Rhizobium Inoculation and Physical Wounding Result in the Rapid Induction of the Same Chalcone Synthase Copy in *Trifolium subterraneum*

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The gene or genes encoding chalcone synthase (*CHS*) in the legume *Trifolium subterraneum* (subterranean clover) were induced within 6 hr after inoculation with *Rhizobium leguminosarum* bv. *trifolii* strain ANU843. No induction was found in uninoculated controls or plants inoculated with either the nodulation-deficient *R. l.* bv. *trifolii* strain ANU845 (pSym$^-$) or *R. meliloti* strain 1021, which is capable of nodulating alfalfa but not clover. Morphological examination of the interaction between the legume and bacteria in this system showed that root hair distortion (a marker of the early events in the interaction) was apparent within 10 hr after inoculation. This indicated that *CHS* induction could occur before any detectable sign of rhizobial penetration of root hairs. The addition of a crude preparation of *R. l.* bv. *trifolii* lipooligosaccharide signals (Nod metabolites) to the plant growth medium had no effect on the expression of *CHS* over 36 hr, although root hair distortion was apparent over this time. These treatments were then contrasted with physical wounding. Wounding the plants led to a rapid induction of *CHS*, occurring within 2 hr. Sequence analysis of cloned *CHS* cDNA from pools sampled after *Rhizobium* inoculation or wounding treatments showed the gene designated *CHS5* was the major *CHS* species in both treatments. Conserved sequences were found in promoters of *CHS5* and soybean *Gmchs7*, a gene which has overlapping expression patterns. These findings support the view that the induction of the phenylpropanoid pathway is involved in the very early events of the *Rhizobium* infection of legumes.

Additional keywords: defense, flavonoids, nodulation, symbiosis.

The enzyme chalcone synthase (CHS) (EC 2.3.1.74) occurs at a key branch point in the phenylpropanoid pathway in plants. The chalcone synthase genes (*CHS*) respond through transcription to a variety of developmental and environmental stimuli (Dixon *et al.* 1983; Hahlbrock and Scheel 1989; Dixon and Lamb 1990). The pathway has been subject to considerable analysis in a wide variety of plants (Stafford 1990). The compounds produced through the CHS branch of the pathway include phenylpropanoid signaling molecules (flavonoids and isoflavonoids), which function as inducers of bacterial *nod* gene expression in the early events of the symbiotic association between legumes and *Rhizobium* (Djordjevic and Weinman 1991). These compounds have also been shown to have a variety of other functions (Stafford 1990).

During the *Rhizobium* infection process, bacterial penetration and proliferation is combined with the induction of a developmental response leading to the formation of a new meristem and nodule structure. The flavonoid products of *CHS* expression could have a role in either or both of these processes (Hirsch 1992). We have therefore focused on the role of *CHS* in the very early preinfection events.

To date *CHS* expression has been examined in a limited number of rhizobial interactions. Wingender *et al.* (1989) found no induction of *CHS* in inoculated soybean root tissues during infection by *Bradyrhizobium japonicum* over 10 hr. However, higher levels of *CHS* expression were found in nodulated root tissue 16 and 28 days after inoculation. Estabrook and Sengupta-Gopalan (1991) examined induction of *CHS* following infection of soybean by *B. japonicum* over 4 days and were able to show that different copies of the *CHS* gene family were specifically induced by 24 hr after inoculation. Use of a plant supernodulation mutant (nts382) provided evidence that the induction of *CHS* copies was the result of postinfection events. Inoculation of soybean with a heterologous strain (*R. meliloti*) was found to increase the expression of the *CHS* gene family, whereas infection by *B. japonicum* led to a specific subset of *CHS* transcripts. Stokkermans *et al.* (1992) also examined *CHS* expression in soybean after inoculation with *B. japonicum* and found only a slight increase in *CHS* expression 6 hr after inoculation, compared to the level of expression 3 hr after inoculation and in control samples. In *Vicia*, *CHS* induction was shown to peak 12 hr after inoculation with *R. l.* bv. *viceae* (Recourt *et al.* 1992b). This induction was correlated with an increased secretion of new phenylpropanoid compounds into the rhizosphere (Recourt *et al.* 1992a).

The *nod* genes code for enzymatic gene products involved in the synthesis of lipooligosaccharides (LOSs) (Lerouge *et al.* 1990), which are essential for successful nodule formation. Isolated LOS has been shown to induce root hair deformation, cortical cell division, and the expression of the early plant nodulin genes (Truchet *et al.* 1991; Bisseling *et al.* 1992; Horvath *et al.* 1993). Recourt *et al.* (1992b) showed that a *Rhizobium* strain without the *nod* genes (pSym-cured and unable to nodulate the host plant) was unable to induce *CHS* expression but that the wild type could induce expres-

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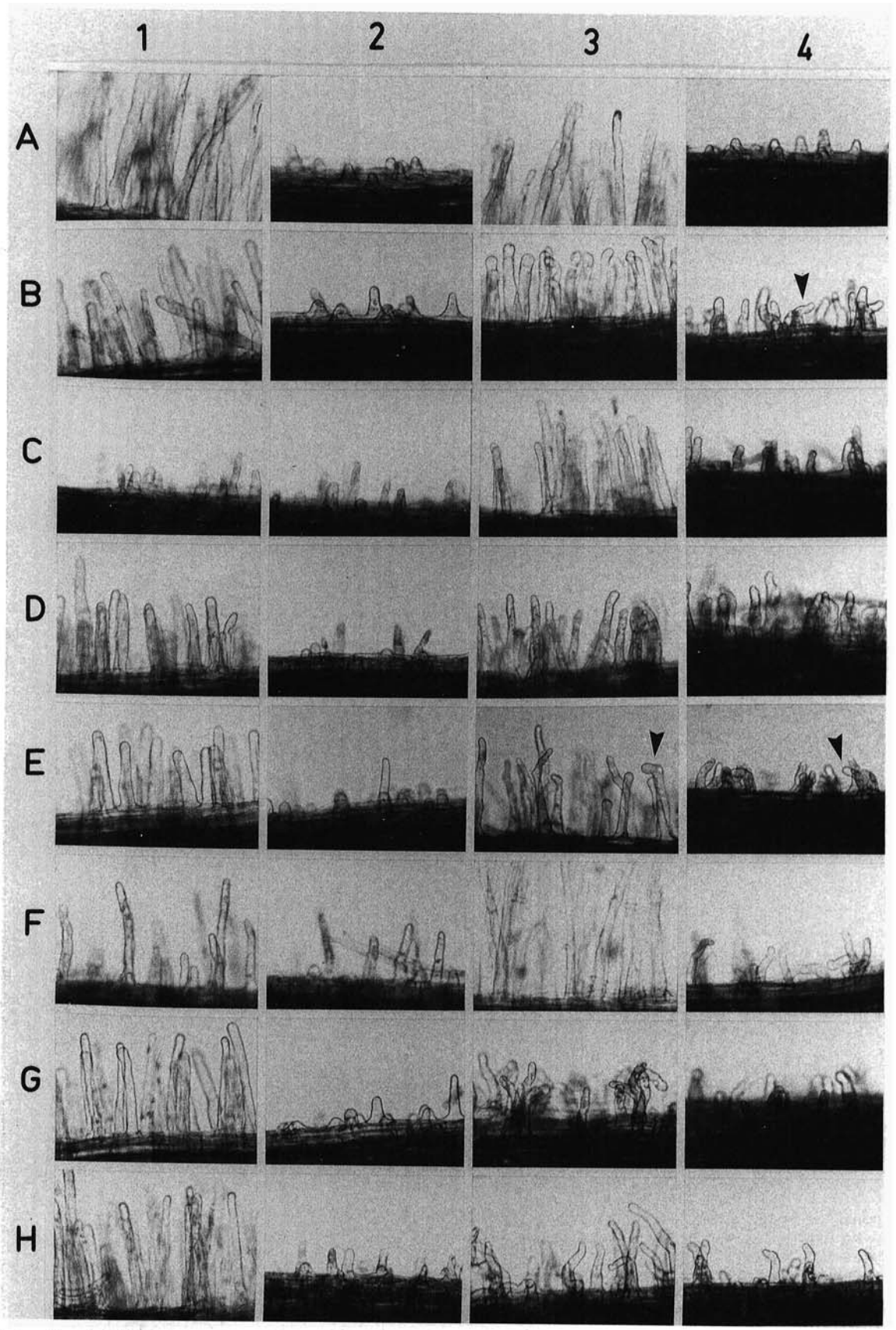


Fig. 1. Root hair curling of *Trifolium subterraneum*. Roots of dark-grown plants **(A)** 2 hr, **(B)** 4 hr, **(C)** 6 hr, **(D)** 8 hr, **(E)** 10 hr, **(F)** 12 hr, **(G)** 18 hr, and **(H)** 24 hr after inoculation with (1 and 2) F medium and (3 and 4) *Rhizobium leguminosarum* bv. *trifolii* strain ANU843 in the region of (1 and 3) nearly fully emerged hairs or (2 and 4) just emerging hairs behind the root tip.

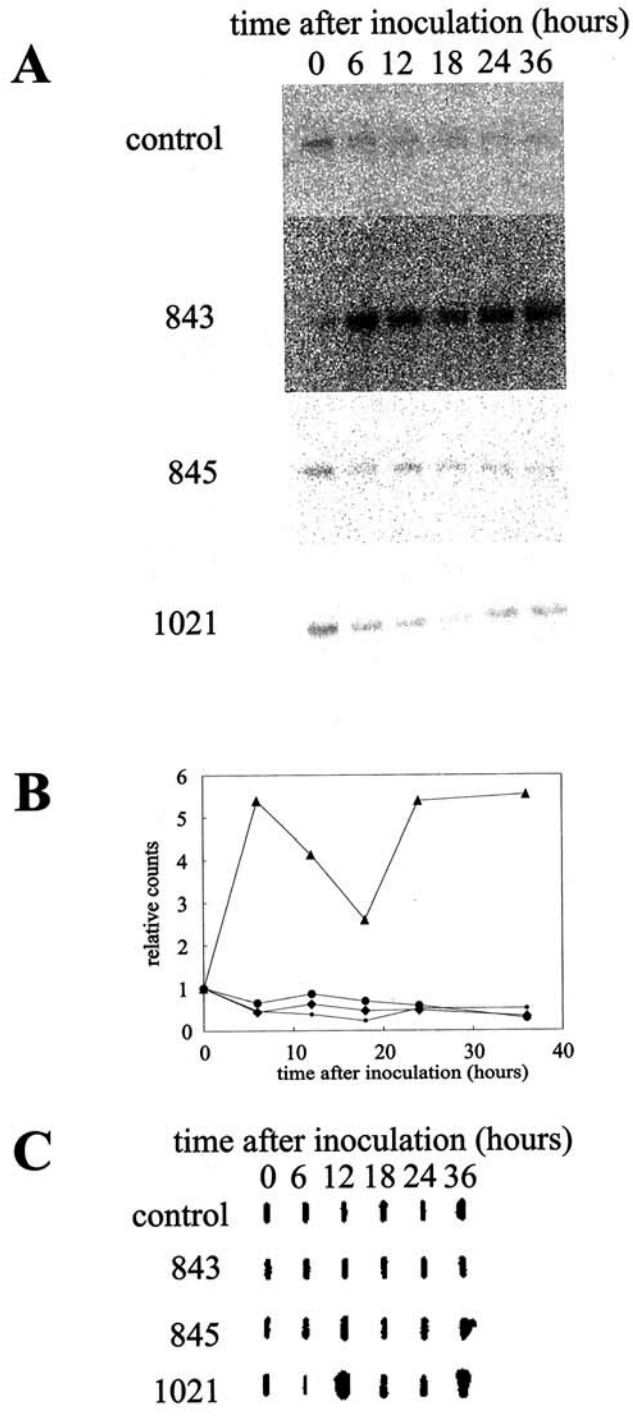


Fig. 2. Induction of total *CHS* expression following *Rhizobium* inoculation of *Trifolium subterraneum*. Blots of total root RNA samples from an uninoculated control and from plants inoculated with *R. leguminosarum* bv. *trifolii* strain ANU843, *R. l.* bv. *trifolii* strain ANU845, and *R. meliloti* strain 1021. Hybridization was analyzed with ImageQuant software of (**A**) northern blots (10 µg) probed with a *CHS2* second-exon fragment, (**B**) represented graphically (large circles = uninoculated control; triangles = strain ANU843; diamonds = strain ANU845; small circles = strain 1021), and compared to (**C**) the same samples (6 µg) hybridized to a constitutive marker, *rRNA*.

sion under the same conditions. One hypothesis for the induction of *CHS* expression was that the bacterial LOSs act as elicitor-type molecules (Spaink 1992; Recourt *et al.* 1992b), but there is as yet no direct evidence showing LOS induces *CHS*.

Earlier work from our laboratory isolated and characterized genomic clones of eight members of the *CHS* multigene family in the subterranean clover cultivar Karridale. This characterization established that *CHS* occurs in this cultivar in a

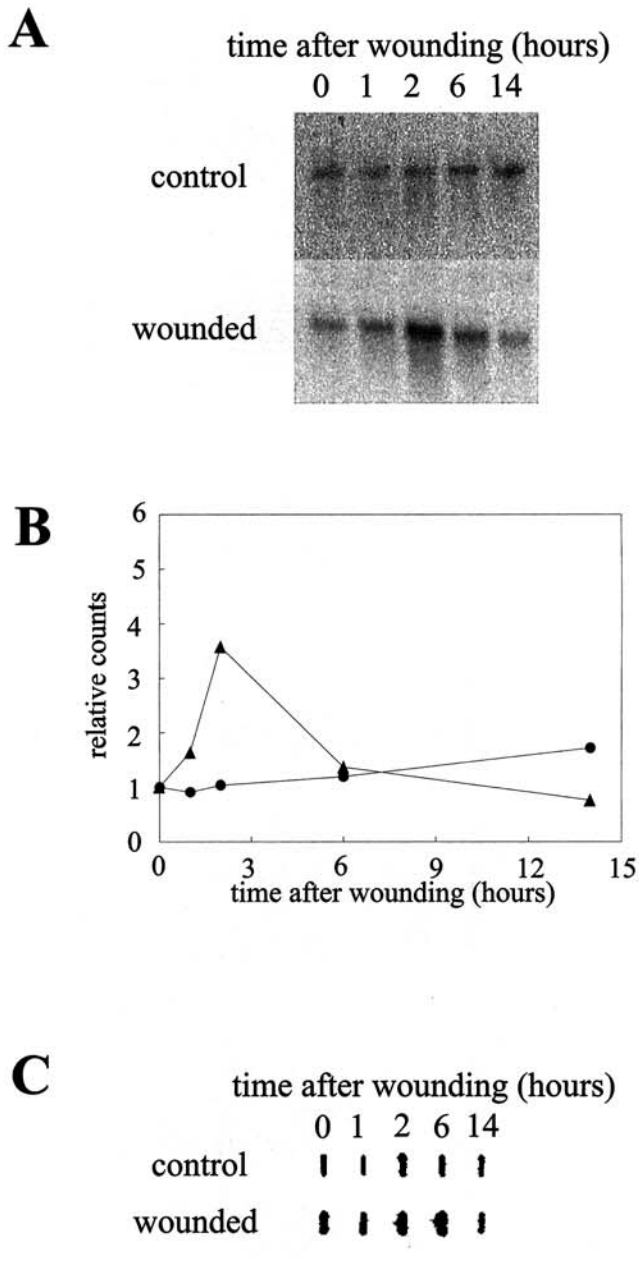


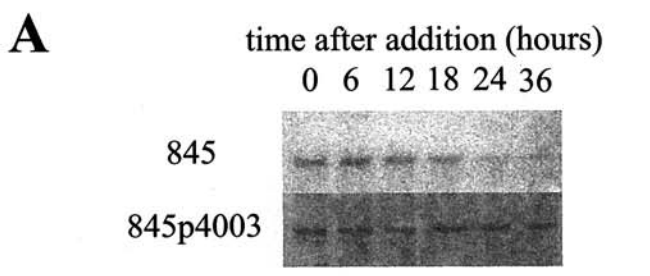
Fig. 3. Total *CHS* induction following wounding of *Trifolium subterraneum*. Blots of total root RNA from control and wounding treatments. Hybridization was analyzed with ImageQuant software of (**A**) northern blots (10 µg) probed with a *CHS2* second-exon fragment, (**B**) represented graphically (circles = control treatment; triangles = wounding treatment), and compared to (**C**) the same samples (6 µg) hybridized to a constitutive marker, *rRNA*.

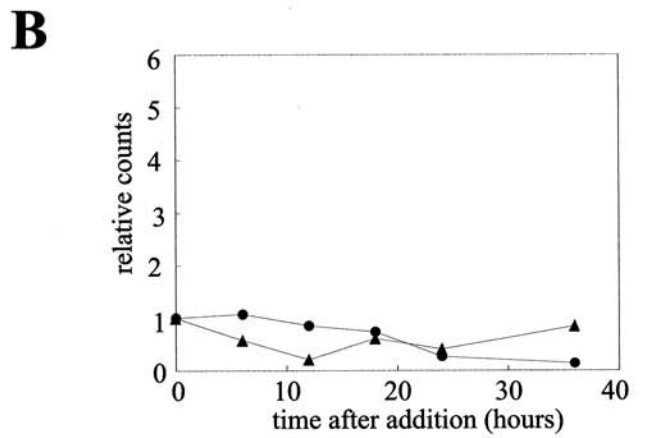
family of at least nine members in a minimum of three clusters (Arioli *et al.* 1994; J. Weinman, unpublished data). This paper presents an analysis of the expression of the *CHS* genes following the interaction between the plant host and both the symbiotic infective bacterium *R. l.* bv. *trifolii* strain ANU843 and the LOS produced by the products of the *nod* genes in this strain. The expression of *CHS* in the symbiotic interaction is then compared and contrasted with the expression of *CHS* following physical wounding.

RESULTS

Root hair curling.

To place the expression of *CHS* genes in a developmental context in our culture system, we conducted a time course of root hair deformation following inoculation with *R. l.* bv. *trifolii* strain ANU843. Root hair deformation in the susceptible root zone (Bhuvaneswari *et al.* 1981) is one of the earliest observable morphological changes in the plant in response to the invading bacterium. Root hair deformation induced by *R. l.* bv. *trifolii* strain ANU843 was readily apparent 10 hr after inoculation, but none occurred in uninoculated controls (Fig. 1). Root hair deformation was apparent at all subsequent sampling times. Experiments were therefore undertaken over the time frame of 36 hr following inoculation.





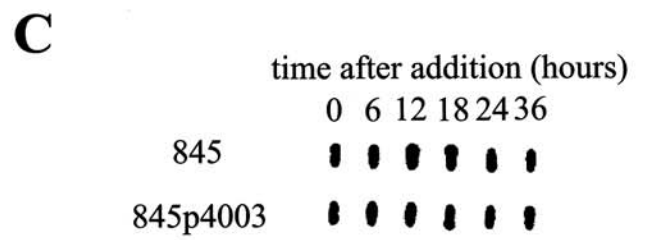


Fig. 4. Total *CHS* induction following lipooligosaccharide treatment. Blots of total root RNA from roots treated with lipooligosaccharide from *Rhizobium leguminosarum* bv. *trifolii* strain ANU845 and *R. l.* bv. *trifolii* strain ANU845(pR14003). Hybridization was analyzed with ImageQuant software of (**A**) northern blots (10 μg) probed with a *CHS2* second-exon fragment, (**B**) represented graphically (circles = ANU845; triangles = ANU845(pRI4003)), and compared to (**C**) the same samples (6 μg) hybridized to a constitutive marker, *rRNA*.

CHS induction following infection.

Total *CHS* expression in dark-grown seedlings was examined over 36 hr following *Rhizobium* infection (Fig. 2). Total RNA was probed with a second-exon fragment from *CHS2* that hybridized to all known subterranean clover *CHS* genes (Arioli *et al.* 1994). *CHS* was induced by the wild-type *R. l.* bv. *trifolii* strain ANU843 within 6 hr of inoculation. In contrast, inoculation with either the nonnodulating *R. l.* bv. *trifolii* strain ANU845 (pSym$^-$) or *R. meliloti* strain 1021 did not induce *CHS* gene expression above background levels. *R. meliloti* strain 1021 nodulates alfalfa but not clover and produces sulfated LOS molecules (Lerouge *et al.* 1990; Truchet *et al.* 1991), which differ from the LOS produced by *R. l.* bv. *trifolii* strain ANU843 (McKay and Djordjevic, 1993).

CHS induction following wounding.

CHS induction following physical wounding was examined to contrast with the response apparent following *Rhizobium* inoculation and to determine the specificity of *CHS* gene induction. Physical wounding was chosen to test the hypothesis that the plant's responses following wounding share some similarities with its responses during *Rhizobium* infection (Brewin 1991). Further, analysis of another inducing treatment acts as a measure of difference for the induction of symbiosis-specific *CHS* genes. Single 5-mm incisions were made in the roots of newly germinated plants, and RNA was extracted at various times following treatment. The conditions were identical to those under which the induction of *CHS* following *Rhizobium* infection was examined. *CHS* expression was rapidly induced within 2 hr after wounding (Fig. 3). The *CHS* mRNA levels declined to background levels by

Table 1. Survey of *CHS* genes expressed[a]

Clover *CHS* gene	Hours after inoculation				Hours after wounding	
	0	6	24	36	2	12
1	...	1	...	...	...	...
2	1	...	...	...	...	...
3	1	...	...	2	...	...
4	...	...	...	...	...	...
5	...	5	5	2	10	2
6	...	1	...	...	...	1
7	...	...	...	...	...	...
8	...	...	...	...	...	...
9	1	...	...	...	...	...

[a] *CHS* mRNA from various time points after inoculation with *Rhizobium leguminosarum* bv. *trifolii* strain ANU843 and wounding treatments was copied into cDNA, amplified by polymerase chain reaction, and then cloned and sequenced. The numbers of clones sequenced corresponding to a particular *CHS* gene are listed according to the time point and treatment pool from which the clone was isolated.

6 hr, in contrast to those of plants inoculated with *Rhizobium* (Fig. 2).

LOS induction of *CHS*.

Isolated LOS applied to roots has been shown to cause root hair curling, cortical cell division, and expression of some of the early nodulin genes (Truchet *et al.* 1991). A crude preparation containing *R. l.* bv. *trifolii* LOS molecules was added to the plate culture medium in sufficient quantities to cause root hair curling after 24 hr in plants grown under the same conditions used to examine the induction of *CHS* after *Rhizobium* inoculation (data not shown). This LOS preparation did not induce *CHS* over the 36-hr assay period (Fig. 4).

Identification of expressed *CHS* copies.

Expressed *CHS* genes were cloned following cDNA synthesis and polymerase chain reaction (PCR) amplification and sequenced to survey their identity when compared to existing genomic sequences (Arioli *et al.* 1994; J. Weinman, unpublished data). The PCR fragment lengths varied between 600 and 700 bp, depending upon which *CHS* gene was being amplified. The cDNA was made from total root RNA isolated from the time points up to 36 hr after inoculation with *R. l.* bv. *trifolii* strain ANU843 and up to 12 hr after wounding. A total of 32 clones from the various treatment time points were examined (Table 1). Sequences identical to the previously designated *CHS2* and *CHS3* were found in uninoculated 0-hr controls in addition to a new *CHS* sequence (designated *CHS9*). *CHS1, CHS5,* and *CHS6* were found in samples 6 hr after inoculation with *R. l.* bv. *trifolii* strain ANU843, and *CHS5* and *CHS6* were found in similar samples 24 and 36 hr after inoculation. *CHS5* and *CHS6* were also found in samples 2 hr after the wounding treatment. It is possible that the C-terminal sequence designated *CHS9* may be that of the previously designated *CHS4* or *CHS8* gene, as the C-terminal sequences of these genes have not been characterized. Sequence comparisons of each of the expressed *CHS* species confirmed the sequence and identity of expressed *CHS* genes and showed the position of the addition of the polyA tail (Fig. 5). These findings indicate that *CHS5* was the predominant *CHS* species in the mRNA pools in our system, in both the inoculation treatments and the wounding treatments, although *CHS1* and *CHS6* were also present.

Sequence comparisons.

A comparison of the promoter regions could identify a (novel) consensus region responsible for copy-specific induction of *CHS*. The promoter regions of *CHS5* and *CHS6* (J. Weinman, unpublished data) were compared to the promoter of the soybean *Gmchs7,* which is the *CHS* gene known to be expressed in the *Bradyrhizobium*-soybean interaction (Estabrook and Sengupta-Gopalan 1991; Akada *et al.* 1993). This comparison revealed four regions of considerable homology between the gene promoters, which we have designated regions I–IV (Fig. 6A and B). Regions I–III can be accounted for by conserved regions established in earlier analysis (Arioli *et al.* 1994; J. Weinman, unpublished data), which are also present in the promoters of *CHS1, CHS2,* and *CHS3* and partially in that of *CHS4* (data not shown). Region IV occurs only in *CHS5* and *CHS6* of the characterized subterranean clover *CHS* genes and has two conserved features, which are also evident in the soybean *Gmchs7* (Fig. 6C). The first is the sequence AA(N)ATAT$(N)_2$CCAAT(N)ATT, which is the same in all three promoters, and the second is an eight-base pair direct repeat, ATAAAATA, which is present only in the promoters of *CHS5* and *Gmchs7*.

DISCUSSION

Hirsch (1992) suggested that the induction of the plant *CHS* gene(s) in the *Rhizobium*-legume interaction could be

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1350                                                                    1429
 1 TAAGA---T---GCTTAGTTATTTTATTTTCATGTATCA-TTTTTAAATTTGCTTGATTTTTATGTAAACATG---AAAA
 2 TGAGA---G---GCTTACTTATTTTATTTTCATGTATCA-TTTTTAAATTTGCTTGATTTTTATGTAACCGTG---AAAA
 3 TGAGA---T-GCGCTTCATTATTTTATTTTCATGTATTA-TTTTTAAATTTGCTTGATTTTTATGTAAGCATG---GAAA
 9 TGAGA-TGT-TTGTTTGTTTGTTTGTTTTCCATGTATTGCTTTTTAAATTTGTCTGATTTTTATGTAAG-ATG---AAAA
 6 TGA-TTTGTG-------TGATTGTAATTTATTT-TAATGTATTACCT---------TCAATCTTGCATGAATTTCCATTA
 5 TGAGATTGAGAGACTTTTGATTGTTTTTTAATTGTATTGTATTACTTTACTTTTAATTAATCTTGCTTGAACTTCCATTT

1430                                                                    1509
 1 GCTCATCTAGA----GTTAAACATGCA-AAATCGTGTTAACATATTAAAGACGACGAAGAATATTGTAACCAGTGAATTA
 2 ACTCATCTAGA----GTTCAACATGGA-CAATCATATTAAAATAATATTTGATT
 3 ACTCATCTAGA----GTTCAACATGGA-CAATCATATTAAAATAATATTCGATTAACTTTGTATTCT
 9 ATTCATCTTCA----TTTCAACATGTACCCATCATATTAAATT
 6 AAAAATAAATATGGAGTTCAATTTGTACCATTGATGTTGAAATATCTTGTATT---CTAGCATTTGTTATTTGTTTAAAT
 5 AAGAATAAATATGGAGTTCAATCTGTACCATCAATGTTAAAATAATATATCGT---TAATAGCTTTT

1510                                        1550
 1 CTATATTTTAAAATGTAATAATATAATGTGCTCTTATAACT
 2
 3
 9
 6 AAATATAAATACTGTC
 5
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Fig. 5. *CHS* cDNA sequence data. *CHS* mRNA was copied into cDNA from various treatment time points, amplified by polymerase chain reaction, and then cloned and sequenced. The sequence was then compared to previously sequenced genomic *CHS* data to determine the identity of the cloned sequence. The cDNA sequence data are presented from the stop codon to the site of the addition of the polyA tail for cDNAs from *CHS1* (1), *CHS2* (2), *CHS3* (3), *CHS5* (5), *CHS6* (6), and *CHS9* (9). The alignment program used was an iterative multiple alignment written by D. K. Smith of the Research School of Biological Sciences Computer Unit, Australian National University, using a unitary nucleotide weight matrix, *k*-tuple size = 2, window = 20, gap penalty = 10, gap penalty per unit length = 8, and a weighted leading edge.

involved in the induction of the *Rhizobium nod* genes (LOS and Ini phenotype) as a defense response to the invading *Rhizobium* (pathogen) or in the regulation of root mitotic activity necessary for nodule development. The separation of these possible events is a complex problem and may only be achieved by dissecting the time of *CHS* gene expression for each of these events in a well-characterized plant-*Rhizobium* interaction. The experiments described in this paper set out to define *CHS* gene expression in the context of well-defined morphological events in the well-characterized interaction between subterranean clover and *R. l.* bv. *trifolii*.

Root hair curling in our system (Fig. 1) indicates that the early events in the interaction take place within 10 hr following inoculation. From previous data it is possible that the initiation of events leading to cortical cell division may have occurred and infection thread formation begun within this time (Djordjevic and Weinman 1991). We therefore designed our experiments to examine the induction of the *CHS* gene(s) by the bacterium and isolated LOS within this time frame. To avoid the known effects of light on *CHS* induction, the plants were incubated in the dark (Koes *et al.* 1989). To counter the possible masking of *CHS* induction by the emergence of lateral roots, newly germinated seedlings were used (Yang *et al.* 1992). In our system lateral roots would not have been initiated until well after the last time point (36 hr). The expression of *CHS* following inoculation was then contrasted with a well-characterized *CHS* induction response (physical wounding) in order to highlight and contrast the responses to *Rhizobium* infection and LOS application.

In our system *CHS* expression was induced within 6 hr after inoculation with the wild-type *R. l.* bv. *trifolii* strain ANU843, while the pSym$^-$ *R. l.* bv. *trifolii* strain ANU845 and *R. meliloti* 1021 (having a different host range) maintained background levels of expression (Fig. 2). The reports of Estabrook and Sengupta-Gopalan (1991) on *Glycine max* and Recourt *et al.* (1992b) on *Vicia sativa* are consistent with the induction of *CHS* by the wild-type strain ANU843, although our results show much faster rates of induction. This rapid induction is significant because the kinetics are similar to those found during incompatible infections with an avirulent bacterium, for example, *Pseudomonas syringae* on bean (Jakobek *et al.* 1993). The absence of induction by the pSym$^-$ strain ANU845 is consistent with the results reported by Recourt *et al.* (1992b). However, we did not find induction of *CHS* by a *Rhizobium* species with a different host range (*R. meliloti* 1021), whereas Estabrook and Sengupta-Gopalan (1991) found *R. meliloti* was able to induce expression of the *CHS* gene family in soybean. It is possible that *R. meliloti* 1021 did not respond to the exudate inducers secreted by subterranean clovers and was therefore unable to synthesize LOS, curl root hairs, or infect roots. Nevertheless we have clearly shown that *CHS* induction occurs before any recognizable *Rhizobium* penetration event, and therefore *CHS* induction is a preinfection event in subterranean clovers and not solely a postinfection phenomenon, which Estabrook and Sengupta-Gopalan concluded it was (1991).

One possible explanation for the observed induction of *CHS* expression by the wild-type strain ANU843, but not by

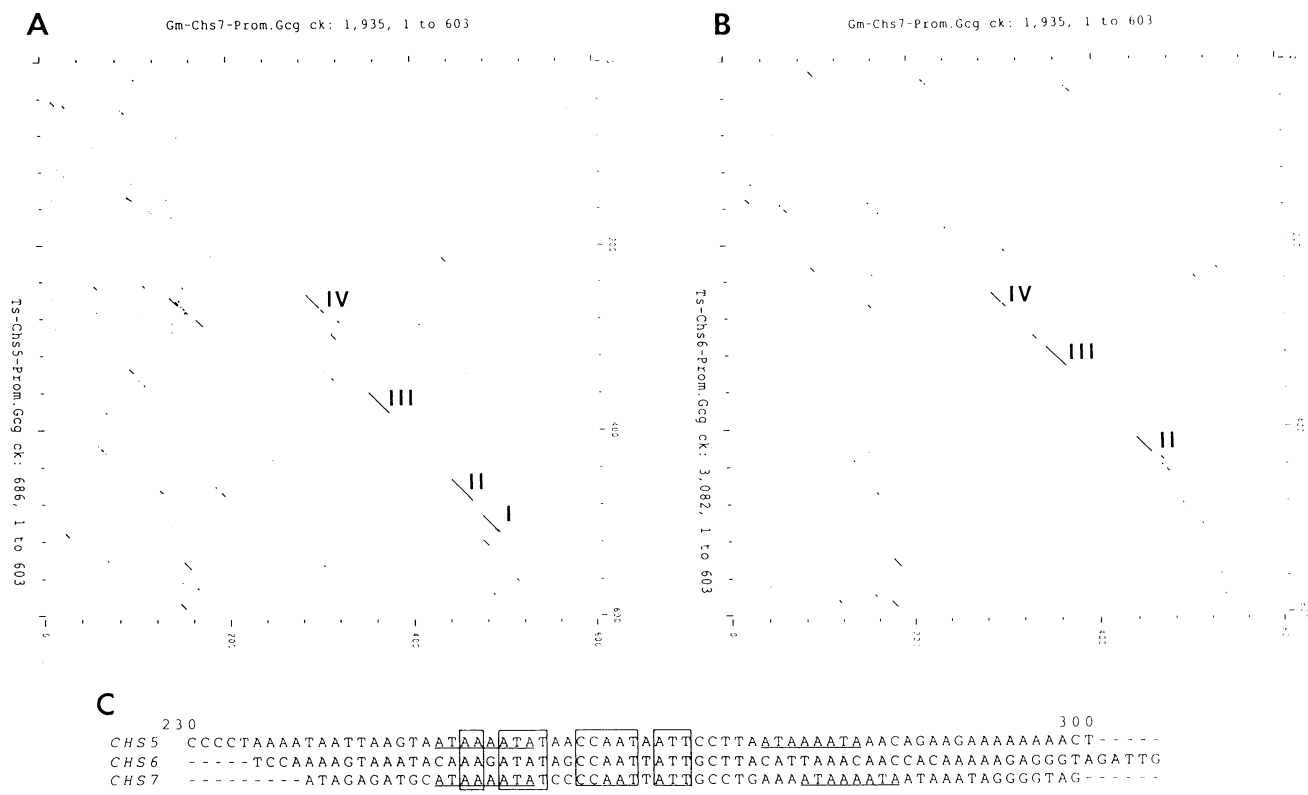

Fig. 6. Comparison of the subterranean clover *CHS5* and *CHS6* promoters and the soybean *Gmchs7* promoter. DotPlot alignments (GCG package) (Devereaux *et al.* 1984) of the *Gmchs7* promoter (horizontal axis) against (vertical axis) (**A**) *CHS5* and (**B**) *CHS6* are presented for the 600 nucleotides upstream of the start codons (601–603). We have designated the areas of homology regions I–IV. Alignment of the sequences corresponding to region IV (**C**) shows conserved sequences (boxed) and direct repeats (underlined).

either the pSym⁻ strain ANU845 or *R. meliloti* 1021, is the LOS molecules excreted by the wild-type strain (Spaink 1992; Recourt *et al.* 1992b). To test this possibility, isolated LOS was applied in sufficient amounts to cause root hair curling within the assay period. The isolated LOS did not induce *CHS* expression (Fig. 4). Therefore, LOS by itself does not account for *CHS* induction following inoculation with the wild type. Junghans *et al.* (1993) treated alfalfa roots with the filtered supernatant from sonicated *R. meliloti* cells as a biotic elicitor and found a rapid induction of a subset of *CHS* transcripts. It is not certain whether the *R. meliloti nod* genes were induced in these experiments. However, the failure of LOS to induce *CHS* in our system and the *Rhizobium* as abiotic elicitor for the induction of *CHS* in the system of Junghans *et al.* (1993) (whether *nod* genes were induced or not) suggests the *Rhizobium* has the potential to be perceived by the host plant as a pathogen (Djordjevic *et al.* 1988). If the *nod* genes (and hence LOS) were absent in the system of Junghans *et al.* (1993), it is then conceivable the LOS may have a role in limiting the plant's responses to elicitation (and infection).

One of the postulated functions of the observed *CHS* expression is the synthesis of new flavonoids to act as auxin regulators in cell division and nodule formation (Hirsch 1992). Flavonoids have been shown to function as auxin transport inhibitors (Jacobs and Rubery 1988), and auxin transport inhibitors have been shown to induce nodule-like structures on alfalfa roots (Hirsch *et al.* 1989). To function in this manner, the flavonoids that are produced as a result of the rapidly induced *CHS* expression would need to be produced in the very early stages of infection, to coincide with cortical cell division in the very early preinfection events. However, in our system the very early expression of *CHS* (Fig. 2) was separated from the early preinfection events by applying isolated LOS (Fig. 4), which causes root hair curling and cortical cell division (Truchet *et al.* 1991). Since LOS alone does not induce *CHS* expression, the flavonoids postulated to be associated with root hair curling and cortical cell divisions cannot be the result of the *Rhizobium*-induced *CHS* expression. In the early preinfection events the mobilization of existing pools of flavonoids in the plant could account for auxin transport inhibition (Recourt *et al.* 1992b) in a manner similar to that found in pollen germination (Mo *et al.* 1992). However, the products of later *Rhizobium*-induced *CHS* expression may have a role in the later stages of nodule development (Yang *et al.* 1992; Grosskopf *et al.* 1993), which is also suggested by the presence of *CHS* transcripts in meristematic tissue (Yang *et al.* 1992). This result also suggests that the signal transduction pathway for the induction of early nodulin genes, which are induced by LOS (Horvath *et al.* 1993), is different from that responsible for the induction of *CHS*.

The contrast between infection and wounding was chosen because of the hypothesis that the plant's responses to *Rhizobium* infection and to wounding share some similarities (Brewin 1991). Our results show some similarities. There is a similar rapid induction of *CHS* gene expression following *Rhizobium* infection and wounding (Figs. 2 and 3) and a predominance of the same *CHS* gene copy in both mRNA pools (Table 1). The absence of *CHS* induction following LOS addition (Fig. 4) and the correlation of similar *CHS* induction following wounding and *Rhizobium* inoculation suggests that some physical "perturbation" of the plant cell wall or membrane may be required for the induction of *CHS*. Such a "perturbation signal" occurs with physical wounding and at the site of infection by a wild-type *Rhizobium* degrading the cell wall soon after attachment (for example, by pectinases) (Mateos *et al.* 1992). The consequence of this "perturbation" is the activation of cell wall repair mechanisms and the formation of the infection thread (Robertson and Lyttleton 1982) with a mobilization of vesicular bodies to the site of perturbation in a manner similar to host-pathogen interactions (Ridge and Rolfe 1985). The differences in outcome between the wound response and the infection response leaves open the possibility that *Rhizobium* is in some way modifying the plant's defense response to enable at least some successful nodule-forming infections (Rolfe *et al.* 1989).

Jakobek *et al.* (1993) showed that a generalized defense response (including the induction of *CHS* expression) could be separated from a hypersensitive response and concluded that there are several levels to the defense response of plants to invading pathogens. It is conceivable that infection by wild-type *Rhizobium* induces one of the levels of the plant defense response and then limits the later levels. Djordjevic *et al.* (1988), using a nonnodulating Tn*5*-induced adenine auxotroph of NGR234, showed that this strain could curl root hairs and initiate an infection thread, but the root hairs and adjacent epidermal cells rapidly collapsed within 24 hr after inoculation. This localized response closely resembles a hypersensitive response. This observation suggests that a wild-type *Rhizobium* induces the plant's generalized defense response and then actively limits the subsequent distinct hypersensitive response to enable further penetration and nodule formation (Djordjevic *et al.* 1987). This *CHS* induction may also have a function in the abortion of infection threads, leading to the plant's limitation of infection reported by Vasse *et al.* (1993).

A comparison of the *CHS5* and *CHS6* promoters with the promoter of the *CHS* induced by *B. japonicum* in soybean (*Gmchs7*) (Estabrook and Sengupta-Gopalan 1991; Akada *et al.* 1993) revealed sequences conserved between these promoters (Fig. 6). Regions I–III are common to all of the characterized clover *CHS* gene promoters (J. Weinman, unpublished data) and contain sequences with possible regulatory roles. These include a TATA box in region I, a G-box (CACGTG) sequence (Giuliano *et al.* 1988) in region II, and a sequence in region III (CCACCAAACTC) homologous to sequences believed to have a role in UV and elicitor induction (Lois *et al.* 1989). The region IV element is found only in *CHS5* and *CHS6* of the characterized clover *CHS* genes. This suggests that region IV (possibly in combination with the other elements) could be responsible for the induction of *CHS* by *Rhizobium* and wounding. Further analysis of these pro-

Table 2. *Rhizobium* strains used in this study

Strain	Description
Rhizobium leguminosarum bv. *trifolii*	
ANU843	Wild type (clover); pSym⁺, Nod⁺ on clover
ANU845	pSym⁻, Nod⁻ on clover
ANU854(pRI4003)	Nod⁺ on clover
R. meliloti 1021	Wild type (alfalfa); pSym⁺, Nod⁺ on alfalfa

moters is necessary, particularly to assess the possible regulatory functions of regions II–IV in the plant.

Analysis of the induction of *CHS* in the very early stages of infection will be necessary to elucidate the kinetics of particular *CHS* genes expressed in the *Rhizobium*-plant interaction. The results presented here suggest that the early *CHS* expression following *Rhizobium* inoculation has similarities with the plant's response to wounding. Experiments are under way to identify quantitatively the expression of individual *CHS* genes in our system and to further dissect the very early interaction events leading to *CHS* expression.

MATERIALS AND METHODS

Bacterial culture and plant inoculation.

The wild-type *R. l.* bv. *trifolii* strain ANU843, the *R. l.* bv. *trifolii* strain ANU845 cured of pSym, and the different host range *R. meliloti* (Table 2) were grown up to OD_{600} 0.6 in BIII medium (Hollingsworth *et al.* 1984) at 28° C and then centrifuged at 5,000 rpm for 5 min in a Sorvall RC5D centrifuge, the supernatant was removed, and the pellet was resuspended in liquid F medium (Rolfe *et al.* 1980). These bacteria (approximately 2×10^8 cells per milliliter in 20 ml) were allowed to recover for 1 hr and then flooded onto germinated seedlings (see below) on F medium plates (Rolfe *et al.* 1980) for 1 hr at 22° C in the dark. The bacterial suspension was then poured off, and the plates were placed in the dark at 22° C for the duration of the experiment.

Plant germination and growth.

Certified seeds of *T. subterraneum* cv. Karridale were surface-sterilized in 3.25% hypochlorite for 5 min and washed four times in sterile distilled water. Seeds were placed on F medium plates in the dark at 4° C for 24 hr (Rolfe *et al.* 1980) to synchronize germination and then were germinated at 22° C for 24 hr in the dark. Selected germinated seedlings were placed on F medium plates. Following *Rhizobium* inoculation or wounding, lids were placed over the plates, which were wrapped in foil and placed in the dark in a growth cabinet at 22° C. Incident light was excluded as much as possible to avoid the interference of light-induced *CHS* expression in the subsequent analysis.

Time course of root hair curling.

Germinated plants were inoculated with *R. l.* bv. *trifolii* strain ANU843 as described above or with F medium alone (control) and then examined and photographed under a microscope at various times after inoculation. Roots were soaked for 5 min in a 0.004% solution of methylene blue prior to examination to aid visualization of the root hairs.

RNA analysis.

For RNA analysis 40 germinated seedlings were inoculated, treated with LOS, or physically wounded with a scalpel, leaving a 5-mm-long incision in the root tissues. At the time of sampling the roots were excised and placed in liquid nitrogen and then stored at –70° C until the extraction of the RNA. Total RNA was isolated from roots by hot phenol extraction and LiCl precipitation (Ausubel *et al.* 1987). The total RNA was quantified spectrophotometrically and checked for degradation on a 1% formaldehyde agarose gel. For northern analysis total RNA was separated in a 1% formaldehyde agarose gel, alkaline-blotted onto Hybond N+ (Amersham International, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions and then probed with a randomly primed [α-^{32}P]dATP-labeled *Pst*I-*Xba*I fragment from the second exon of *CHS2* (Arioli *et al.* 1994). Membranes were prehybridized for 1 hr at 42° C in 50% formamide, 5× SSPE (20× stock: 3.6 M NaCl, 0.2 M sodium phosphate, and 0.02 M EDTA, pH 7.7), 5× Denhardt's solution (100× stock: 2% bovine serum albumin, 2% Ficoll, and 2% polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate (SDS) and then hybridized for 12–16 hr at 42° C in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 0.5 mg of salmon sperm DNA. For slot blots (Hybri-slot manifold, Bethesda Research Laboratories, Gaithersburg, MD) total RNA was blotted onto Hybond N+ according to the manufacturer's instructions and then probed with a randomly primed [α-^{32}P]dATP-labeled *Bam*HI fragment containing the wheat ribosomal RNA gene (Appels and Dvorak 1982). Following hybridization membranes were washed for 20 min in 2× SSPE at 42° C and then twice for 20 min in 0.2× SSPE and 0.1% SDS at 42° C. Hybridization was analyzed with a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Isolation of bacterial signal molecules (LOS).

R. l. bv. *trifolii* strain ANU845(pRI4003) (Innes *et al.* 1988), a strain with the multicopy plasmid carrying the *nod* gene region, and a strain without this plasmid (*R. l.* bv. *trifolii* strain ANU845) were grown in BIII medium (Hollingsworth *et al.* 1984), and Nod metabolites were extracted from the entire culture in water-saturated butanol as described by McKay and Djordjevic (1993). A 100-µl sample of the extract was placed on plates and allowed to air-dry before molten F medium (55° C) (Rolfe *et al.* 1980) was added and allowed to set. Ten selected germinated plants were placed on each of four plates per treatment for each time point, and the plants were cultured as described above. A 100-µl sample of dried extract was sufficient to cause root hair curling on the plates within 24 hr.

cDNA synthesis and cloning.

cDNA was synthesized from 8 µg of total RNA by SuperScript Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD); a polyT(17) primer was used, incorporating a *Bam*HI site and a T3 primer sequence (5′-ATTAACCCTCACTAAAGGATCC$(T)_{17}$-3′) in a total volume of 25 µl according to the manufacturer's instructions. Following the cDNA synthesis, 175 µl of H_2O was added. Aliquots (10 µl) of this dilution were then used directly in a 30-cycle PCR (1 min at 95° C, 2 min at 52° C, and 3 min at 72° C, with a final extension of 15 min at 72° C) with 100 pmol CHS-BAG-2 primer (5′-TCGAATTCCATTGTTGGTTCTGATCC-3′) and 100 pmol Long-T3 primer (5′-GCTCGGAATTAACCCTCACTAAAGG-3′) in a reaction tube containing 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.1 mM tetramethylammonium chloride, and 2 U of *Taq* DNA polymerase and the supplied reaction buffer. The CHS-BAG-2 sequence is homologous to all the known *CHS* genes of the subterranean clover cultivar Karridale (J. Weinman, unpublished data). The products were cloned according to standard protocols (Ausubel *et al.* 1987) into pBluescript SK/KS(+) vectors (Stratagene, La Jolla, CA).

Sequencing and analysis.

Clones were sequenced with the dye primer PRISM cycle sequencing kit (Applied Biosystems, Forster City, CA) and analyzed with an Applied Biosystems 373A instrument. Sequence data were analyzed by the SEQ suite of sequencing programs available at the Research School of Biological Sciences, Australian National University. This includes the GCG package (Devereaux *et al.* 1984).

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LITERATURE CITED

Akada, S., Kung, S. and Dube, S. 1993. Nucleotide sequence and putative regulatory elements of a nodule-development-specific member of the soybean (*Glycine max*) chalcone synthase multigene family, *Gmchs7*. Plant Physiol. 102:321-323.

Appels, R., and Dvorak, J. 1982. The wheat ribosomal RNA spacer region: Its structure and variation in populations and among species. Theor. Appl. Genet. 63:337-348.

Arioli, T., Howles, P. A., Weinman, J. J., and Rolfe, B. G. 1994. In *Trifolium subterraneum,* chalcone synthase is encoded by a multigene family. Gene 138:79-86.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., and Smith, J. A. 1987. Current Protocols in Molecular Biology. John Wiley and Sons, New York.

Bisseling, T., Frassen, H., Heidstra, R., Horvath, B., Katinakis, P., Moerman, M., Spaink, H., van Bussel, T., and Vijn, I. 1992. *Rhizobium nod* metabolites and early nodulin gene expression. Advances in Molecular Genetics of Plant Microbe Interactions. E. W. Nester and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Bhuvaneswari, T . V., Bhagwat, A. A., and Bauer, W. D. 1981. Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. Plant Physiol. 68:1144-1149.

Brewin, N. J. 1991. Development of the legume root nodule. Annu. Rev. Cell Biol. 7:191-226.

Devereaux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12: 387-395.

Dixon, R. A., Dey, P. M., and Lamb, C. J. 1983. Phytoalexins: Enzymology and molecular biology. Adv. Enzymol. 55:1-136.

Dixon, R. A., and Lamb, C. J. 1990. Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Physiol. Plant Mol. Biol. 41:339-367.

Djordjevic, M. A., Gabriel, D. W., and Rolfe, B. G. 1987. *Rhizobium*—The refined parasite of legumes. Annu. Rev. Phytopathol. 25:145-168.

Djordjevic, M. A., and Weinman, J. J. 1991. Factors determining host recognition in the clover-*Rhizobium* symbiosis. Aust. J. Plant Physiol. 18:543-557.

Djordjevic, S. P., Ridge, R. W., Chen, H., Redmond, J. W., Batley, M., and Rolfe, B. G. 1988. Induction of pathogenic-like responses in the legume *Macroptilium atropurpureum* by a transposon-induced mutant of fast-growing, broad-host-range *Rhizobium* NGR234. J. Bacteriol. 170:1848-1857.

Estabrook, E. M., and Sengupta-Gopalan, C. 1991. Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development. Plant Cell 3:299-308.

Giuliano, G., Pichersky, E., Malik, V. S., Timko, M. P., Scoinick, P. A., and Cashmore, A. R. 1988. An evolutionary conserved protein binding sequence upstream of a plant light-regulated gene. Proc. Natl. Acad. Sci. USA 85:7089-7093.

Grosskopf, E., Cam Ha, D. T., Wingender, R., Rohrig, H., Szecsi, J., Kondorosi, E., Schell, J., and Kondorosi, A. 1993. Enhanced levels of chalcone synthase in alfalfa nodules induced by a Fix$^-$ mutant of *Rhizobium meliloti.* Mol. Plant-Microbe Interact. 6:173-181.

Hahlbrock, K., and Scheel, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:347-349.

Hirsch, A. M. 1992. Developmental biology of legume nodulation. New Phytol. 122:211-237.

Hirsch, A. M., Bhuvaneswari, T. V., Torrey, J., and Bisseling, T. 1989. Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA 86:1244-1248.

Hollingsworth, R. L., Abe, M., Sherwood, J. E., and Dazzo, F. B. 1984. Bacteriophage-induced acidic heteropolysaccharide lyases that convert the acidic heteropolysaccharides of *Rhizobium trifolii* into oligosaccharide units. J. Bacteriol. 160:510-516.

Horvath, B., Heidstra, R., Lados, M., Moerman, M., Spaink, H. P., Prome, J.-C., van Kammen, A., and Bisseling, T. 1993. Lipo-oligosaccharides of *Rhizobium* induce infection-related early nodulin gene expression in pea root hairs. Plant J. 4:727-733.

Innes, R., Hirose, M., and Kuempel, P. 1988. Induction of nitrogen-fixing nodules on clover requires only 32 kilobase pairs of DNA from *Rhizobium trifolii* symbiosis plasmid. J. Bacteriol. 170:3793-3802.

Jacobs, M., and Rubery, P. H. 1988. Naturally occurring auxin transport regulators. Science 241:346-349.

Jakobek, J. L., Smith, J. A., and Lindgren, P. B. 1993 Suppression of bean defence responses by *Pseudomonas syringae.* Plant Cell 5:57-63.

Junghans, H., Dalkin, K., and Dixon, R. A. 1993. Stress responses in alfalfa (*Medicago sativa* L.). 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol. Biol. 22:239-252.

Koes, R. E., Spelt, C. E., and Mol, J. N. M. 1989. The chalcone synthase multigene family of *Petunia hybrida* (V30): Differential, light-regulated expression during flower development and UV light induction. Plant Mol. Biol. 12:213-225.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C., and Denarie, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature. 344:781-784.

Lois, R., Dietrich, A., Hahlbrock, K., and Schulz, W. 1989. A phenylalanine ammonia-lyase gene from parsley: Structure, regulation and identification of elicitor and light responsive *cis*-acting elements. EMBO J. 8:1641-1648.

Mateos, P. F., Jimenez-Zurdo, J. I., Chen, J., Squartini, A. S., Haack, S. K., Martinez-Molina, E., Hubbel, D. H., and Dazzo, F. B. 1992. Cell-associated pectinolytic and cellulolytic enzymes in *Rhizobium leguminosarum* biovar *trifolii.* Appl. Environ. Microbiol. 58:1816-1822.

McKay, I. A., and Djordjevic, M. A. 1993. Production and excretion of nod metabolites by *Rhizobium leguminosarum* bv. *trifolii* are disrupted by the same environmental factors that reduce nodulation in the field. Appl. Environ. Microbiol. 59:3385-3392.

Mo, Y., Nagel, C., and Taylor, L. P. 1992. Biochemical complementation of chalcone synthase mutant defines a role for flavonols in functional pollen. Proc. Natl. Acad. Sci. USA 89:7213-7217.

Recourt, K., Schripsema, J., Kijne, J. W., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1992a. Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* bv. *viciae* results in the release of *nod* gene activating flavanones and chalcones. Plant Mol. Biol. 16:841-852.

Recourt, K., van Tunen, A. J., Mur, L. A., van Brussel, A. A. N., Lugtenberg, B. J. J., and Kijne, J. W. 1992b. Activation of flavonoid biosynthesis in roots of *Vicia sativa* subsp. *nigra* plants by inoculation with *Rhizobium leguminosarum* bv. *viciae.* Plant Mol. Biol. 19:411-420.

Ridge, R. W., and Rolfe, B. G. 1985. *Rhizobium* sp. degradation of legume root hair cell wall at the site of infection thread origin. Appl. Environ. Microbiol. 50:717-720.

Robertson, J. G., and Lyttleton, P. 1982. Coated and smooth vesicles in the biogenesis of cell walls, plasma membranes, infection threads and peribacteroid membranes in root hairs and nodules of white clovers. J. Cell Sci. 58:63-78.

Rolfe, B. G., Gresshoff, P. M., and Shine, J. 1980 Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci. Lett. 19: 277-284

Rolfe, B. G., Weinman, J. J., and Djordjevic, M. A. 1989. Host specificity in *Rhizobium.* Pages 327-341 in: Plant-Microbe Interactions: Molecular and Genetic Perspectives. T. Kosuge and E. Nester, eds. McGraw-Hill, New York.

Spaink, H. P. 1992. Rhizobial lipo-oligosaccharides: Questions and answers. Plant Mol. Biol. 20:977-986.

Stafford, H. A. 1990. Flavonoid Metabolism. CRC Press, Boca Raton,

FL.
Stokkermans, T. J. W., Sanjuan, J., Ruan, X., Stacey, G., and Peters, N. K. 1992. *Bradyrhizobium japonicum* rhizobitoxine mutants with altered host-range on *Rj4* soybeans. Mol. Plant-Microbe Interact. 5: 504-512.
Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Prome, J.-C., and Denarie, J. 1991. Sulfated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351:670-673.
Vasse, J., de Billy, F., and Truchet, G. 1993. Abortion of infection during the *Rhizobium meliloti*–alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. Plant J. 4:555-566.
Wingender, R., Rohrig, H., Horicke, C., Wing, D., and Schell, J. 1989. Differential regulation of soybean chalcone synthase genes in plant defence, symbiosis and upon environmental stimuli. Mol. Gen. Genet. 218:315-322.
Yang, W.-C., Canter-Cremers, H. C. J., Hogendijk, P., Katinakis, P., Wijffelman, C. A., Frassen, H., van Kammen, A., and Bisseling, T. 1992. *In situ* localization of chalcone synthase mRNA in pea root nodule development. Plant J. 2:143-151.