Induction of Chalcone Synthase Expression in White Spruce by Wounding and Jasmonate

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The phenylpropanoid pathway has important functions in angiospermous plants following exposure to environmental stresses, such as wounding and pathogen attack, that lead to production of compounds including lignin, flavonoids and phytoalexins. Chalcone synthase (CHS) is a key enzyme in this pathway, catalyzing the first step in flavonoid biosynthesis, whose expression can be induced in response to environmental stress. To explore the response of conifers to environmental stress, expression of spruce CHS and its inducibility were investigated. A partial spruce CHS cDNA clone was isolated using PCR. Examination of the expression patterns of the CHS gene family in white spruce revealed accumulation of CHS mRNA in needle tissue following mechanical wounding, or application of signal molecules like jasmonic acid or methyl jasmonate. Repeated mechanical wounding or jasmonate applications had an enhancing effect on transcript accumulation in needles. A systemic accumulation of CHS mRNAs following wounding was also observed. Conifers thus appear to possess a general wound response similar to that found for angiosperms, which includes CHS induction as well as its inducibility by jasmonic acid and airborne methyl jasmonate.

Key words: Environmental stress — Flavonoid — Gene — Picea glauca.

Abbreviations: CHS, chalcone synthase; JA, jasmonic acid; MeJ, methyl jasmonate.

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During their life cycle, plants are exposed to numerous environmental stresses. These stresses can result from injuries caused by the attacks of insects and microbes or by mechanical wounding, and can induce many distinctive biochemical changes. These include the production of protective compounds either at the site of injury, or systemically in distant unwounded tissues (reviewed in Bowles 1990, Ryan 1990). Several groups of defense-related genes have also been identified with respect to insect and microbial pathogens (Smith 1996, Hammond-Kosack and Jones 1997), and include protease inhibitors (Koiwa et al. 1997), chitinase (Oppenheim and Chet 1992), phenylalanine ammonia lyase and chalcone synthase (Dixon and Paiva 1995), all of which accumulate systemically.

Flavonoids play many important roles in flowering plants, such as providing floral pigments, antibiotics, UV protectants and insect repellents (Hahlbrock and Scheel 1989). Chalcone synthase (CHS) is a key enzyme of the phenylpropanoid pathway catalyzing the first step in flavonoid biosynthesis and is by far the most abundant protein in the phenylpropanoid biosynthetic pathway. CHS gene expression is influenced by many stresses and environmental factors (reviewed in Dixon and Paiva 1995). Jasmonates are proposed to be signaling molecules associated with the activation of an increasing number of plant genes (reviewed in Creelman and Mullet 1997, Wasternack and Parthier 1997). Jasmonic acid (JA) was first associated in the activation of genes encoding protease inhibitors following wounding (Farmer et al. 1992). Jasmonate levels also increase rapidly but transiently in wounded plants (for a review on jasmonate see Sembdner and Parthier 1993) and induce gene expression through a lipid-based signaling pathway called the octadecanoid pathway (MueIler 1997). Such gene induction has been correlated with induction of messenger RNA encoding for flavonoid biosynthetic enzymes (Dittrich et al. 1992, Gundlach et al. 1992) and other phenylpropanoid genes such as 4-coumarate: CoA ligase (Ellard-Ivey and Douglas 1996). Methyl jasmonate (MeJ) treatment of soybean cell suspension was also reported to induce CHS transcript accumulation (Creelman et al. 1992). This suggests the important role of jasmonates both in the wound response as well as in the pathogen attack response in higher plants.

Flavonoids are also important compounds in conifers. Picea sitchensis possesses both CHS and stilbene synthase activities. Following stress treatments (nicking of the hypocotyl with a knife and in vacuo infiltration of a solution of yeast extract), Fliegmann et al. (1992) observed an augmentation of CHS as
PCR amplification, cloning and sequencing

Single-stranded cDNA was made from 15 spruce seedlings following wounding, MeJ or JA treatment. System, Promega) from pooled fractions of total RNA isolated and poly(dT) 15 primer (First Strand cDNA Synthesis Kit, Boehringer Mannheim). Degenerated primers (forward: 5′-CGGAATTCACIACI T/A C/G IGGIGTIGA T/C A TG; reverse: 5′-GGGGGAGTA T- C′) were used in an amplification reaction containing approximately 50 ng of first-strand cDNA. Reaction conditions were: 0.1 µM of each primer, 0.2 mM of each dNTP, 0.025 unit µl−1 Taq DNA polymerase (Boehringer Mannheim) and 1× of the supplied reaction buffer, 35 cycles (94°C, 1 min; 50°C, 45 s; 72°C, 1 min) followed by 10 min at 72°C in a DNA thermal cycler (Perkin Elmer). Amplicons were cloned into pBluescript™ SK+ (Stratagene) and sequenced using the dideoxy chain termination method (Sanger et al. 1977) with an ABI 373 automated DNA sequencer stretch (Perkin-Elmer).

RNA blot analysis

Total RNA was isolated from pooled material of needles, roots and stems from 2-year-old seedlings according to Chang et al. (1993). Non-stressed root material from 2-year-old potted plants was harvested, immediately frozen in liquid nitrogen and stored at −80°C. Total RNA from five to ten pooled vegetative, male and female buds tissues of 15-year-old field-planted trees was extracted using the Chang et al. (1993) extraction buffer together with the QIAGEN RNeasy total RNA kit. Equal amounts of total RNA (10 µg) were denatured with formamide/ formaldehyde and fractionated on denaturing 1.2% agarose formaldehyde gel, blotted onto Nytran Plus positively charged nylon membranes (Schleicher and Schuell), and UV crosslinked. Probes were radioactively labeled by random priming with the High Prime labeling kit (Boehringer Mannheim) and purified with QIAquick nucleotide removal kit (QIAGEN). Membranes were prehybridized in hybridization buffer (Church and Gilbert 1984) for 2 h prior to adding [α-32P]-labeled probe (1× 106 c.p.m. ml−1 buffer). Genomic DNA blot hybridizations were performed at 60°C and washed in 2× SSC and 0.1% SDS at 60°C. High-stringency hybridization conditions for RNA blot analyses were achieved by hybridizing the membranes at 65°C for 16 h and washing them twice with 1× SSC and 1% SDS at 65°C for 30 min. To correct for differences in RNA loading, membranes were stripped by boiling for 15 min in 0.1 × SSC and 0.1% SDS and rehybridized with a white spruce 400 bp 18 S ribosomal probe. Ribosomal probes were generated by PCR using NS51 and NS61 primers (5′-GGGGGAGTAGTGTCGCCTAAAGGC-3′ and 5′-TCAGTGATGCCGGCGTTCGC-3′ respectively; Simon et al. 1992). Hybridized membranes were exposed to X-OMAT™ AR Scientific Imaging Film (Kodak) for 12–36 h for RNA blots and 7–10 d for genomic DNA blots, and/or exposed to a Storage Phosphor Screen. Quantification of RNA hybridization signals was evaluated with a Fuji Bio imaging analyzer/Phosphor imager analyzer and by densitometric measurement of autoradiograms using the RFLPscan Plus 3.0 program (Scanalytics, CSP Inc.). Results were normalized for an equal loading quantity of 18 S ribosomal RNA hybridization intensity. The histogram represents scanning values of the most representative hybridization experiment (shown) and is a mean of four different quantification results obtained from densitometry scan analyses as well as phosphor imager quantification of replicate induction experiments. In the analyses, we considered a difference of magnitude of transcript accumulation of 2-fold or greater to be significant.

Wounding and jasmonate treatments

All experiments were conducted with 2-year-old plants, five plants per experiment. Wounding consisted in trimming off the first 5–15 mm of each needle tip. For systemic wounding, the needles of five bottom branches were wounded and needles...
were harvested from five non-wounded branches toward the apical region of the seedlings. Multiple mechanical wounding treatments were carried out over the entire plant and repeated at 4-h intervals. JA treatments consisted in spraying plants to saturation with a 100 μM JA-solution (Farmer et al. 1992) prepared in 0.05% Tween 20. Control plants were sprayed with 0.05% Tween 20 in an adjacent greenhouse to avoid cross contamination. Treatment with MeJ (Bedoukian Research Inc.) was carried out by placing plants in plastic bags with a cotton swab containing 500 µl of a 10% MeJ in ethanol (Farmer et al. 1992) or ethanol alone for control plants, with care taken to ensure that the plants did not come in contact with the swab. Seedlings were kept under normal greenhouse conditions throughout the experiments and all treatments were performed in duplicate.

Isolation and characterization of PgCHS1 partial cDNA clone

A 222 bp partial CHS cDNA clone (PgCHS1) was isolated using a PCR-cloning strategy employing degenerate primers derived from conserved regions of other known CHS proteins. Translation of PgCHS1 yielded an open reading frame of 74 amino acids (Genbank accession number AF22922), which is highly homologous to that of other CHS proteins, sharing 99–100% identity with coniferous CHS and 95% with angiosperm CHS. PgCHS1 thus represents a very well conserved region of the CHS gene. Baker and White (1996) showed the presence of many fragments with sequence homology to CHS in white spruce and several other gymnosperms using a probe very similar to PgCHS1. In fact, the probe utilized by these authors was obtained with the same set of oligonucleotides described in the present study for amplification of genomic fragment from *Pinus monticola*. These results are also consistent with an extensive PCR-cloning survey that revealed a large CHS gene family within black spruce (Rutledge, unpublished results).

General expression pattern of Picea glauca CHS

RNA blot analysis indicated that CHS transcript levels are highest in female buds, and in the early stages of vegetative and male bud development (Fig. 1). Levels were approximately 5-fold greater than those present in needles from 2-year-old seedlings maintained under normal greenhouse conditions. Moderate levels of CHS mRNA were also observed in all other tissues examined, except roots where CHS mRNA was barely detectable. These results are consistent with the observation that both female and male buds become intensively pigmented following bud break, and that male cones lose their pigmentation as they mature, whereas female cones maintain their intense pigmentation well after pollination. Vegetative buds also often become pigmented following bud break. These results thus support an association of CHS expression with flavonoid biosynthesis (e.g. anthocyanin biosynthesis) similar to that within flowers (Mol et al. 1996), in addition to the possibility that CHS plays a defensive role in protecting buds during early development.

Changes in CHS mRNA accumulation induced by mechanical wounding or jasmonate treatments

To examine the effects of stress treatments on CHS mRNA levels in needles of 2-year-old white spruce seedlings, a series of experiments were conducted to compare endogenous response induced via wounding, and exogenous induction via application of JA or MeJ. Local versus systemic effects and single versus multiple treatments were also evaluated. Figure 2 summarizes changes in CHS mRNA levels following a single treatment at time 0. Local wounding induced accumulation of CHS transcripts by more than 5-fold (Fig. 2A). Increased CHS gene expression after wounding has been previously described in soybean (Creelman et al. 1992) and alfalfa (Junghans et al. 1993). In both cases, wounding by cutting leaves (alfalfa) or hypocotyl tissue (soybean) induced maximal CHS mRNA accumulation approximately 4–8 h after treatments. In the present case, maximal mRNA level occurred after 4 h (Fig. 2A). A similar response was observed for systemic wounding (Fig. 2B), reaching a maximal induction of about 5-fold. The time of maximal induction was slightly different however (4 vs. 8 h respectively), and was more persistent over the 24-h time period examined. Systemic wound response has been mostly characterized with respect to the production of defense products such as protease inhibitors; however, systemic accumulation of CHS genes has also been reported (reviewed in Dixon and Paiva 1995). Such a systemic induction of CHS was also observed here and, as expected, this activation was less immediate than...
The local wound response (Fig. 2A). As reported by others, we also observed that application of JA had a similar effect to wounding, although it produced both a stronger effect and a significantly different induction profile, with a 10-fold maximal induction at 10 h that only dropped slightly after 24 h (Fig. 2C). Endogenous synthesis of JA in conifer has been previously demonstrated by Mueller et al. (1993), who showed rapid synthesis of JA in *Taxus baccata* cell cultures after challenge with a fungal elicitor preparation. However, only a limited number of studies have clearly identified a relationship between jasmonates and changes in gene expression within gymnosperms. These include enhanced expression of genes encoding late embryogenesis abundant (LEA) protein after exposure of Douglas-fir seeds to MeJ (Jarvis et al. 1997) and induction of a Douglas-fir heat shock protein (HSP) gene in seed also by MeJ exposure (Kaukinen et al. 1996). We have also reported the induction of a white spruce glycine-rich RNA binding protein by exogenous JA applications (Richard et al. 1999), which is similar to JA induction of a white spruce dehydrin recently isolated in our laboratory (Richard et al. unpublished).

Multiple wounding or jasmonate treatments were also examined to see if enhanced *CHS* transcripts accumulation could be obtained and are summarized in Fig. 3. Multiple wounding produced a profile similar to that of a single wounding, reaching a slightly higher maximal level of about 5- to 8-fold (relative to time 0) at 8 h. Note, however, that the differences observed in the time course accumulation probably reflect the additive effect of both a local and a systemic response. This enhancement of transcription activation following multiple wounds has also been observed with protease inhibitors (Gra-

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**Fig. 2** *CHS* mRNA accumulation following a single stress treatment. Changes in *CHS* mRNA levels over time within the needles of 2-year-old seedlings determined following a single wound or JA treatment. (A) Local response directly within wounded needles. (B) Systemic wound response within unwounded needles collected from the apical branches of plants in which the needles within the basal branches were wounded. (C) JA response within needles from plants sprayed with elicitor. Time indicates the harvesting period following the beginning of the treatment. *CHS* mRNA quantification was conducted as described in the Materials and Methods section, and is expressed as percentage of the highest value.

**Fig. 3** *CHS* mRNA accumulation following multiple wound or JA treatments, or continuous MeJ exposure. Changes in *CHS* mRNA levels within the needles were determined over time following wounding or JA application at time 0, 4 and 8 h. For continuous MeJ exposure, plants were placed in plastic bags with a cotton swab containing a MeJ solution. Time 0 represents needles prior to any of the mentioned treatments. *CHS* mRNA quantification was conducted as described in the Materials and Methods section, and is expressed as percentage of the highest value.
HAM et al. 1986). Similar to the enhancing effect of multiple wounding, an enhancing effect was observed following the second application of JA, although CHS mRNA levels were much higher in response to JA treatment than in response to wounding. Multiple JA treatments produced the strongest induction of CHS mRNA accumulation, reaching greater than 20-fold at 8 h, but falling to about 8-fold at 24 h. Continuous exposure to MeJ produced a gradual accumulation of CHS mRNA that reached greater than 15-fold at 24 h. Results from JA and MeJ control plants showed no significant induction in CHS transcript (data not shown). MeJ response induced transcript level of CHS to levels intermediate to that of wounding and JA treatments. However, mRNA levels were still rising at the last time point examined, and could thus possibly reflect a postponed response for MeJ compared to JA simply on the basis of the concentration and speed of jasmonate reaching the plant tissues. Addition of MeJ to soybean suspension cultures also increased transcript level of chalcone synthase gene, suggesting a role for jasmonates in mediation of several changes in gene expression associated with wound response. Several other studies have also reported initiation of de novo transcription of genes involved in the phenylpropanoid pathway following jasmonate treatments (Dittrich et al. 1992, Gundlach et al. 1992, Mueller et al. 1993, Ellard-Ivey and Douglas 1996), which correlate with Creelman et al. (1992), who reported increased internal levels of both JA and MeJ after wounding of hypocotyl tissue.

In conclusion we have shown that the tissue distribution pattern of expression, response to wounding and jasmonate treatments in spruce are all consistent with previous studies on CHS expression. Although CHS transcript accumulation is clearly visible in response to all treatments (wounding or jasmonates), the induction kinetics differ. Further studies will be needed to gain a complete understanding of the role for CHS expression in spruce defense mechanisms as well as the function of jasmonate in mediating this response in spruce.

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References


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