} M) (these roots were incubated in a higher substrate concentration which resulted in a higher than normal expression, this was done to enhance the effect of local downregulation of *GH3*:*gusA*); (g) an unsubstituted chitin pentamer (10^{-6} M); (h) the flavonol quercetin (50 μM). Bars = 1 mm.

et al., 1980). Therefore, we used lateral root formation as a control to examine the changes in auxin responsiveness during nodule formation in clover. We also compared the effect of LCOs and LCO derivatives upon *GH3*:*gusA* expression to that of compounds known to interfere with the plant auxin balance such as the auxin transport inhibitor, NPA. Two micro-inoculation techniques, spot-inoculation (Bhuvaneswari *et al.*, 1981) and microtargeting (Sautter *et al.*, 1991; Schlaman *et al.*, 1997), were used to localize a microbe or compound to marked sites on, or inside, the root, respectively. Both these methods made it possible to relate any temporal or spatial changes in reporter gene expression to the inoculation site as a reference point. Our results suggest that nodulating rhizobia, clover-specific LCOs, NPA and flavonoid aglycones transiently inhibit auxin transport and that this leads to a transient accumulation of auxin at the site where a nodule will be initiated.

Results

Localization of GH3:gusA *in untreated roots detected by histochemical GUS staining and immunohistochemistry*

A comparison of histochemical GUS staining and immunohistochemistry showed that histochemical GUS staining occurred only in the cells in which the GUS enzyme was located (Figure 1a, b). In control roots, *GH3*:*gusA* expression and the GUS enzyme were localized in the vascular bundle with little or no expression in the cortex (Figures 1c and 2a). Expression inside the vascular bundle occurred in the pericycle and in the parenchyma, especially around the xylem poles (Figure 2a).

Expression of GH3:gusA *during lateral root organogenesis*

Undivided pericycle cells in the young root stele expressed *GH3*:*gusA* (Figure 2a). The earliest dividing pericycle cells, which are likely to be the progenitors of a lateral root, maintained *GH3*:*gusA* expression (Figure 2b). Expression of *GH3*:*gusA* in the dividing pericycle cells clearly preceded that seen in the cortex in front of lateral root primordia at a later stage of development. After several rounds of pericycle divisions, the expression of *GH3*:*gusA* in the lateral root primordium diminished and cortex cells overlying the lateral root primordium strongly expressed *GH3*:*gusA* (Figure 2c). After lateral root emergence, the newly formed vascular bundle expressed *GH3*:*gusA* (Figure 2d), thus re-establishing in the lateral root the same pattern of *GH3*:*gusA* expression seen in the main root. The cortex region, through which the lateral root had penetrated, retained *GH3*:*gusA* expression (Figure 2d).

GH3:gusA *expression during early nodule initiation and development*

Roots spot-inoculated with strain ANU843, a wild-type *Rhizobium leguminosarum* biovar *trifolii*, showed temporal and spatial changes in *GH3*:*gusA* expression during different stages of nodule formation. The confined spot-inoculation of strain ANU843 resulted in a high (84%) probability of a nodule occurring at the inoculated site, which enabled us to confidently relate changes in *GH3*:*gusA* expression patterns to the inoculation point.

The changes in *GH3*:*gusA* expression observed could be categorized into distinct stages. Compared to control roots

Table 1. Bacterial strains used for spot-inoculation

Bacterial strain	Relevant characteristics		Source/reference
R.l. bv. *trifolii*			
ANU843	Wild-type strain	Nod$^+$ clover	Djordjevic *et al.*, 1985
Mutants			
ANU277	ANU843 *nodC*::*Tn*5*	Nod$^-$ clover	Djordjevic *et al.*, 1985
ANU845	ANU843 pSym cured†	Nod$^-$ clover	Schofield *et al.*, 1983
R. meliloti			
1021	Nod$^+$ *Melilotus*	Nod$^-$ clover	Meade *et al.*, 1982
B. japonicum			
USDA110	Wild-type	Nod$^+$ soybean Nod$^-$ clover	Nieuwkoop *et al.*, 1987
A. tumefaciens			
C58	Wild-type tumour-forming bacterium	Nod$^-$	Gift from Professor Ann G. Matthysse (University of North Carolina)

*The *nodC* mutant is deficient in a chitin synthase and does not make LCOs.
†All known nodulation genes are deleted in this strain.

Table 2. Effects of bacterial strains and compounds on *GH3*:*gusA* expression patterns resulting from spot-inoculation

Bacterial strain	Local expression (± 1 mm) at bead*	Expression 1–5 mm acropetal from bead*	Expression 1–5 mm basipetal from bead*	% of treated roots with altered phenotype†	% of control roots with altered phenotype	Total number of analysed roots
ANU843, 1 h	–	–	–	0	0	10
ANU843, 5 h	↓	↓	–	10	0	29
ANU843, 10 h	–	↓	↑	5 (5)	0	23
ANU843, 20 h	↑	↑	↑	50 (5)	5	37
ANU843, 30 h	↑↑	↑	↑↑	40 (11)	5	35
ANU843, 50 h	↑↑	–	–	70 (10)	0	20
ANU845, 5 h	–	–	–	0	0	16
ANU845, 24 h	–	–	–	0	0	12
ANU277, 5 h	–	–	–	0	0	10
ANU277, 24 h	–	–	–	0	0	9
Rm 1021, 5 h	–	↓	↑	40	0	12
Rm 1021, 24 h	–	–	–	0	0	12
USDA 110, 5 h	–	–	–	0	0	7
USDA110, 24 h	–	–	–	0	0	6
C58, 24 h	–	–	–	0	0	10
Compound						
LCOs (10^{-8} M) 5 h	–	–	–	0	0	8
LCOs 10 h	–	↓	–	20	0	30
LCOs 20 h	↓	↑	↑	55 (18)	0	11
LCOs 30 h	↑↑	↑↑	↑↑	100	0	9
LCOs 50 h	–	–	–	0	0	10
LCOs 80 h	–	–	–	0	0	14
NAA 10^{-4} M, 20 h	–	–	–	0	0	9
NAA 10^{-6} M, 20 h	–	–	–	0	0	7
IAA 10^{-4} M, 20 h	–	–	–	0	0	9
IAA 10^{-6} M, 20 h	–	–	–	0	0	9
NPA 10^{-6} M, 5 h	↓	↓↓	↑	35	0	15
NPA 10^{-6} M, 24 h	↓	↑	↑	35	5	15
Quercetin 20 h	↓	↓	↑	25	5	15
Apigenin 20 h	↓	↑	↑	35	10	19

* '↓' reflects downregulation of the *GH3* promoter compared to control roots, and '↑' reflects upregulation of the GH3 promoter. The number of arrows indicates the intensity of the described changes in GUS expression. '–' indicates that no change in GUS expression was observed compared to controls.

†The response of each root was not uniform. Roots were only scored positive when they showed the phenotype indicated in columns 2, 3 and 4. Some roots at each time-point showed the phenotype of an earlier or in some cases a later time-point, especially roots inoculated with rhizobia. These roots were not scored as positive. The numbers in parentheses indicate the additional percentage of roots that had a phenotype of an earlier or later timepoint. The result of each treatment was compared to the response in an equal number of negative (solvent) and positive (*R. l.* bv. *trifolii*) controls.

(Figure 1c), no changes in *GH3*:*gusA* expression occurred within 1 h of inoculation (Figure 1d). Between 1 and 5 h post-inoculation (p.i.), *GH3*:*gusA* expression was reduced in the vascular bundle between the inoculation site and the root tip (Figure 1e; stage 1). After 10 h, increased *GH3*:*gusA* expression occurred immediately basipetally from the inoculation site in the cortex, and the reduced expression in the vascular bundle remained between the inoculation site and the root tip (Figure 1f; stage 2). At 20 h p.i., *GH3*:*gusA* expression was high in a zone of about 2 mm to either side of the inoculation site in the cortex. At this time, expression in the vascular bundle began to reappear between the inoculation site and the root tip (Figure 1g). However, the expression just basipetal from the root tip was in most cases lower than in control roots. At 30 h p.i., the root cortical and vascular cells showed enhanced *GH3*:*gusA* expression in a more extended zone around the inoculation bead than after 20 h (Figure 1h; stage 4). Transverse sections at the site of inoculation confirmed high *GH3*:*gusA* expression in all cortical and vascular cells at the inoculation site (Figure 2e). *GH3*:*gusA* expression in the vascular bundle occurred towards the root tip, but in 50% of the roots it remained visibly reduced compared to controls. After about 50 h, the first cell divisions were apparent in the inner cortex. At this stage, *GH3*:*gusA* expression was focused in the cells undergoing

Table 3. Effects of compounds on *GH3*:*gusA* expression patterns, assayed 24 h after microtargeting

Compound	Expression at application site (± 1 mm)*	Expression 1–5 mm acropetal from site*	Expression 1–5 mm basipetal from site*	% of treated roots with altered phenotype	% of control roots with altered phenotype	Total number of analysed roots
LCOs (10^{-8} M)	↓↓	↓↓	↑↑	90	0	10
O-Ac. IV (10^{-6} M)†	–	↓	↑	20	0	10
O-Ac. V	↓↓	↓↓	↑↑	65	0	6
O-Ac. VI	↓	↓	↑	65	0	9
Chitin II (10^{-6} M)‡	–	–	–	0	0	10
Chitin IV	–	–	–	0	0	10
Chitin V	–	–	–	0	0	10
Chitin VI	–	–	–	0	0	10
Quercetin (50 μM)	↓↓	↓	↑↑	65	0	11
Quercetin-3-glucoside	↓	↓	↑	10	0	10
Kaempferol	↓	↑	↑	65	0	12
Kaempferol- 3-glu§	–	–	–	0	0	10
Apigenin	↓	↑	↑	40	0	14
Apigenin-7-rha-glu§	–	–	–	0	0	5
Naringenin	↑	↓↓		80	0	5
Naringenin-7-rha-glu§	–	–	–	0	0	5
Fisetin	↓↓	↑↑	↑↑	80	0	5
Genistein	–	–	–	0	0	5
*Ms*ENOD40–1, 10^{-6}–10^{-10} M	–	–	–	0	0	40
IAA 10^{-4} M	↑↑	↑↑	–	70	10 +	10
IAA 10^{-6} M	↑↑	↑↑	–	50	0	10
NAA 10^{-4} M	↑	↑		50	0	10
NAA 10^{-6} M	↑↑	↑↑		70	0	10
NPA 10^{-4} M	↓↓	↓	↑↑	45	0	9
NPA 10^{-6} M	↓↓↓	↓↓	↑↑	55	0	9

* '↓' reflects downregulation of the *GH3* promoter compared to control roots, and '↑' reflects upregulation of the promoter. The number of arrows indicates the intensity of the described changes in GUS expression. '–' indicates that no changes in GUS expression were observed compared to controls.
†*O*-Ac.- IV, V, VI = *O*-acetylated chitin tetramer, pentamer and hexamer, respectively.
‡Chitin II, IV, V, VI = chitin dimer, tetramer, pentamer and hexamer, respectively.
§glu = glucoside; rha = rhamnoside.
+In one plant microtargeted with 2% methanol, a positive response was observed. The result of each treatment was compared to the response in an equal number of negative (solvent) and positive (*R. l.* bv. *trifolii*) controls and in most cases independently repeated at least once.

division, but was very low or not detectable in the surrounding non-dividing cortical cells (Figure 1i and Figure 2f; stage 5). By about 70 h, when the nodule primordium began to differentiate, no *GH3*:*gusA* expression was detected in the centre of the primordium, but expression remained at the base and periphery (Figure 2g; stage 6). Examination of nodules 4–5 days after inoculation showed that *GH3*:*gusA* expression occurred in a bifurcated pattern (Figure 2h; stage 7).

Non-nodulating Rhizobium *strains do not alter* GH3:gusA *expression*

To determine if the change in *GH3*:*gusA* expression was specifically induced by clover-nodulating bacteria, we spot-inoculated non-nodulating mutant and non-host bacteria onto the transgenic plants (Table 1). The inoculation of two non-nodulating ANU843 derivatives that are unable to synthesize LCOs, ANU277 (*nodC*::Tn*5*) and ANU845 (pSym-cured), did not induce any changes in *GH3*:*gusA* expression (Table 2). At 5 h p.i., the non-host *R. meliloti* strain Rm1021 initially induced similar changes to the pattern of *GH3*:*gusA* expression as that seen with ANU843; however, no further changes in *GH3*:*gusA* expression were seen at later time-points. The more distantly related bacterial species, *Bradyrhizobium japonicum* USDA110 and *Agrobacterium tumefaciens* C58, failed to induce any detectable changes in *GH3*:*gusA* expression (Table 2).

LCOs and O*-acetylated chitin oligosaccharides cause a similar local downregulation of* GH3:gusA *expression as ANU843 and NPA*

The microtargeting or spot-inoculation of control solutions (water or solvents) did not alter the expression of *GH3*:*gusA* (Figure 3a). As positive controls, we examined the effect of

indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), NPA and the ENOD40–1 peptide on *GH3*:*gusA* expression. The microtargeting of IAA and NAA increased *GH3*:*gusA* expression at and acropetal from the application site within 24 h of incubation (Figure 3b and Table 3), whereas the spot-inoculation of IAA or NAA was ineffective (Table 2). Microtargeted or spot-inoculated NPA (Figure 3c) induced strong downregulation of *GH3*:*gusA* expression acropetal from the inoculation site from 5 h onwards (Tables 2 and 3). Microtargeted *Ms*ENOD40–1 peptide (at 10^{-6}, 10^{-7}, 10^{-9} or 10^{-10} M) did not induce any changes to *GH3*:*gusA* expression after 24 h (Figure 3d, Table 3).

Given that ANU843 induces changes in *GH3*:*gusA* expression during nodule initiation (Figure 1e–i), we expected that LCOs inoculation would cause similar effects. Consequently, purified LCOs of strain ANU843 were either spot-inoculated onto or microtargeted into roots at 10^{-8} M. The LCOs induced changes in *GH3*:*gusA* expression that were qualitatively and spatially similar to the changes in expression induced by ANU843 over the first 30 h p.i. (Figure 1e–h and Figure 3e), but had a slightly delayed onset (up to 5 h; Table 2). The local downregulation of *GH3*:*gusA* by LCOs was more enhanced in microtargeted roots compared to spot-inoculated roots, but in both cases resembled the effects observed with NPA. In contrast to results obtained with strain ANU843 inoculation, no further effect of LCOs application on *GH3*:*gusA* expression was apparent from 50 h p.i. onwards (Table 2). The microtargeting of LCOs into the mature root (several centimetres from the root tip) did not alter *GH3*:*gusA* expression (data not shown).

O-acetylated chitin oligosaccharides were microtargeted into roots because they form the LCOs backbone. Tetra-, penta- or hexamers of the *O*-acetylated chitin oligosaccharides induced a similar local downregulation of *GH3*:*gusA* expression as LCOs and NPA (Figure 3f and Table 3). The pentamer had the strongest effect (Table 3). Microtargeted non-*O*-acetylated chitin oligosaccharides including either di-, tetra-, penta- or hexamers failed to induce alterations to *GH3*:*gusA* expression (Figure 3g and Table 3).

Flavonoid aglycones cause similar changes to GH3:gusA *expression as NPA*

We tested the effect of flavonoids on *GH3*:*gusA* expression because certain flavonoids have been reported to act as endogenous auxin transport regulators (Jacobs and Rubery, 1988). We observed that the structure of the microtargeted flavonoids affected the ability to influence *GH3*:*gusA* expression. The flavonols, quercetin (Figure 3h), fisetin and kaempferol, the flavone, apigenin, and the flavanone, naringenin, caused a similar local and acropetal downregulation of *GH3*:*gusA* expression as LCOs, *O*-acetylated chitin fragments and NPA after 24 h (Table 3). Spot-inoculation of quercetin and apigenin had a similar effect as microtargeting (Table 2). In contrast to the aglycones, the microtargeting of isoflavonoid, genistein, and the flavonoid glucosides, quercetin-3-glucoside, kaempferol-3-glucoside, apigenin-7-rhamnoglucoside and naringenin-7-rhamnoglucoside, failed to alter *GH3*:*gusA* expression after 24 h (Table 3).

Discussion

How reliable is GH3:gusA *expression as a marker for the activity of auxin?*

A major limitation of plant biology is the inability to accurately assess the levels of active phytohormones in tissues and individual cells. We have used an indirect method to assess the auxin responsiveness of clover root tissues. We used the auxin responsive *GH3* promoter that was originally isolated from soybean hypocotyls in conditions promoting auxin-stimulated cell elongation (Hagen *et al.*, 1984). However, several results suggest that the transgenic white clover plants containing *GH3*:*gusA* are reliable indicators of changes in auxin activity during development. First, the expression patterns obtained for lateral root formation (Figure 2) and gravi-stimulated roots (Larkin *et al.*, 1996) are consistent with the physiological role of auxin in these processes, both temporally and spatially (Pelosi *et al.*, 1995; Sussex *et al.*, 1995). Furthermore, the expression pattern indicates that *GH3*:*gusA* expression is not restricted to cells during either auxin-stimulated division or elongation, but that every cell in the root is able to express *GH3*:*gusA* (Figure 2e), as reported previously by Guilfoyle *et al.* (1993). Second, *in vitro* studies show the selectivity of the *GH3* promoter for auxin and not other phytohormone classes (Larkin *et al.*, 1996). In addition, the auxin inducibility increases linearly with rising auxin concentrations over a range from 10^{-6} to 10^{-3} M (Guilfoyle *et al.*, 1993). Third, the histochemical localization of the indigo dye product coincides with the immunolocalization of β-glucuronidase (Figure 1a, b). Fourth, *in situ* hybridization of *GH3* transcripts in soybean roots shows the vascular bundle, especially around the xylem poles, as the main site of *GH3* mRNA accumulation (Guilfoyle *et al.*, 1993), similar to *GH3*:*gusA* expression patterns described in this study. Furthermore, as would be expected, *GH3*:*gusA* was upregulated by local application of auxins (Figure 3b) and acropetally downregulated by the auxin transport inhibitor, NPA (Figure 3c), and flavonoid aglycones, but not flavonoid glycosides (Jacobs and Rubery, 1988). Therefore, interpretations about the effects of bacteria and signal molecules used in this study were based on comparisons with the effect of auxins and established auxin transport inhibitors. Notwithstanding these findings, it is possible that potential artifacts may occur due to indirect detection

of the GUS enzyme (Guivarc'h *et al.*, 1996), the nature of the *GH3* regulatory regions (Taylor, 1997) or the differential perception of auxin by cells via separate signalling pathways (Guilfoyle *et al.*, 1993). Therefore, future studies will attempt to verify, by different methods, that auxin transport inhibition occurs early during root nodule formation.

Auxin transport inhibition precedes the earliest stages of root nodule formation

Rhizobium inoculation leads to temporal and spatial changes in *GH3*:*gusA* expression in white clover roots. Using the inoculation site as a reference point, two main patterns of *GH3*:*gusA* expression were recognized before initiation of cell divisions (stages 1–4). A rapidly induced (stage 1), local and transient (until stage 2) downregulation of *GH3*:*gusA* expression occurred in the vascular bundle and cortex (Figure 1e, f), followed by a basipetal upregulation in the cortex and vascular bundle (stage 2–4; Figure 1f–h). Both acropetal downregulation and basipetal upregulation of *GH3*:*gusA* was mimicked by the auxin transport inhibitor NPA, flavonoid aglycones and LCOs (Tables 2 and 3 and Figure 3c,e,h).

We conclude from a comparison of the effects of auxins and NPA treatments with that of *Rhizobium* inoculation and LCOs addition, that an inhibition of the acropetal auxin flow leads to the downregulation of *GH3*:*gusA* expression between the inoculation site and the tip. Concomitantly, the basipetal upregulation of *GH3*:*gusA* is most probably due to an accumulation of shoot-derived auxin that is stopped at the inoculation site. By 20–30 h p.i., the auxin transport inhibition presumably weakens, because *GH3*:*gusA* gradually reappears acropetally from the inoculation site, although at a slower rate in the cortex than in the vascular bundle (Figure 1g, h). However, even at 30 h p.i., *GH3*:*gusA* expression is still reduced behind the root tip, compared to untreated controls.

In contrast to ANU843, non-nodulating ANU843 derivatives (which do not make LCOs) and non-host rhizobia (which make incorrect LCOs) were unable to induce sustained auxin transport inhibition (Table 2). These results suggest that local auxin transport inhibition is necessary for nodule initiation. The exceptional result was the change in *GH3*:*gusA* expression that occurred 5 h after the inoculation with *R. meliloti*, which did not occur at later timepoints. Because the inoculation of *R. meliloti* strain 1021 led to root hair distortions at the inoculation site but not to root nodules or infection threads, this finding may suggest that the *R. meliloti* LCOs are recognized by clover roots but that all of the changes needed to induce nodule initiation do not occur. Indeed, recent studies have shown that non-legume roots show some responsiveness to LCOs (Spaink, 1996).

LCOs, O*-acetylated chitin fragments and flavonoid aglycones induce auxin transport inhibition*

LCOs, the necessary signal molecules of rhizobia, are the most likely mediators of the effect of ANU843 on auxin transport, because LCOs caused similar changes in *GH3*:*gusA* expression as application of ANU843 and of NPA (Tables 2 and 3 and Figure 3e). This would be expected if auxin transport inhibition was a prerequisite for nodulation. Interestingly, *O*-acetylated chitin oligosaccharides induced similar changes in *GH3*:*gusA* expression as LCOs (Figure 3f), indicating that the chitin backbone of LCOs is the biologically active moiety. Recently, *O*-acetylated chitin fragments were shown to induce cortical cell divisions in roots of *Vicia sativa* following microtargeting (Schlaman *et al.*, 1997). Our results indicate that LCOs are especially active in the susceptible zone near the root tip, because the microtargeting of LCOs into the mature part of the root did not induce changes in *GH3*:*gusA* expression. Bhuvaneswari *et al.* (1981) showed that the area behind the root tip was particularly sensitive to *Rhizobium* inoculation.

Flavonoids are potential candidates for endogenous inhibitors of auxin transport in the early stages of nodule initiation (Hirsch *et al.*, 1989; Hirsch, 1992). Our results support this hypothesis, because introduction of flavonoids into the roots had a similar effect upon *GH3*:*gusA* expression as LCOs, *O*-acetylated chitin fragments and NPA (Figure 3h). Flavonols, flavanones and flavones were most active, whereas their respective glycosides and also an isoflavonoid were inactive (Table 3), which indicates a specificity for certain flavonoid aglycones as auxin transport inhibitors. Similar results were obtained by Jacobs and Rubery (1988), who used the same flavonoids in their study. Also consistent with the biological activity of flavonoids is the specific induction of flavonoid genes and end products by compatible rhizobia on their respective hosts (clover; Lawson *et al.*, 1994, 1996; *Vicia*, Recourt *et al.*, 1992). We have obtained additional data to support these findings (U. Mathesius *et al.*, unpublished data).

Changes in GH3:gusA *expression during nodule differentiation are similar to changes during lateral root differentiation*

Only the clover nodulating strain ANU843 (Figure 2e–h) was able to induce stage 1–6 changes in *GH3*:*gusA* expression, whereas *R.l.* bv. *trifolii* LCOs were only able to induce the stage 1–4 changes (Table 2). The activity of plant chitinases (Staehelin *et al.*, 1994) could explain why LCOs alone were unable to induce nodule initiation. In contrast, compatible bacteria continually produce LCOs during the infection process. Between stage 4 and 5, a reduction in *GH3*:*gusA* expression occurred in all cortex cells except a specific subset of inner cortical cells (Figure 2f) that con-

tinued to divide to form the nodule primordium. The specific retention of *GH3*:*gusA* expression in these dividing inner cortical cells may infer that an additional signal is required to trigger these particular cells to divide. One possible signal, the ENOD40–1 peptide, is reported to change the response of cells to auxin (van de Sande *et al.*, 1996). However, our results did not support any role for the ENOD40–1 peptide in perturbing the auxin balance when added exogenously (Figure 3d).

Because high levels of *GH3*:*gusA* expression were observed before initiation of cell division (Figure 2e) and in the progenitor cells of a nodule primordium (Figure 2f), a period of high auxin exposure and responsiveness is likely to be necessary in these cells prior to and during the first divisions (stages 4 and 5). Since it has also been found that cytokinin application can induce nodule formation (Bauer *et al.*, 1996; Cooper and Long, 1994), it is unlikely that the auxin levels alone determine the conditions for nodule initiation, but that the ratio of cytokinin to auxin levels is important for nodule initiation. Similar to nodule progenitor cells, high *GH3*:*gusA* expression was also detected during lateral root initiation in pericycle cells prior to and during division (Figures 1d and 2a). The difference between progenitor cells of nodule and lateral root primordia is that pericycle cells were always situated in a zone of high *GH3*:*gusA* expression and did not undergo a transient phase of *GH3*:*gusA* downregulation (i.e. no stages 1–2). High auxin requirements in cells prior to division could reflect the role of auxin in the accumulation of $p34^{cdc2}$-like proteins needed to activate mitosis (John *et al.*, 1993; Zhang *et al.*, 1996).

After the initiation of nodule and lateral root primordia, we found further similarities in *GH3*:*gusA* expression patterns at parallel developmental stages. In both organs, expression was high in dividing cells of the early primordium (Figure 2b, f) but reduced during later stages of development and differentiation (Figure 2c, d and g, h). These observations are supported by the results of Pelosi *et al.* (1995) and Sussex *et al.* (1995), which show that lateral root morphogenesis is a two-step process (requiring high auxin concentration during the initiation of the primordium and subsequently lower auxin concentrations during primordium differentiation). After the initiation of the lateral root primordium, a pulse of *GH3*:*gusA* expression was seen in the outer cortex overlying the primordium. This resolves the issue raised in Larkin *et al.* (1996), where it was unclear whether this characteristic pulse preceded or followed lateral root initiation. Because the *GH3*:*gusA* expression patterns in developing nodules are very similar to patterns during lateral root formation, our results suggest that nodule formation also requires high auxin levels for initiation of cell divisions (Figure 2e) and establishment of the primordium (Figure 2f), but lower levels after the establishment of the nodule primordium (Figure 2g, h).

Overall, our results show for the first time that rhizobia cause a localized, temporary and early inhibition of auxin transport, which subsequently leads to an accumulation of auxin at the site of nodule initiation. Although the later stages of nodule and lateral root formation are similar in their apparent requirements for auxin, the changes in *GH3*:*gusA* expression preceding nodule initiation are unique. Both lateral root and nodule formation appear to be a two-step process in their auxin requirement. Possible endogenous signal molecules that could mediate the effect of LCOs on auxin transport are certain flavonoid aglycones. Current experiments also indicate that localized flavonoid induction occurs inside the root following *Rhizobium* inoculation (U. Mathesius *et al.*, unpublished data). Our results also show that the *O*-acetylated chitin moiety of the LCO molecule is biologically active.

Experimental procedures

Bacterial strains and test compounds

Table 1 lists the bacterial strains used. Test compounds used for spot-inoculation or microtargeting were NPA, NAA and IAA (Sigma Chemicals Co., St Louis, MO), dissolved in 0.01 or 1% methanol; and kaempferol, apigenin, apigenin-7-rhamnoglucoside, genistein (Roth, Karlsruhe, Germany), quercetin, naringenin, naringenin-7-rhamnoglucoside (Sigma), quercetin-3-glucoside, kaempferol-3-glucoside (Indofine Chemicals, France), dissolved in 5% methanol. The *Ms*ENOD40–1 peptide was provided by Henk Franssen and dissolved in 5% DMSO. Chitin fragments composed of five or six glucosamine residues were obtained from Seika-Gaku (Tokyo, Japan) and dissolved in Milli-Q purified water. Chitin fragments were *O*-acetylated at the C6 position of the non-reducing sugar using the NodL enzymatic assay, and purified using HPLC as described previously (Bloemberg *et al.*, 1994). LCOs were dissolved in 1% DMSO or 5 mM cyclodextrin (Sigma).

Transgenic plants

The transgenic white clover (*Trifolium repens* cv. Haifa) plants used have been described previously (Larkin *et al.*, 1996). Five first generation transgenic lines were used for analysis. Mature first generation transgenic plants were grown in soil under natural light conditions at 26°C, 60% average humidity during the day, and 19°C, 80% average humidity during the night, and were maintained by regrowth from shoot cuttings.

Generation of rooted leaves

Axenically grown rooted leaves were generated from mature plants (Rolfe and McIver, 1996) using Jensen's medium (Jensen, 1942). The roots were kept dark by covering the lower half of the plates with brown paper or specifically designed black plastic boxes. Growth conditions for the rooted leaves were, 16 h day (24–25°C), 8 h night (19°C) with a mixture of fluorescent and incandescent light with an intensity of ~ 140 μmol m^{-2} s^{-1} and 80% average humidity. Under these conditions, roots formed visible nodules after 4–5 days p.i.

Spot-inoculation of transgenic plants

Two transgenic lines were primarily used for the experiments. Of each line, 5–20 rooted leaves were used for each treatment and time-point. Equal numbers of roots were inoculated with either a test or a (negative) control solution consisting of the solvent only. In the case of bacterial inoculations, Bergensens Modified Medium (BMM) (Rolfe *et al*., 1980) was used as a negative control. Positive controls consisted of roots inoculated with *R. l.* bv. *trifolii*. One rooted leaf each with one to four roots exceeding 1 cm (8–10 days growth) was transferred to a fresh, dry Jensen's plate and grown overnight. The fresh dry plate was needed to avoid spreading of the inoculum due to excessive moisture. The spot-inoculation technique of Bhuvaneswari *et al.* (1981) was used. One root per rooted leaf was spot-inoculated with ~ 20 nl of the dissolved compound or solvent. For bacterial inoculations, bacteria were grown in liquid BMM at 28°C over night, to OD (600 nm) of 0.2–0.4 before inoculation. Bacteria, 5000–10 000, (as tested by plate counts) were applied thus avoiding any effects of over-inoculation.

Microtargeting of transgenic plants

Microtargeting was carried out with a modified microtargeting microprojectile accelerator (Gisel *et al*., 1996; Sautter *et al*., 1991). Each test compound was dissolved in solvent at the final concentration and mixed with an equal volume of gold particles (average size of 1 μm diameter), suspended in sterile water. To introduce the aerosol, a 60 nl suspension load with a restriction size of 140 μm was used at 50 bar nitrogen. Rooted leaves with 1 cm long roots were used. The leaf was excised 5 mm above the emerged roots and the roots mounted on Jensen's plates containing 2% agarose with 2% maltose as an osmoticum, and incubated for 2 h prior to treatment. One root per root system was microtargeted in the same area used for spot-inoculation and that spot was marked with sterile red ink. Control solutions were microtargeted into an equal number of roots as the test compounds. After microprojectile bombardment, the roots were incubated a further 24 h at 25°C in a horizontal position and subsequently used for histochemical GUS localization.

Isolation of LCOs

LCOs were isolated from *Rhizobium leguminosarum* bv. *trifolii* strain ANU843 either following the method of Spaink *et al.* (1995) or as a modification of this technique by L. F. Roddam, J. W. Redmond and M. A. Djordjevic (manuscript in preparation).

GUS assays

Histochemical GUS assays were carried out as described in Larkin *et al.* (1996).

Microscopy

Whole, histochemically stained roots were examined using a Leica or a Nikon SMZ-10 stereomicroscope. For sectioning, fixed and histochemically stained material was embedded in 3% agarose and sectioned (80 μm thickness) on a vibratome (Lancer series 1000). Alternatively, roots were fixed overnight at 4°C in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), dehydrated in increasing concentrations of ethanol and infiltrated with historesin following the manufacturer's protocol (Historesin Embedding Kit, Jung). Sections of 10 μm were cut on a Reichert Jung Ultra Microtome and analysed and photographed using a Nikon Optiphot light microscope fitted with a Nikon FX 35 camera. Kodak Ektachrome 100 ASA or EPY 64 T colour slide film was used.

Immunohistochemistry

Histochemically stained roots were fixed in 4% paraformaldehyde in phosphate buffer at 4°C over night and embedded in historesin as described above. Root sections were washed with BSA buffer (containing 20 mM Tris–HCl, 0.1% BSA, 0.02% fish skin gelatine) and incubated with a polyclonal rabbit anti-β-glucuronidase antiserum (Clontech Laboratories) overnight in BSA buffer at 4°C. Sections were rinsed with BSA buffer and incubated for 2 h with a secondary pig anti-rabbit–FITC labelled antibody (Dako, Denmark) and viewed and photographed using a Leitz Diaplan fluorescent microscope. Kodak 400 ASA film was used for fluorescent photographs.

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