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Regulatory roles of benzyl adenine and sucrose during wound response of the ribosomal protein gene, *rpL34*

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ABSTRACT

We previously reported that a large subunit ribosomal protein gene, *rpL34*, was wound-inducible in tobacco leaves, and that benzyl adenine (BA) enhanced the wound-inducible expression of the gene. Here we report that the wound-inducible expression of the *rpL34* gene is enhanced by sucrose. The regulatory roles of BA and sucrose in wound-signalling pathways were investigated using transgenic plants expressing a fusion molecule between the *rpL34* promoter and the chloramphenicol acetyltransferase (*cat*) gene. Wounding caused a progressive increase in CAT activity; BA and sucrose enhanced the response. This indicates that regulation occurs at the transcriptional level. Diethyldithiocarbamic acid (DIECA) and other potent inhibitors of the octadecanoid pathway inhibited the *rpL34* promoter, and also reduced BA inducibility of that wound response. However, inhibitors of the octadecanoid pathway did not affect the sucrose response. Protein kinase inhibitors increased BA enhancement while decreasing the sucrose effect, suggesting that protein kinases differentially regulate BA- and sucrose-signalling in the promoter's wound response. Taken together, BA and sucrose enhanced the wound response of the *rpL34* promoter via different signalling pathways, although they exerted overlapping effects on wound-induction of the promoter.

Key-words: *rpL34* promoter; BA; ribosomal protein gene; sucrose; wound-signalling pathway.

INTRODUCTION

Plants undergo various environmental stresses, including shade or high light levels, nutrient imbalance, drought, flooding, disease, predation and wounding. Wounding is one of the most severe stresses, and can lead to the induction of a large number of genes, such as stress-response genes, defense genes, and wound-repair genes (Bowles 1990; Dixon, Harrison & Lamb 1994; Yang, Shah & Klessig 1996). The octadecanoid pathway leading to the synthesis of jasmonic acid (JA) and its methyl ester derivative, methyl jasmonate (MJ), has been identified as a central part of the signalling cascades triggered by wounding (Ryan & Farmer 1991; Creelman & Mullet 1997). JA and MJ signals are transduced to affect the expression of a number of genes (Farmer & Ryan 1992; Ellard-Ivey & Douglas 1996; Rickauer *et al*. 1997; Menke *et al*. 1999). An alternative wound-signalling, octadecanoid-independent pathway has also been found. Its existence is supported by the wound-induced expression of the glutathione S-transferase, tyrosine amino transferase homologs, choline kinase, and acyl CoA oxidase in a JA-deficient or JA-insensitive *Arabidopsis* mutant (McConn *et al*. 1997; Titarenko *et al*. 1997; Rojo *et al*. 1998).

Whether a plant can live within unfavourable conditions depends on how a metabolic pathway is adjusted via the modulation of expression in sets of genes (Schmulling, Schafer & Romanov 1997; Bohnert & Sheveleva 1998; Roitsch 1999). This implies that many hormonal and metabolic factors are involved in the plant adaptive response to wound stress. It has been shown that cytokinins influence various phases of growth and development by altering gene expression in concert with environmental factors (Miller 1956; Simmons *et al*. 1992; Sano & Youssefian 1994; Schmulling *et al.* 1997; Sakakibara *et al*. 1998). Sucrose is also thought to be an important signalling molecule that controls the expression of many plant genes, thereby regulating metabolic and developmental processes (Jang & Sheen 1994; Koch 1996; Smeekens & Rook 1997; Yu 1999). There is evidence that cytokinins, sucrose, and wounding cooperate to induce a set of genes. The *pmas1′* promoter and the ribosomal protein promoter *rpL34* exhibit an additive interaction between cytokinins and wounding signals (Dai *et al*. 1996; Guevara-Garcia *et al*. 1998). Sucrose-induced gene expression has also been demonstrated for proteins involved in wound response (Salanoubat & Belliard 1989; Johnson & Ryan 1990; Tsukaya *et al*. 1991). Sucrose enhances the expression of proteinase inhibitor (*pin*) genes under wound stress, and plays a regulatory role for the *pin* gene promoter (Johnson & Ryan 1990; Kim, Costa & An 1991). In soybean hypocotyls, the presence of sucrose is prerequisite for wound-induction of the hydroxyproline-rich glycoprotein gene *SbHRGP3* (Ahn *et al*. 1996). Although these studies suggest interaction between wounding signals and cytokinin or sucrose stimuli, the

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molecular mechanism of interaction has not been understood to date.

Some wound-inducible genes show striking homologies to housekeeping genes, which may function in wound repair (Bowles 1990; Dixon *et al*. 1994; Yang *et al*. 1996; Titarenko *et al*. 1997; Iyer *et al*. 1999). The ribosomal protein (r-protein) genes are considered housekeeping genes (Nomura, Gourse & Baughman 1984), and their usual role is for protein synthesis in proliferating tissues, including shoot and apical meristems, anthers, ovaries, embryos, and young seedlings (Larkin *et al*. 1989; Lebrun & Freyssinet 1991; Bonham-Smith, Oancia & Moloney 1992; Kohler *et al*. 1992; Taylor *et al*. 1992; Devitt & Stafstrom 1995; Williams & Sussex 1995; Dai *et al*. 1996). However, these r-protein genes can also respond to various factors, e.g. growth hormones, nutrients, genotoxic stress, ozone, and wounding (Gantt & Key 1985; Crowell *et al*. 1990; Gao *et al*. 1994; Dai *et al*. 1996; Wool 1996; Brosche & Strid 1999; Revenkova *et al*. 1999). In the present study, we examined the effects of BA and sucrose on the induction of the wound-inducible *rpL34* gene, and whether the regulation of r-protein gene expression occurred at the transcriptional level. We also studied the regulatory roles of BA and sucrose in wound-signalling pathways leading to *rpL34* promoter induction.

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants were grown in soil in a temperature-controlled greenhouse at 25 °C under natural sunlight. Transgenic tobacco plants carrying the *rpL34* promoter-*cat* (chloramphenicol acetyltransferase) fusion, which was composed of the 1·5 kb-long promoter region of the gene for *rpL34* from tobacco (Dai *et al*. 1996) and the coding sequence for *cat* (An *et al*. 1988). Kanamycin-resistant transformants were selected on agar plates and grown in soil under greenhouse conditions. All of the experiments were performed with the second generation of the transgenic plants.

Plant treatments

Fully expanded tobacco leaves from 8-week-old plants were wounded by cutting them into 1-cm^2 segments. As a wounding control, leaf segments were incubated on a sucrose-free Murashige and Skoog (MS) medium (Murashige & Skoog 1962) in Petri dishes under dim light (80–100 $\mu mol\ m^{-2}s^{-1}$). BA and sucrose treatments were performed by adding each of those compounds to the incubation medium. The BA was dissolved in DMSO at 5 mM as a stock solution, then diluted to a final concentration of 5 μM. Sucrose was dissolved in the incubation medium from a 10% stock solution. After incubation, the samples were harvested, frozen in liquid nitrogen, and stored at – 80 °C until processing.

For the individual stock solutions, diphenylene iodium (DPI), ibuprofen, genistein, H-7, *n*-propyl-gallate, and phenylbutazone were dissolved in DMSO at 5 mM as a stock solution. Diethyldithiocarbamic acid (DIECA) and salicylic acid were dissolved in an MS medium at a final concentration of 100 μM and 10 μM, respectively.

RNA preparation and gel blot analysis

Total RNA was extracted using the Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. Twenty micrograms of total RNA per sample were separated in an agarose-formaldehyde gel and blotted onto a Hybond-N^+ nylon membrane (Amersham, Buckinghamshire, UK) by capillary blotting (Sambrook, Fritsch & Maniatis 1989). The *rpL34* probe was prepared from the *Eco*R1 fragment (394 bp) of the *TSC40* cDNA clone (Gao *et al*. 1994), while the *rpL25* probe was prepared from the *Eco*RI fragment (524 bp) of the *TSC 29* clone (Gao *et al*. 1994). A 542-bp cDNA fragment encoding *pin II* gene was PCR-amplified from a tobacco cDNA library using the 5′ primer (5′-TTGATGCCAAGGCTTGTACCA-3′) and the 3′ primer (5′-CAGCACTTTGAGGCTCCCCAC-3′). Probes were labelled with [α-^{32}P]dCTP, using the Rediprime system (Amersham). Hybridization was performed at 60 °C according to Church & Gilbert (1984). The blots were washed twice in 2 × SSPE at 60 °C for 10 min, followed by a final wash in 0·2 × SSPE at 60 °C for 5 min. The blots were scanned and quantified with the Fuji bio-imaging analyzer BAS 1000 (Fuji Photo Film, Japan). The relative RNA accumulation was calculated by dividing the relative expression of the 18S rRNA. The relative expression was calculated by dividing each spot quantified value by the highest value of the autoradiograph.

Soluble sugar assay

Approximately 100 mg of the frozen leaf material was ground to a fine powder in liquid N_2, then homogenized with a mortar and pestle in 0·8 mL of 0·2 M KOH containing 0·08% Triton X-100. Soluble sugars were extracted on ice for 15 min. Sucrose, glucose, and fructose contents in the supernatant were determined by using a sucrose/D-glucose/D-fructose assay kit (Boehringer Mannheim, Germany).

CAT assay and other procedures

The CAT assay was performed with 20 μg of soluble protein and [^{14}C]chloramphenicol at 37 °C for 30 min, and the reaction products were separated by TLC (An 1987). The amount of soluble protein was measured by the Bradford method, using bovine serum albumin as a standard (Bradford 1976). This experiment was repeated at least three times with separately prepared samples.

RESULTS

Dosage effects of BA and sucrose on wound-inducible expression of r-protein genes

We previously studied the effects of BA and sucrose on the r-protein gene expression in leaf segments prepared from

wild-type tobacco leaves. The large subunit ribosomal protein genes in tobacco, *rpL25* and *rpL34,* were inducible by incubating leaf segments on an MS salt medium; this induction was due to mechanical wounding (Gao *et al.* 1994). Because the mRNA accumulation was saturated after 6 h of incubation (Gao *et al.* 1994), we selected that time period for the present study.

Figure 1(a) shows the dosage effects of BA and sucrose on wound-inducible expression of *rpL25* and *rpL34*. The transcript levels of the *rpL25* and *rpL34* genes reached their maximum at 5 μM BA and 1% sucrose, respectively. However, treatment with 1% sorbitol or 1% mannitol as an osmotic agent had little influence on the rpL gene induction (data not shown). The data in Fig. 1(b,c) indicate that the BA and sucrose effects were neither synergistic nor additive.

Enhancement of wound-inducible expression of the rpL34 promoter by BA and sucrose

To elucidate whether the increase in the r-protein gene expression occurred at transcriptional level, we examined transgenic tobacco plants expressing a chimaeric fusion between the *rpL34* promoter and the *cat* gene. Wounding

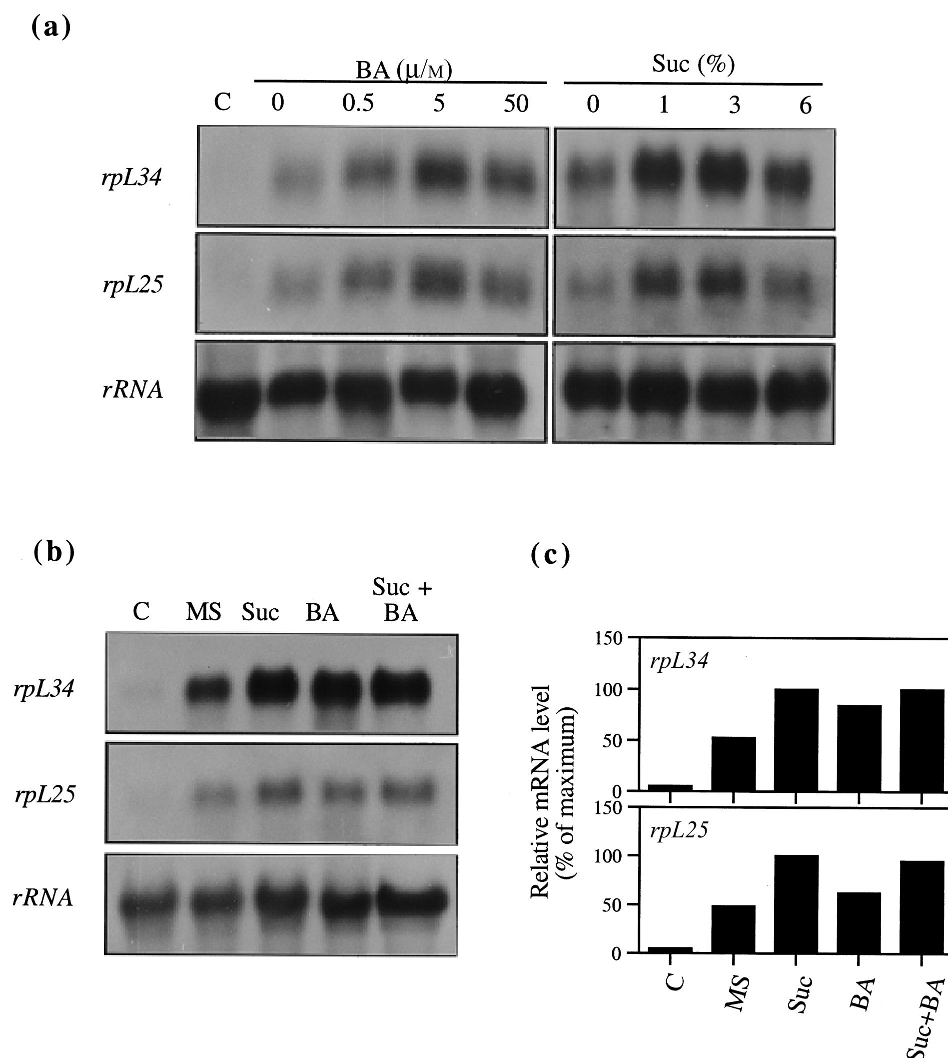

Figure 1. Effects of BA and sucrose on accumulations of mRNAs encoding *rpL34* and *rpL25* in wounded leaves. Fully expanded tobacco leaves were wounded by cutting them into 1-cm^2 segments and incubating them for 6 h on an MS medium (MS) or MS media supplemented with various concentrations of BA (BA) or sucrose (Suc). When BA dissolved in DMSO were used, all treatments of leaf segments were carried out in the presence of 0·1% DMSO. For the unwounded control (C), mature leaves were harvested from tobacco plants and immediately frozen in liquid nitrogen. Equal RNA loading was determined by hybridizing the gel blot with a labelled 18S rRNA probe. The experiment was repeated three times with similar results. (a) RNA gel blot analysis for the effects of various concentrations of BA and sucrose on *rpL34* and *rpL25* expressions. (b) RNA gel blot analysis for the effect of cotreatment of 1% sucrose or 5 μM BA on the expression of *rpL34* and *rpL25*. (c) Quantitation of RNA blot analysis shown in Figure 1(b). Relative RNA levels on the basis of the 18S rRNA signals were plotted.

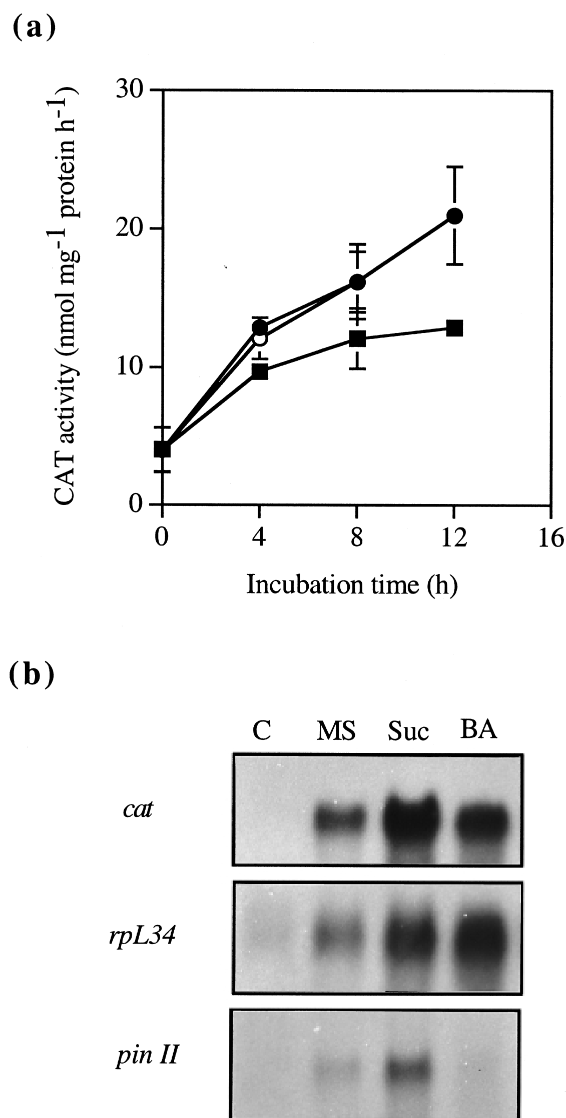

Figure 2. Induction of the *rpL34* promoter by wounding alone, and in combination with BA or sucrose. Fully expanded transgenic tobacco leaves were wounded by cutting them into 1-cm^2 segments and incubating them on MS media with or without 5 μM BA, or 1% sucrose. (a) Time course of CAT activity in wounded control (■), and BA-added (○) or sucrose-added (●) samples. Values are means ± SE, $n = 3$. (b) The mRNA levels of *cat*, *rpL34*, and *pin II*. Leaf segments were incubated for 6 h on an MS medium (MS) or MS media supplemented with 5 μM BA (BA) or 1% sucrose (Suc). For the unwounded control (C), mature leaves were harvested from tobacco plants and immediately frozen in liquid nitrogen.

caused a progressive increase in CAT activity driven by the *rpL34* promoter, and treatments with BA and sucrose enhanced that response (Fig. 2a). Treatment with 5 μM BA elevated the CAT activity level by 1·6-fold over the control. Adding 1% sucrose also resulted in an increase in CAT activity, to the level induced by BA. The *cat* transcript level was increased by wounding and enhanced by addition of sucrose and BA (Fig. 2b). Similar results were observed when the *rpL34* transcript level was measured. These results suggest that the various effects on the *rpL34* gene expression occur at the transcriptional level.

To examine whether the BA and sucrose effects observed from the rpL genes were general phenomena for wound-inducible genes, we also studied expression patterns of the *pin II* gene. It was previously reported that the *pin II* gene transcript was inducible by wounding and further enhanced by sucrose (Johnson & Ryan 1990; Kim *et al.* 1991). Here, we also observed that *pin II* gene expression was enhanced by sucrose, but not by BA. Therefore, it appears that BA induction is not a general phenomenon for wound-inducible genes.

Involvement of octadecanoid pathway in regulation of rpL34 expression by BA and sucrose

The octadecanoid pathway and the reactive oxygen species (ROS)-generating pathway have been implicated in wound-induced signalling pathways that lead to the expression of a number of genes (Scheel 1998). DIECA is a potent inhibitor of jasmonate production in the octadecanoid pathway (Farmer *et al.* 1994), whereas DPI inhibits NADPH oxidase that is involved in ROS generation (Cross & Jones 1986, 1991). The wound-inducible CAT activity driven by the *rpL34* promoter was severely inhibited by 100 μM DIECA (Fig. 3a). Application of 100 μM DIECA also inhibited BA enhancement (Fig. 3b), but did not affect sucrose enhancement (Fig. 3c). Similar results were obtained from the RNA gel blot analysis of the *cat* and *rpL34* transcripts.

All of these results indicate that the octadecanoid pathway is involved in wound-inducible expression of the *rpL34* promoter and enhancement of the expression by BA. In contrast, treatment with 100 μM DPI had little effect on the inducibility of the *rpL34* promoter, which suggests that this promoter is not inducible by ROS. Alternatively, ROS is not generated by wounding as had been observed in strips of tobacco and grape leaves (Papadakis & Roubelakis-Angelakis 1999). We also reported previously that ROS may not be involved in MJ-inducible expression of the nopaline synthase promoter (Yu, Sung & An 1998).

We further tested four inhibitors (ibuprofen, *n*-propyl-gallate, phenylbutazone, salicylic acid) for their possible effects on the BA- and sucrose-signalling pathways that lead to enhancement of wound-inducible activation by the promoter (Fig. 4). These inhibitors are suggested to inhibit lipoxygenase or hydroperoxide dehydrase, respectively, which are involved in the biosynthesis of JA (Staswick, Huang & Rhee 1991; Farmer 1994). At the concentrations used, all of the inhibitors reduced *rpL34* promoter activity in the wounded samples as well as in the BA-treated samples. However, as seen with the DIECA treatment, these chemicals did not affect sucrose-mediated enhancement of the promoter activity. These results suggest that the octadecanoid pathway is involved in wound response of the

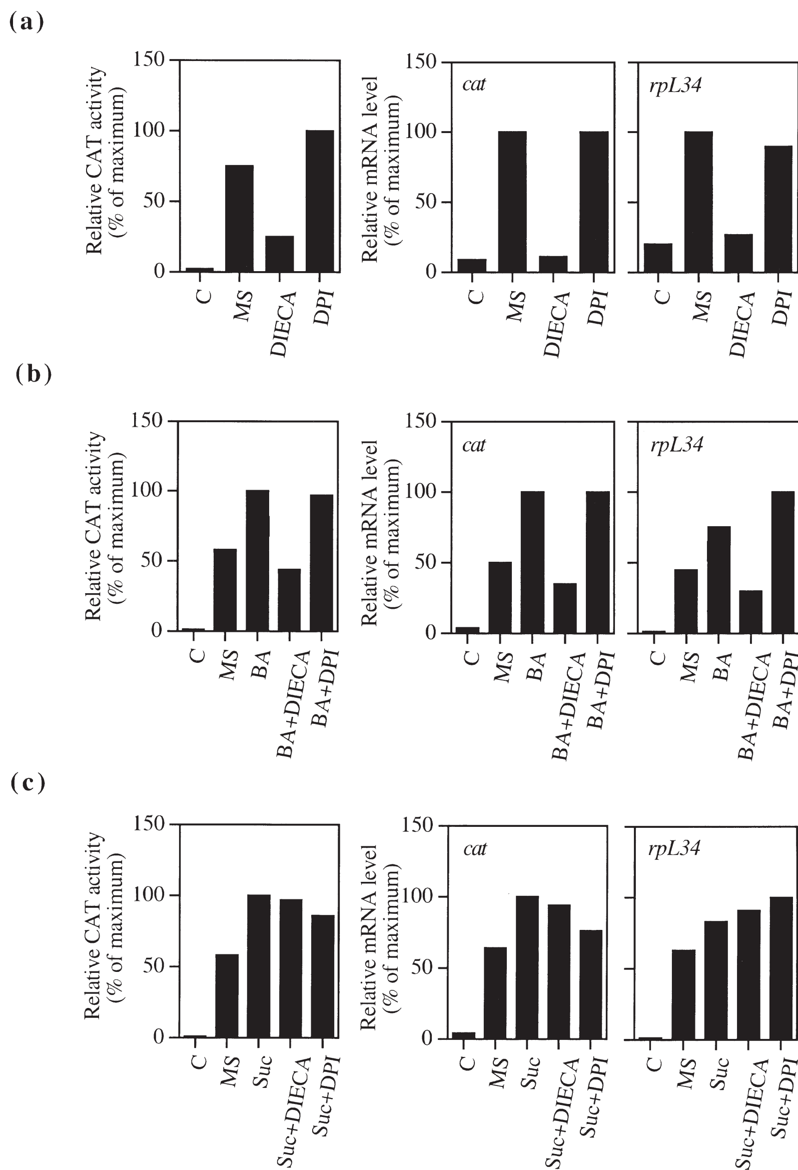

Figure 3. Effects of DIECA and DPI on *rpL34* promoter induction by wounding (a), BA response (b), and sucrose response (c). Fully expanded transgenic tobacco leaves were treated as described in Figure 2. The incubation period was 10 h for the CAT assay (left) and 6 h for RNA gel blot analysis (right). Inhibitors were included in the incubation media at the following concentrations: DIECA, 100 μM; or DPI, 100 μM. Relative RNA levels on the basis of the 18S rRNA signals were plotted. The experiment was repeated three times with similar results.

rpL34 promoter and BA enhancement of that response, but this pathway is not involved in sucrose enhancement.

Involvement of protein kinases in the BA and sucrose effects

We tested the effects of protein kinase inhibitors, i.e. genistein for the Tyr protein kinase and H-7 for the Ser/Thr protein kinase, on the *rpL34* promoter response to BA or sucrose. After 6 h of incubation, the *cat* mRNA level controlled by the *rpL34* promoter was examined in each sample. Although the inhibitors had little effect on *rpL34* promoter activity in the wounded control, they caused a striking increase in *rpL34* expression in the BA-treated samples (Fig. 5). The inhibitor treatment severely reduced the *cat* transcript levels of the sucrose-treated samples, but did not affect the endogenous sucrose level (data not shown). These results suggest that protein kinases, of either Tyr- or Ser/Thre-type, positively regulate the sucrose-signalling that leads to enhancement of wound-inducible expression of the *rpL34* promoter, and negatively regulate the BA-signalling pathway.

DISCUSSION

Plants react to wound stress by activating a set of genes. Some of the wound-responsive genes have a defensive role against insect predation and pathogen infection, while others, such as housekeeping genes, may function in wound repair (Bowles 1990; Dixon *et al*. 1994; Yang *et al*. 1996; Titarenko *et al*. 1997; Iyer *et al*. 1999). Ribosomal protein genes, which are considered housekeeping genes (Nomura *et al*. 1984), are known to respond to wound stress; a prominent example is the tobacco r-protein genes, *rpL25* and *rpL34* (Gao *et al*. 1994; Dai *et al*. 1996).

Upon wounding, lipid hydrolysis and peroxidation

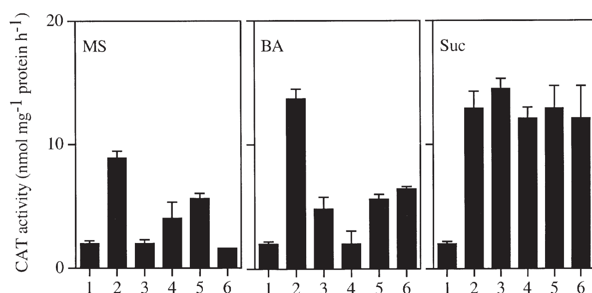

Figure 4. Effects of octadecanoid pathway inhibitors on the CAT activity level controlled by the *rpL34* promoter in wounded leaves treated with BA or sucrose. Tobacco leaves were treated as described in Figure 2. The incubation period was 10 h. Inhibitors were included in the incubation media at the following concentrations: ibuprofen, 10 μM; *n*-propyl-gallate, 10 μM; phenylbutazone, 100 μM; or salicylic acid, 10 μM. Values are means ± SE, *n* = 3·1, unwounded control; 2, wounded leaves without an inhibitor; 3, ibuprofen; 4, *n*-propyl-gallate; 5, phenylbutazone; and 6, salicylic acid.

increase, which leads to synthesis of the signal molecules, JA and MJ. These molecules then induce expression of a number of plant genes (Ellard-Ivey & Douglas 1996; Rickauer *et al.* 1997; Menke *et al.* 1999). Although the involvement of JA on regulating gene activation upon wounding has been well established, the processes occurring immediately after wounding are poorly characterized. Therefore, it is still unknown how hormones and metabolites influence the signalling cascades triggered by wound stress.

Here, we have demonstrated that BA and sucrose enhance wound-inducible expression of *rpL34*. We also have shown that DIECA inhibits wound-inducible expression of the *rpL34* gene and reduces BA enhancement of that wound-inducible expression. DIECA converts 13-hydroperoxylinoleic acid, the product of lipoxygenase acting on linolenic acid, to 13-hydroxylinolenic acid, which is not a signalling molecule, thus shutting down the octadecanoid pathway (Farmer *et al.* 1994). These results support that JA (and/or MJA) plays a role for an endogenous signal in regulating the *rpL34* promoter. Indeed, exogenously applied 50 μM MJ increased the *rpL34* promoter activity (data not shown). We found that several inhibitors of lipoxygenase or hydroperoxide dehydrase (salicylic acid, *n*-propyl-gallate, ibuprofen, and phenylbutazone) affect induction of the *rpL34* promoter by wounding and BA. These chemicals are suggested to inhibit lipoxygenase or hyroperoxide dehydrase, which are involved in JA production (Doherty, Selvendran & Bowles 1988; Staswick *et al.* 1991; Pena-Cortes *et al.* 1993; Doares *et al.* 1995). One of these chemicals, *n*-propylgallate, is also known to inhibit the alternative respiratory pathway (Siedow & Girvin 1980). We cannot rule out the possibility that inhibitors acted by a mechanism other than inhibition of the octadecanoid pathway, although structural similarities among them suggest otherwise.

Our results suggest that cytokinin is involved in wound response, probably by enhancing the octadecanoid pathway. It was reported that abscisic acid, MJ, and electrical activity could activate p44^{MMK4} kinase only under wounding stress (Bogre *et al.* 1997). Physiological and developmental studies have indicated that an overlap with other stimuli is a frequent feature of cytokinin-regulated gene expression and a common theme of plant hormone activity (Schmulling *et al.* 1997). For example, the parsley *pmas1′* promoter is inducible by an additive interaction between cytokinin and wounding (Guevara-Garcia *et al.* 1998).

As with BA, sucrose also enhanced wound-inducible expression of *rpL34*. However, DIECA and the other inhibitors of the octadecanoid pathway did not reduce the sucrose effect, which indicates that an octadecanoid-independent signalling pathway mediates the sucrose response. Our results are consistent with studies on the promoters of the *pin II* gene and soybean *vspB* genes (Kim *et al.* 1991: Mason, deWald & Mullet 1993; Sadka *et al.* 1994). These illustrated that DNA elements capable of modulating the gene expression by sucrose are separated from the wound-response elements (An *et al.* 1989; Kim *et al.* 1991; Mason *et al.* 1993).

Rojo *et al.* (1998) suggested that protein phosphorylation may regulate the wound signal transduction that branches into at least two signalling pathways, i.e. octadecanoid-dependent and octadecanoid-independent pathways. To test whether phosphorylation is involved in the BA-and sucrose-signal transduction of the *rpL34* promoter, we used specific inhibitors of protein kinases. Protein kinases can

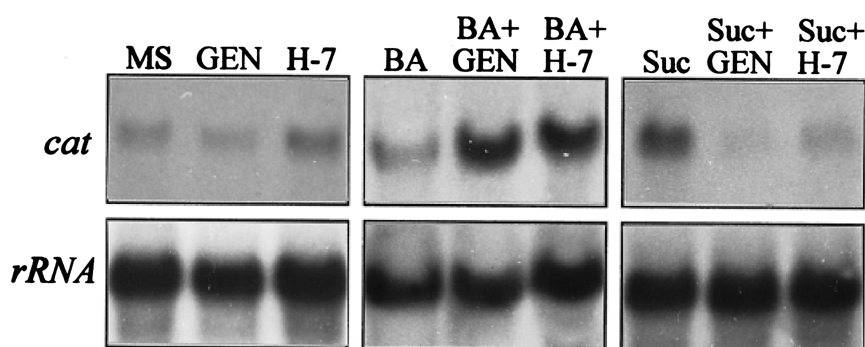

Figure 5. Effects of protein kinase inhibitors on *rpL34* induction by wounding alone, and in combination with BA or sucrose. Leaves were treated as described in Figure 2. The incubation period was 6 h. Chemicals were included in the incubation media at the following concentrations: genistein (GEN), 1 μM; or H-7, 1 μM. The experiment was repeated three times with similar results.

control cytokinin-regulated gene expression via diverse signalling pathways (Dominov *et al*. 1992; Crowell 1994; Silver *et al*. 1996). In this study, protein kinase inhibitors, of either the Tyr-or Ser/Thre-type, led to an enhanced BA effect, which suggests that reversible phosphorylation of a protein may negatively regulate BA-signalling.

In contrast, we observed that protein kinase inhibitors blocked the sucrose-mediated enhancement of *rpL34* expression. Protein phosphorylation is apparently required for sugar-mediated changes in gene expression, which indicates a possible role of hexokinase as a putative sugar sensor in plants (Jang & Sheen 1994; Koch 1996; Smeekens & Rook 1997; Yu 1999). Several studies have also suggested the possible existence of hexokinase-independent signal transduction pathways for sugar regulation of gene expression (Koch 1996; Jang & Sheen 1997). Both hexokinase-dependent and hexokinase-independent signal transduction pathways involve reversible protein phosphorylation (Lue & Lee 1994; Takeda *et al*. 1994). This is consistent with our results that the *rpL34* promoter response to sucrose is positively regulated by protein kinases.

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