

Fumonisin B1–Induced Cell Death in Arabidopsis Protoplasts Requires Jasmonate-, Ethylene-, and Salicylate-Dependent Signaling Pathways

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We have established an Arabidopsis protoplast model system to study plant cell death signaling. The fungal toxin fumonisin B1 (FB1) induces apoptosis-like programmed cell death (PCD) in wild-type protoplasts. FB1, however, only marginally affects the viability of protoplasts isolated from transgenic NahG plants, in which salicylic acid (SA) is metabolically degraded; from *pad4-1* mutant plants, in which an SA amplification mechanism is thought to be impaired; or from *jar1-1* or *etr1-1* mutant plants, which are insensitive to jasmonate (JA) or ethylene (ET), respectively. FB1 susceptibility of wild-type protoplasts decreases in the dark, as does the cellular content of phenylalanine ammonia-lyase, a light-inducible enzyme involved in SA biosynthesis. Interestingly, however, FB1-induced PCD does not require the SA signal transmitter NPR1, given that *npr1-1* protoplasts display wild-type FB1 susceptibility. Arabidopsis *cpr1-1*, *cpr6-1*, and *acd2-2* protoplasts, in which the SA signaling pathway is constitutively activated, exhibit increased susceptibility to FB1. The *cpr6-1* and *acd2-2* mutants also constitutively express the JA and ET signaling pathways, but only the *acd2-2* protoplasts undergo PCD in the absence of FB1. These results demonstrate that FB1 killing of Arabidopsis is light dependent and requires SA-, JA-, and ET-mediated signaling pathways as well as one or more unidentified factors activated by FB1 and the *acd2-2* mutation.

INTRODUCTION

Plant cell death is often the consequence of plant–pathogen interactions in both compatible and incompatible relationships (Greenberg, 1997). A notable example is localized cell collapse, called the hypersensitive response (HR), which is induced rapidly in a resistant plant at the infection site of an avirulent pathogen (Staskawicz et al., 1995; Bent, 1996; Dangl et al., 1996; Hammond-Kosack and Jones, 1996). Hypersensitive cell death, which is distinct from necrosis caused by metabolic toxins or severe trauma, is genetically programmed (programmed cell death [PCD]) and requires active host cell metabolism (Mittler and Lam, 1996; Morel and Dangl, 1997; Pennell and Lamb, 1997; Gilchrist, 1998; Gray and Johal, 1998; Heath, 1998; Richberg et al., 1998). In fact, plant cells undergoing the HR display several molecu-

lar and morphological markers characteristic of animal apoptosis (a specialized form of PCD), including systematic DNA degradation and formation of apoptotic-like bodies, which suggests that the terminal steps in PCD are well conserved in animals and plants (Levine et al., 1996; Mittler et al., 1996; Ryerson and Heath, 1996; Wang et al., 1996). It remains to be determined, however, whether the signal transduction mechanisms leading to the onset of PCD are also equally conserved between the two kingdoms.

Salicylic acid (SA) is the best-characterized signaling molecule in plant defense responses (Ryals et al., 1996; Delaney, 1997; Durner et al., 1997). Application of exogenous SA or SA analogs activates the expression of a variety of pathogenesis-related (*PR*) genes and enhances resistance to a variety of pathogens (White, 1979; Ward et al., 1991; Uknes et al., 1992). Conversely, transgenic plants expressing the bacterial *nahG* gene, which encodes an enzyme that degrades SA to catechol, display reduced or no expression of *PR* genes and fail to establish systemic acquired resistance (SAR; Gaffney et al., 1993; Delaney et al., 1994). Phenylalanine ammonia-lyase (PAL) is a key enzyme in the synthesis of SA (Bate et al., 1994; Mauch-Mani and Slusarenko, 1996; Coquoz et al., 1998; Smith-Becker et al., 1998), and genes encoding PAL are induced by a variety of

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pathogens and pathogen-derived elicitors (Lois et al., 1989; Wanner et al., 1995, and the references therein).

An important advance in our understanding of SA-mediated signaling response pathways was the isolation of Arabidopsis *npr1* (*nim1*, *sai1*) mutants, which fail to express *PR* genes or fail to induce SAR in response to exogenously supplied SA or its analogs (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). The recessive nature of these mutations suggests that NPR1 is a positive regulator that transmits SA signals to downstream components in a signaling pathway. In addition to *NPR1*, at least one other defense-related gene, *PAD4*, is directly involved in SA signaling. *pad4* (phytoalexin deficient) mutants, which were isolated by screening for enhanced susceptibility to virulent strains of *Pseudomonas syringae* or *Erysiphe orontii* (Glazebrook et al., 1996; Jirage et al., 1999), exhibit decreases in *PR-1* expression and SA accumulation after pathogen infection (Reuber et al., 1998; Zhou et al., 1998). Because the *pad4* mutant shows no defects in defense responses when infected with avirulent pathogens that elicit a strong HR, *PAD4* is thought to amplify weak SA signals, such as those resulting from infection by a virulent pathogen, to higher amounts sufficient for activation of SA signaling (Zhou et al., 1998; Jirage et al., 1999). In contrast to the *npr1* and *pad4* mutants, a different group of defense-related mutants, including *cpr1* (for constitutive expresser of *PR* genes; Bowling et al., 1994), *cpr6* (Clarke et al., 1998), and *acd2* (for accelerated cell death; Greenberg et al., 1994), accumulates greater amounts of SA constitutively and expresses SAR and *PR* genes continuously. As a consequence, these mutants exhibit enhanced resistance to various pathogens.

Recent evidence shows that in addition to SA, jasmonate (JA) and ethylene (ET) also play important roles as signal molecules mediating disease resistance, especially in response to necrotrophic fungal pathogens (Penninckx et al., 1996; Dong, 1998; Reymond and Farmer, 1998; Thomma et al., 1998; Glazebrook, 1999; Lam et al., 1999; Pieterse and van Loon, 1999). Interestingly, most of the JA/ET-dependent defense responses analyzed to date are SA independent. For instance, the *PDF1.2* gene encoding an antifungal plant defensin is induced concomitantly by the JA and ET signaling pathways (Penninckx et al., 1998), and the induction is enhanced in transgenic NahG plants (Penninckx et al., 1996). Induced systemic resistance (ISR) in Arabidopsis, triggered by the biocontrol bacterium *P. fluorescens* in association with Arabidopsis roots, also depends on both JA and ET but is independent of SA (Pieterse and van Loon, 1999). Interestingly, although ISR is SA independent, it is dependent on *NPR1*, indicating that the JA/ET- and SA-dependent resistance pathways share at least one key regulatory component (Pieterse et al., 1998; Pieterse and van Loon, 1999). Additional evidence for interaction of the SA and JA/ET pathways is that the *cpr6* and *acd2* mutations cause constitutive expression of the JA/ET-responsive *PDF1.2* gene as well as the SA-responsive *PR* genes (Penninckx et al., 1996; Clarke et al., 1998).

Useful model systems for the study of plant cell death in pathogen response pathways use PCD-eliciting mycotoxins such as AAL toxin or fumonisin B1 (FB1; Gilchrist et al., 1995; Gilchrist, 1997, 1998). AAL toxin is secreted by the tomato pathogen *Alternaria alternata* f sp *lycopersici*, and the structurally related toxin FB1 is produced by the maize pathogen *Fusarium moniliforme*. Gilchrist and co-workers have shown that these toxins induce apoptosislike cell death in tomato leaflets and protoplasts and that the cell death in the leaflets induced by AAL toxin is suppressed by inhibitors of ET biosynthesis or its action (Moussatos et al., 1994; Wang et al., 1996; Moore et al., 1999). They have further demonstrated that leaflets of the tomato *never ripe* mutant, which is deficient in ET perception, display fewer lesions in response to these toxins (Moore et al., 1999). These results indicate that an ET-dependent pathway is involved in the tomato cell death signaling triggered by AAL toxin or FB1. Using FB1, we have developed a pathogen-free model system to study disease response pathways in Arabidopsis plants (Stone et al., 2000). Leaves infiltrated with FB1 or seedlings transferred to FB1-containing media develop HR-like lesions and express *PR* genes.

In this article, we describe an Arabidopsis protoplast model for the analysis of defense-related cell death in Arabidopsis. We demonstrate that FB1 induces light-dependent PCD in Arabidopsis protoplasts and that this PCD requires SA, JA, and ET as well as one or more unknown factors activated by FB1 and the *acd2* mutation.

RESULTS

Induction of Apoptosis-like Cell Death in Arabidopsis Protoplasts

Protoplasts isolated from Arabidopsis leaves were treated with different concentrations of FB1 and analyzed for viability after various incubation times by using an Evans blue staining assay. Evans blue dye is excluded from viable cells and early stage apoptotic cells that retain intact plasma membranes (Gaff and Okong'O-Ogola, 1971; Turner and Novacky, 1974; O'Brien et al., 1997). As shown in Figure 1A, although a little cell death was observed in the absence of FB1, the toxin reduced protoplast viability in a dose-dependent manner. In the following experiments, protoplasts were treated with 70 nM FB1 for 16 hr before Evans blue staining. These conditions were chosen because they consistently reduced protoplast viability by 30 to 40% and because this proportion of cell death permitted detection of both increases and decreases in death caused by various treatments and mutations analyzed in this work.

FB1 killing was efficiently suppressed by cordycepin and cycloheximide, inhibitors of transcription and translation, respectively, and by staurosporine, a broad-spectrum inhibitor of protein kinases (Figure 1B). These results suggest that

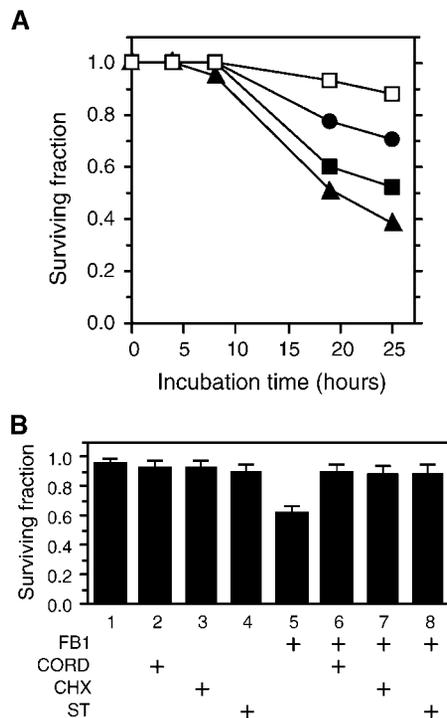


Figure 1. FB1-Induced PCD in Arabidopsis Protoplasts.

(A) Time and dose dependency of FB1-induced cell death. Protoplasts were prepared from wild-type plants and incubated in the light with various concentrations of FB1 (open squares, 0 nM; filled circles, 0.7 nM; filled squares, 70 nM; and filled triangles, 7 μM). Samples were withdrawn at 4, 8, 19, and 25 hr after the addition of FB1, and surviving fractions were determined as described in Methods. For control protoplasts (0 nM FB1), methanol was added instead of the FB1 solution at the final concentration of 0.1%. The methanol did not affect protoplast viability (not shown). The largest standard error in this experiment was 0.05.

(B) Requirement of plant cell activities for FB1-induced cell death. Protoplasts isolated from wild-type plants were incubated in the light for 16 hr in the presence of 70 nM FB1 (+FB1) or various inhibitors (100 μM cordycepin [+CORD], 10 μM cycloheximide [+CHX], and 1 μM staurosporine [+ST]), alone or in combination. In experiments 1 to 5, methanol was added instead of FB1 or inhibitor solutions. The final concentrations of methanol were as follows: experiment 1, 0.101%; experiments 2 to 4, 0.001%; and experiment 5, 0.1%. Surviving fractions were determined as described above. Error bars indicate SD.

FB1 does not induce necrosis in Arabidopsis protoplasts. Rather, FB1 most likely elicits cell death by activating a controlled program that requires active plant metabolism. As with penicillin, however, FB1 may require active host functions to express its killing effect (discussed in Greenberg et al., 1994). In the following sections, we show that protoplasts isolated from certain defense-related mutants are as insensitive to FB1 as protoplasts treated with the above in-

hibitors. Unlike the inhibitor-treated protoplasts, the mutant protoplasts would not be expected to exhibit the global shutoff of cellular metabolism. Thus, we conclude that FB1 triggers PCD in Arabidopsis protoplasts.

One hallmark of apoptosis is the fragmentation of nuclear DNA with disintegration of the nucleus and the subsequent formation of distinct apoptotic bodies containing the fragmented DNA (Martin et al., 1994). Typically, the TUNEL method, which labels free 3'-OH groups of DNA, is used to detect fragmented DNA and visualize apoptotic bodies in situ (Gorczyca et al., 1993). We applied this method to Arabidopsis protoplasts treated with FB1. As shown in Figure 2A, control protoplasts incubated for 6 hr without FB1 contained a single nucleus in each cell, as visualized by non-specific DNA staining with Hoechst 33342 (Wang et al., 1996). Specific labeling of fragmented DNA, however, was undetectable in the control protoplasts (Figure 2B). In contrast, protoplasts treated with 70 nM FB1 contained nuclei of various sizes and shapes, frequently accompanied by multiple distinct bodies containing DNA (Figure 2C). Furthermore, application of the TUNEL method to the same FB1-treated protoplasts clearly detected free 3'-OH groups of DNA within the nuclei and the distinct bodies (Figure 2D). The TUNEL assays described here were conducted 6 hr

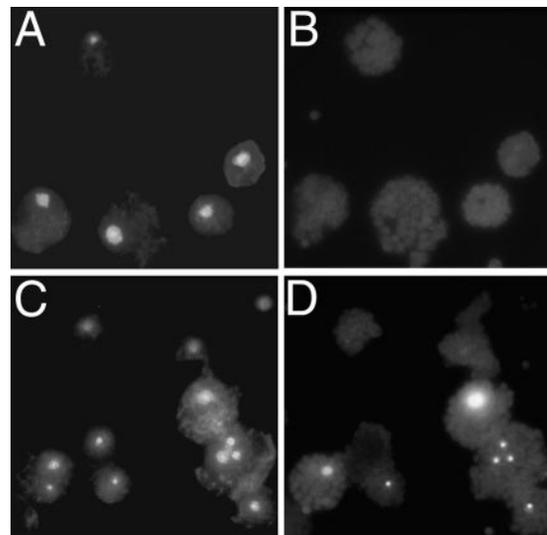


Figure 2. Detection of Fragmented DNA in FB1-Treated Protoplasts.

Protoplasts were prepared from wild-type plants and incubated for 6 hr in the light in the presence of 0.001% methanol or 70 nM FB1. DNA molecules in the protoplasts then were stained with Hoechst 33342, and free 3'-OH groups in the molecules were labeled by the TUNEL technique as described in Methods.

(A) Hoechst 33342 staining of methanol-treated protoplasts.

(B) TUNEL staining of methanol-treated protoplasts.

(C) Hoechst 33342 staining of FB1-treated protoplasts.

(D) TUNEL staining of FB1-treated protoplasts.

after FB1 treatment. Because FB1-elicited Evans blue staining did not occur until later (Figure 1A), nuclear DNA fragmentation apparently precedes the loss of membrane integrity, suggesting that the observed DNA fragmentation was caused by apoptotic rather than necrotic cell death (Martin et al., 1994; O'Brien et al., 1997). FB1-treated Arabidopsis leaves displaying systemic FB1-induced lesions (Stone et al., 2000) also showed TUNEL-positive cells (data not shown). Similar results were reported by Wang et al. (1996) after treating tomato leaves and protoplasts with an FB1 analog, AAL toxin. Although the presence of 3'-OH groups should not be used as a sole criterion for the non-random DNA fragmentation characteristic of apoptosis (Collins et al., 1992), these results are consistent with the conclusion that FB1 induces PCD in Arabidopsis protoplasts.

As shown in Figure 1A, ~40% of the protoplasts maintained membrane integrity, even after a 25-hr incubation with 7 μ M FB1; the addition of greater concentrations of FB1 did not markedly reduce the survival rate (data not shown). Although FB1-elicited killing could not be correlated directly with nuclear fragmentation (because the TUNEL assay was performed several hours before the protoplasts became Evans blue positive), we think it likely that ~40% of the cells may be unable to respond to FB1 or FB1-induced signals. In tomato, fully expanded leaves produce fewer lesions in response to AAL toxin than do rapidly expanding leaves (Moussatos et al., 1993a, 1993b). Thus, Arabidopsis protoplasts derived from older leaves may be less sensitive to FB1 than are protoplasts from younger leaves.

Mutational Analysis of Cell Death Signaling

To determine whether JA-, ET-, or SA-dependent signaling pathways are involved in FB1-induced cell death, we used the JA-insensitive mutant *jar1-1* (JA-resistant; Staswick et al., 1992), the ET-insensitive mutant *etr1-1* (ET-resistant; Bleecker et al., 1988), the SA-depleted mutant *pad4-1*, and the SA-depleted transgenic plant NahG. As shown in Figure 3A, protoplasts isolated from all four of these plants exhibited much less susceptibility to FB1, suggesting that all three signaling pathways are required for cell death. In contrast, *npr1-1* protoplasts were just as susceptible as wild-type protoplasts to FB1 (Figure 3A), even though NPR1 is required for both SAR and ISR, which are SA-dependent and JA/ET-dependent defense responses, respectively. These results suggest that FB1-elicited cell death requires SA and JA/ET signaling components upstream of NPR1 and are consistent with the observation that the *npr1-1* mutant develops the HR normally when infected with avirulent bacterial pathogens (Cao et al., 1994). Similar to FB1-elicited cell death, the constitutive expression of *PR* genes conferred by the *cpr6-1* and *ssi1* (for suppressor of SA insensitivity) mutations and the induction of *PDF1.2* by *Alternaria brassicicola* are also NPR1 independent, although they require the SA

and JA/ET signaling pathways, respectively (Penninckx et al., 1996; Clarke et al., 1998; Shah et al., 1999).

The *cpr1-1* mutant accumulates high amounts of SA constitutively (Bowling et al., 1994). As shown in Figure 3A, FB1-induced cell death of *cpr1-1* protoplasts was substantially greater than with wild-type protoplasts. When treated with 70 nM FB1, only 40% of the *cpr1-1* protoplasts survived, whereas 65% of the wild type survived. As described above, ~40% of the total protoplasts are not killed even with higher concentrations of FB1 and may be FB1 insensitive. Thus, one interpretation of the result obtained with *cpr1-1* protoplasts is that although SA, JA, and ET are all required for FB1-induced PCD, SA may be the limiting factor. This idea is compatible with the observation that in soybean

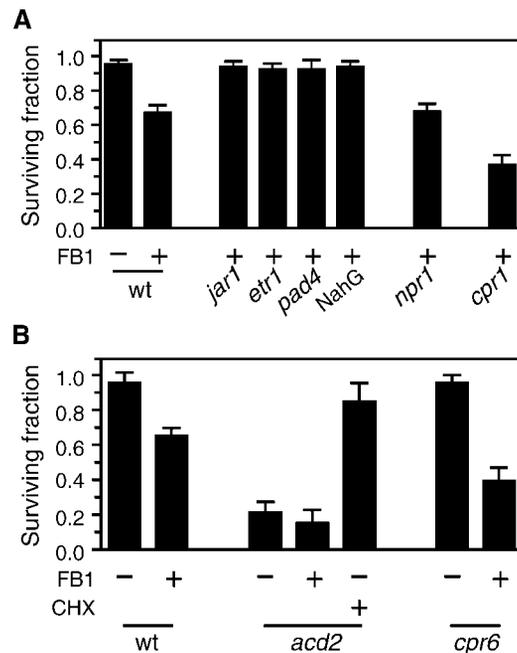


Figure 3. Mutational Analysis of PCD.

(A) Effects of various defense-related mutations on FB1-induced cell death. Protoplasts isolated from the indicated plants were incubated for 16 hr in the light, and surviving fractions were determined as described in Methods. FB1 was added at the final concentration of 70 nM (+FB1). To the control experiment (wild type [wt], -FB1), methanol was added instead of FB1 at the final concentration of 0.001%. In the presence of methanol alone, surviving fractions of these mutant protoplasts were similar to that of wild-type protoplasts (not shown).

(B) Effects of the *acd2-2* and *cpr6-1* mutations on protoplast viability. Protoplasts were isolated from the indicated plants and incubated for 16 hr in the light in the presence of 70 nM FB1 (+FB1) or 0.001% methanol (-FB1). Cycloheximide was added at the final concentration of 10 μ M (+CHX). Surviving fractions were determined as described in Methods. Error bars indicate SD.

cell suspensions, addition of exogenous SA greatly potentiates hypersensitive cell death triggered by a pathogen (Shirasu et al., 1997; Tenhaken and Rübeler, 1997). In our protoplast system, however, exogenous SA did not enhance FB1-induced cell death (T. Asai and F.M. Ausubel, unpublished data), suggesting that exogenous SA may not always increase cellular SA concentrations.

Several lines of evidence suggest that the JA-, ET-, and SA-dependent signaling pathways necessary for FB1-induced cell death are constitutively activated in the *cpr6-1* and *acd2-2* mutants (Penninckx et al., 1996; Clarke et al., 1998). In addition, similar to *cpr1-1*, SA accumulates constitutively to high concentrations in the *cpr6-1* and *acd2-2* mutants (Greenberg et al., 1994; Clarke et al., 1998). Therefore, in light of the results described above indicating that JA, ET, and SA are all required for FB1-induced PCD and that SA was most likely the limiting factor, we tested whether FB1 is dispensable for the death of protoplasts isolated from *cpr6-1* and *acd2-2* leaves. As predicted and as shown in Figure 3B, *acd2-2* protoplasts showed only ~20% of the viability of wild-type protoplasts, even in the absence of FB1. The toxin-independent death of *acd2-2* protoplasts is a programmed event because it was effectively suppressed by cycloheximide (Figure 3B). In contrast to *acd2-2*, however, the viability of *cpr6-1* protoplasts was similar to that of wild-type protoplasts in the absence of FB1. These results are consistent with the observation that the *acd2-2* mutant, but not the *cpr6-1* mutant, spontaneously develops HR-like lesions (Greenberg et al., 1994; Clarke et al., 1998). Enhanced FB1 susceptibility of *cpr6-1* protoplasts most probably results from high concentrations of SA in this mutant (see above). We conclude that induction of PCD in Arabidopsis protoplasts requires, in addition to SA, JA, and ET, a cell death factor or factors that are activated by FB1 or the *acd2-2* mutation but are missing in *cpr6-1* protoplasts (see Discussion).

Because of the toxin-independent cell death of *acd2-2* protoplasts, it is not clear whether high amounts of SA in *acd2-2* plants also potentiate FB1-induced cell death (Figure 3B). We therefore infiltrated FB1 into *acd2-2* plants and monitored the development of lesions. In these experiments, we infiltrated healthy leaves of *acd2-2* plants that had developed spontaneous lesions on the lower leaves (Greenberg et al. [1994] has demonstrated that SA contents are increased in such healthy leaves). For a control, we also infiltrated FB1 into wild-type plants. As shown in Figure 4, *acd2-2* leaves collapsed after 3 days, whereas wild-type leaves took 6 days to collapse. This accelerated lesion formation was not simply the result of the spontaneous cell death phenotype of the *acd2-2* mutant or of wounding (Greenberg et al., 1994). When infiltrated with 0.14% methanol (carrier for the FB1 infiltrate), *acd2-2* plants showed little lesion development after 3 days (Figure 4A). Moreover, in contrast to the *acd2-2* mutant, SA-depleted NahG plants infiltrated with FB1 produced much less severe lesions than the corresponding wild-type plants (Figure 4B). These observations are consistent with the results obtained with Arabi-

dopsis protoplasts and suggest that SA is an important regulator that determines the extent of cell death in Arabidopsis leaves.

Induction of JA-, ET-, and SA-Responsive Genes in FB1-Treated Protoplasts

The results described above suggested that FB1 increases cellular contents of JA, ET, and SA. Direct measurement of cellular concentrations of these molecules, however, is not practical, given the difficulty of obtaining a sufficient number of protoplasts from Arabidopsis leaves. Therefore, as an alternative to direct measurement of JA, ET, and SA concentrations in protoplasts, we monitored the expression of JA-, ET-, and SA-regulated genes instead. The rationale of this approach is that if FB1 increases the cellular concentrations of JA, ET, and SA, genes that are responsive to these molecules should be induced in FB1-treated protoplasts. To obtain a profile of FB1-induced gene expression in protoplasts, we created a subtracted cDNA library to represent mRNA species that are present at relatively high amounts in FB1-treated protoplasts but in relatively low amounts in untreated protoplasts (see Methods). As listed in Table 1, among the ~200 clones from this library that were sequenced, eight corresponded to genes (or homologs of genes from

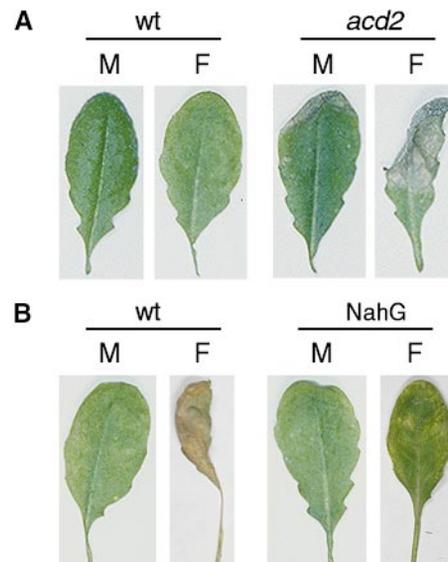


Figure 4. Lesion Formation by FB1 in Arabidopsis Leaves.

Five-week-old plants (wild type [wt], *acd2-2*, and NahG) were infiltrated with 10 μ M FB1 (F) or 0.14% methanol (M) as described by Greenberg et al. (1994) and incubated under a 12-hr light/dark cycle. Leaves were excised 3 or 6 days after infiltration for photographing.

(A) After 3 days.

(B) After 6 days.

other species) described in the literature as being induced by SA, JA, or ET. Although it is still necessary to confirm that the genes identified by subtractive hybridization actually are induced by FB1, the data in Table 1 are consistent with the conclusion that FB1 increases cellular concentrations of JA, ET, and SA. Interestingly, several of the FB1-induced genes encode proteins that are expressed during senescence, in which ET plays an important role (Pennell and Lamb, 1997; Gray and Johal, 1998; Johnson and Ecker, 1998). Some of these senescence-related genes induced by FB1 also are listed in Table 1. A description of the genes in the subtracted library that are not known to be SA, JA, or ET inducible will be published separately.

One of the genes that was present in the subtracted library was the Arabidopsis glucosyltransferase gene (Table 1), the homologs of which in tobacco are known to be induced by SA treatment with immediate-early kinetics (Horvath and Chua, 1996). To substantiate the conclusion that the glucosyltransferase gene is induced in protoplasts by FB1, we used a protoplast transient expression system (Kovtun et al., 2000) in which a luciferase (*LUC*) reporter gene was fused to the glucosyltransferase promoter to monitor the amounts of FB1-induced expression. To normalize the *LUC* activity for variation in transfection efficiency and cell viability, we cotransfected the protoplasts with a control plasmid carrying the constitutive ubiquitin1 (*UBQ1*) promoter (Holtorf et al., 1995) fused to the β -glucuronidase (*GUS*) reporter gene (Kovtun et al., 2000). The relative *LUC/GUS* activities shown in Figure 5 therefore represent relative promoter activities per viable protoplast. When wild-type protoplasts were incubated with 70 nM FB1 for 16 hr in the light, the promoter activity increased approximately fivefold. In NahG protoplasts, on the other hand, FB1 did not affect the promoter activity. Moreover, the basal promoter activity was reduced in NahG protoplasts. Although various factors could be affecting the expression of the glucosyltransferase

promoter in this system, the results are consistent with the idea that induction of the glucosyltransferase promoter is SA dependent and that FB1 increases SA concentrations in wild-type protoplasts.

The FB1-mediated accumulation of SA in Arabidopsis protoplasts does not appear to require de novo synthesis of *PAL1* or *PAL2* mRNAs because the *PAL1* and *PAL2* promoters fused to the same reporter gene were not induced substantially by FB1 (data not shown). As a control, we showed that the *PAL1* and *PAL2* promoters were strongly induced in the same assay by a different elicitor, Flg22 (Felix et al., 1999), derived from bacterial flagellin (T. Asai and F.M. Ausubel, unpublished data). At this time, how FB1 increases the intracellular SA concentrations is unclear. FB1 may activate PAL enzymes post-translationally. Or perhaps FB1 indirectly stimulates the hydrolysis of the glucose-conjugated form of SA (SA β -glucoside), increasing the cellular SA concentrations independently of PAL.

Effects of Light on Cell Death

Light has been shown to activate expression of the Arabidopsis *PAL* genes (Ohl et al., 1990). In fact, as shown in Figure 6A, *PAL1* promoter activity decreased approximately fivefold when wild-type protoplasts were incubated in the dark. The decrease in the promoter activity was reflected in a decrease in PAL enzyme activity (Figure 6B). SA concentrations in protoplasts as monitored by the activity of the glucosyltransferase promoter also were notably reduced in the dark, and the FB1 inducibility of the promoter was abolished (Figure 5). Indeed, the activity of the glucosyltransferase promoter in the dark was similar to that obtained in NahG protoplasts. This suggested that incubated in the dark wild-type protoplasts should be less susceptible to FB1, and indeed, this was demonstrated by the experiment

Table 1. Examples of FB1-Induced Genes and SA, JA, ET, and Senescence Inducibility

Gene	Identity/Similarity	Inducibility ^a				Reference
		SA	JA	ET	Senescence	
2B11	Glucosyltransferase	+	+	ND	ND	Horvath and Chua (1996)
2E8	Extensin	+	+	ND	ND	Merkouropoulos et al. (1999)
2E11	α -Vacuolar processing enzyme	+	-	+	+	Kinoshita et al. (1999)
1C11	Proteinase inhibitor I	ND	+	-	ND	Heitz et al. (1999)
1G9	Polyphenol oxidase	ND	+	ND	ND	Constabel et al. (1995)
2H5	ACC oxidase	ND	ND	+	+	Johnson and Ecker (1998)
5G5	Senescence-associated gene 21	ND	ND	+	+	Weaver et al. (1998)
1A5	Blue copper binding protein	ND	ND	+	+	Weaver et al. (1998)
1A6	Metallothionein	ND	ND	-	+	Butt et al. (1998)
1H12	ATP sulfurylase	ND	ND	ND	+	Buchanan-Wollaston (1997)
3E1	B12D protein	ND	ND	ND	+	To et al. (1999)

^a(+), induced; (-), not induced; ND, not determined.

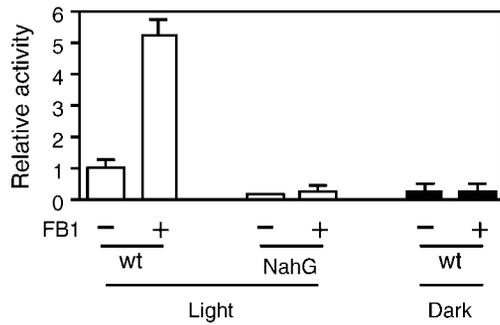


Figure 5. Inducibility of an SA-Responsive Promoter by FB1.

Activity of the Arabidopsis glucosyltransferase promoter was determined in wild-type (wt) and NahG protoplasts by using a transient expression system as described in Methods. The protoplasts were incubated for 16 hr in the light or in the dark in the presence of 70 nM FB1 (+FB1) or 0.001% methanol (-FB1). Promoter activities were normalized to the value obtained with wild-type protoplasts incubated in the light without FB1. Error bars indicate SD.

shown in Figure 6C. Interestingly, we also found that the toxin-independent death of *acd2-2* protoplasts is light dependent (Figure 6D).

DISCUSSION

In this article, we have shown that the Arabidopsis protoplast system is a useful tool for characterizing plant cell death signaling related to defense responses. By using the pathogen-derived cell death elicitor FB1, we can control the extent of cell death and create a situation in which cell death is semisynchronized. The number of protoplasts undergoing cell death is sufficient for pharmacological and molecular biological analyses. Unlike whole-plant systems, cell death can be quantified in the protoplast system similarly to its quantification in plant suspension cell systems. The advantage of the protoplast system over the suspension cell system is that the role of a variety of Arabidopsis defense-related genes in PCD can be readily assessed by preparing protoplasts from the corresponding mutants.

We propose the model shown in Figure 7 for the signal transduction pathways leading to cell death in Arabidopsis protoplasts. The conclusion that SA mediates FB1-induced PCD is based on the result that NahG protoplasts exhibit markedly less susceptibility to FB1, which is consistent with the observation that the HR against avirulent pathogens observed in wild-type Arabidopsis is impaired in transgenic NahG plants (Delaney et al., 1994). The *nahG* gene also suppresses spontaneous lesion formation in certain defense-related mutants such as *Isd6* and *Isd7* (for lesion-simulating disease; Weymann et al., 1995), *ssi1* (Shah et al., 1999), and *acd6* (Rate et al., 1999). FB1 also appears to elicit an in-

crease in SA concentrations in protoplasts, because it induces the SA-dependent glucosyltransferase promoter. However, because FB1 susceptibility is decreased in *pad4* protoplasts, in which a regulatory mechanism that amplifies SA signals is thought to be compromised (Zhou et al., 1998; Jirage et al., 1999), our results are also consistent with the conclusion that FB1 can induce only relatively small amounts of SA accumulation. This latter conclusion is supported by the observation that FB1 susceptibility increases in protoplasts isolated from the *cpr1*, *cpr6*, and *acd2* mutants,

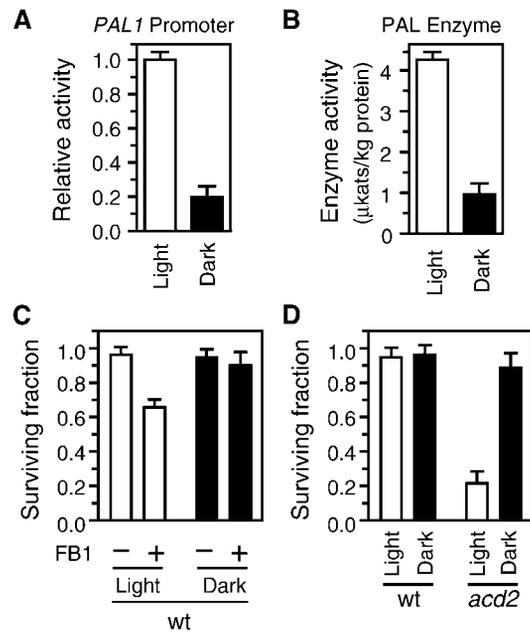


Figure 6. Effects of Light on PAL Expression, PAL Activity, and Cell Death.

(A) *PAL1* promoter activity in wild-type protoplasts analyzed with a transient expression system. Protoplasts transformed with a *PAL1* promoter-LUC construct were divided into two identical portions; one was incubated in the light and the other in the dark. LUC activities were assayed 16 hr after transformation, and the activity obtained in the dark was normalized to the activity in the light. See Methods for details of this assay system.

(B) PAL enzyme activities in wild-type protoplasts. Protoplasts isolated from wild-type plants were divided into two identical portions; one was incubated in the light and the other in the dark. After 16 hr, samples were taken and the PAL enzyme activity in each sample was determined.

(C) Effects of light on FB1-induced cell death. Protoplasts isolated from wild-type (wt) plants were incubated for 16 hr in the light or dark in the presence of 70 nM FB1 (+FB1) or 0.001% methanol (-FB1). Surviving fractions were determined as described in Methods.

(D) Effects of light on toxin-independent cell death. Protoplasts were isolated from wild-type (wt) and *acd2-2* plants and incubated for 16 hr in the light or dark before determination of the surviving fractions. Error bars indicate SD.

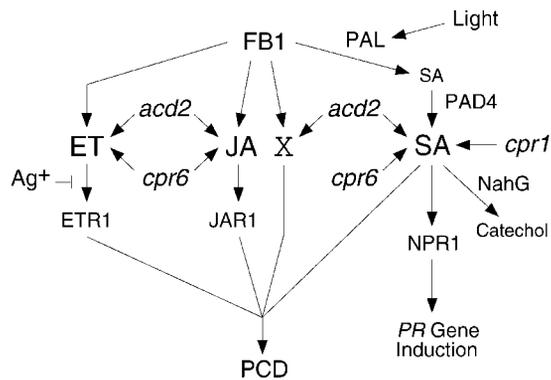


Figure 7. A Model for Cell Death Signaling in Arabidopsis Protoplasts.

Weak and strong SA signals are denoted by smaller and larger letters, respectively. As alternatives to the light-dependent PAL-mediated generation of SA shown in the model, SA might be produced from SA β -glucoside through a pathway independent of PAL and light, and the primary role of light in PCD may be to generate ROS rather than to activate the transcription of PAL genes. See text for details.

in which SA is overproduced (Bowling et al., 1994; Greenberg et al., 1994; Clarke et al., 1998).

Induction of cell death by FB1 and the *acd2* mutation requires light, suggesting that FB1- and *acd2*-inducible signals are insufficient to activate cell death signaling. The generation of reactive oxygen species (ROS) has been implicated in the HR as well as in lesion formation in the Arabidopsis *Isd1* mutant (Jabs et al., 1996; Lam et al., 1999; McDowell and Dangl, 2000). Therefore, one explanation for the observed light requirement for FB1-induced PCD is that ROS generated during photosynthesis are involved, either directly or indirectly, in the cell death process. For example, increased amounts of SA elicited by FB1 and the *acd2* mutation may inhibit H₂O₂-scavenging enzymes such as ascorbate peroxidase and catalase, leading to the enhanced sensitivity of protoplasts to the ROS produced during photosynthesis (Durner et al., 1997). A different, but not mutually exclusive, model for the light requirement of FB1-induced PCD is presented in Figure 7. As shown in Figure 5, FB1 appears to increase SA contents in wild-type protoplasts incubated in the light. However, under the same conditions, FB1 does not substantially induce the PAL promoter (data not shown). Thus, SA-mediated cell death triggered by FB1 may depend on light-induced PAL enzyme activity. Consistent with this hypothesis, wild-type protoplasts incubated in the dark display a concomitant reduction in PAL activity and FB1 susceptibility. The importance of PAL in SA-mediated cell death has been demonstrated previously (Mauch-Mani and Slusarenko, 1996; Shirasu et al., 1997).

The model shown in Figure 7 predicts that FB1 should induce SA-responsive *PR* genes in addition to the SA-responsive genes such as those that encode glucosyltransferase that were identified in the subtracted cDNA library (Table 1).

This was confirmed in FB1-treated seedlings, in which *PR-1*, *PR-2*, and *PR-5* mRNAs accumulated to high amounts 4 days after treatment (Stone et al., 2000). Preliminary experiments in protoplasts, however, showed that induction of a *PR1-LUC* fusion was undetectable within a 20-hr incubation with FB1—the longest treatment time feasible before a substantial number of the FB1-treated protoplasts died (T. Asai and F.M. Ausubel, unpublished data). In addition, *PR-1*, *PR-2*, and *PR-5* were not among the putative FB1-induced genes identified in the subtracted cDNA library (Table 1). In an accompanying paper (Stone et al., 2000), we analyzed the pattern of expression of the *PR-1* gene with a transgenic plant harboring a *PR-1* promoter–*GUS* fusion construct and found that *PR-1* expression was restricted to the cells surrounding the FB1-induced lesions. This suggests that *PR* gene expression may occur in response to a signal (or signals) emerging from dead or dying cells, rather than from within the cells undergoing cell death. Unless the signal is diffusible, the *PR* gene induction would not be expected in protoplast suspensions, in which individual cells are not directly attached to each other.

In addition to the SA signaling, Figure 3 shows that FB1-induced cell death requires the JA- and ET-dependent signal transduction pathways. To our knowledge, this is the only defense-related response for which induction clearly requires the concomitant activation of the SA-, JA-, and ET-dependent pathways. As is the case with SA, our data and the model shown in Figure 7 predict that FB1 should cause the cellular contents of JA and ET to increase. Indeed, in a separate study, we have demonstrated that the JA/ET-responsive *PDF1.2* gene is induced in FB1-treated seedlings 4 days after treatment (Stone et al., 2000). Moreover, analysis of cDNA molecules derived from FB1-induced mRNAs suggests that FB1 induces a variety of JA- and ET-responsive genes within 20 hr (Table 1; see Methods), although we have not identified the *PDF1.2* gene among the ~200 cDNA molecules sequenced to date. Involvement of ET in FB1-induced cell death is further supported by the observation that the ET action inhibitor Ag⁺ suppresses FB1-mediated cell death in protoplasts (T. Asai and F. Ausubel, unpublished data) and that FB1 induces the ET-forming enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (Table 1). Finally, induction of ET and its biosynthetic genes by AAL toxin and FB1 has been reported in tomato leaflets (Moussatos et al., 1994; Moore et al., 1999). ET has been well documented to be involved in developmentally controlled PCD such as senescence (Pennell and Lamb, 1997; Gray and Johal, 1998; Johnson and Ecker, 1998). Interestingly, a fraction of the FB1-induced genes in protoplasts encode senescence-related proteins (Table 1), suggesting that senescence and pathogen-elicited PCD may share a common molecular basis (Butt et al., 1998; Navarre and Wolpert, 1999; Pontier et al., 1999; Quirino et al., 1999).

The signaling pathways described above that are necessary for FB1-induced cell death are activated in the *cpr6* and *acd2* mutants. However, unlike the *acd2* mutant, the

cpr6 mutant does not exhibit spontaneous lesions, and protoplasts isolated from the mutant still require FB1 to undergo cell death. Perhaps in the *cpr6* mutant, activation of the pathways may not be strong enough to induce cell death. Another possibility is that the *acd2* mutation, but not the *cpr6* mutation, can activate an additional cell death factor or factors in the absence of FB1, bypassing the requirement of this elicitor for triggering cell death. This putative factor is denoted by the letter X in Figure 7, which cannot be constitutively activated in *acd2-2* plants because the mutant is viable. In *acd2* plants, spontaneous HR-like lesions develop primarily on the cotyledons and the older leaves (Greenberg et al., 1994). Therefore, in the absence of pathogens or pathogen-derived elicitors such as FB1, the unknown cell death factor may be regulated developmentally in a sporadic fashion. The factor could be a component of a signaling pathway normally involved in senescence. In the presence of the *acd2* mutation, the factor may be overproduced in the older tissue, accelerating natural senescence. This idea is consistent with the observation that FB1 induces many senescence-related proteins (see above). Greenberg et al. (1994) also have demonstrated that HR-like lesions can be initiated in *acd2* plants by mechanical stress such as wounding. Because plant tissue suffers mechanical stress during the course of protoplast isolation, the cell death factor is probably overproduced in *acd2* protoplasts, explaining why they are not viable.

The HR is closely correlated with disease resistance. However, a determinative role of the HR in restricting pathogen growth and spread has not been conclusively demonstrated (Richael and Gilchrist, 1999). An Arabidopsis mutant has been isolated in which effective pathogen resistance is expressed in the absence of HR cell death (Yu et al., 1998). Likewise, it is unclear whether the cell death signaling described here is involved in actual pathogen resistance. However, protoplasts isolated from the plants with enhanced pathogen resistance exhibit enhanced FB1 susceptibility, and protoplasts from the plants that show enhanced pathogen susceptibility often exhibit reduced FB1 susceptibility. These results may indicate that the cell death signaling identified in our protoplast system plays a critical role in developing defense responses in the entire plant. Protoplast systems are ideal for characterizing plant signaling components such as mitogen-activated protein kinases (Kovtun et al., 2000). Identification of the signaling pathways located upstream and downstream of SA, JA, and ET should help elucidate the role of PCD in pathogen resistance.

METHODS

Plants and Growth Conditions

All plants used in this work were *Arabidopsis thaliana* ecotype Columbia (Col-0) and its derivatives. Transgenic NahG plants were con-

structed in our laboratory (Reuber et al., 1998). The *pad4-1* (Glazebrook et al., 1996) and *acd2-2* (Greenberg et al., 1994) mutants were isolated in our laboratory; the *npr1-1* (Cao et al., 1994), *cpr1-1* (Bowling et al., 1994), and *cpr6-1* (Clarke et al., 1998) mutants were obtained from X. Dong (Duke University, Durham, NC); and the *jar1-1* (Staswick et al., 1992) and *etr1-1* (Bleecker et al., 1988) mutants were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Plants were grown in a greenhouse as described by Reuber et al. (1998).

Chemicals

Stock solutions of fumonisin B1 (FB1) and other chemicals were prepared as follows: FB1, 7 mM in methanol; cordycepin, 50 mM in 50% ethanol; cycloheximide, 0.8 M in methanol (all from Sigma); and staurosporine (Calbiochem, San Diego, CA), 1 mM in methanol. Stock solutions were diluted with water (FB1 and cycloheximide) or 50% ethanol (cordycepin).

Protoplast Preparation and Determination of Surviving Fractions

Protoplasts were isolated, under normal light conditions, from a mixture of rapidly expanding and fully expanded young leaves of ~4-week-old plants. We used the protocol described by Kovtun et al. (2000) except that leaf strips were digested for 3 hr in 1.5% cellulase and 0.4% Macerozyme (both from Yakult Pharmaceutical Co., Tokyo, Japan). Isolated protoplasts were suspended in Murashige and Skoog complete medium (Gibco BRL, Gaithersburg, MD) supplemented with 0.4 M glucose. After taking three samples for the initial cell count, we treated the protoplast suspension with FB1 or other chemicals, singly or in combination, and incubated them at 22°C either in the light ($40 \mu\text{mol m}^{-2} \text{sec}^{-1}$) or in the dark. After incubation, three samples were withdrawn, and Evans blue dye (Sigma) was added to the samples to a final concentration of 0.04%. The suspension was incubated for 10 min, and the number of unstained cells was determined under the light microscope with a hemacytometer. The fraction of surviving cells was calculated by dividing the number of viable cells after incubation by the initial cell count.

Detection of Nuclear DNA Fragmentation and Apoptotic Bodies

Total cellular DNA was stained with Hoechst 33342 (Sigma; $5 \mu\text{g mL}^{-1}$ in PBS), and the free 3'-OH groups in the DNA were labeled by the TUNEL method, using the In Situ Cell Death Detection Kit (Boehringer Mannheim) as instructed by the manufacturer (see also Wang et al., 1996). DNA was visualized with a fluorescent microscope.

Construction of a Subtracted cDNA Library

Protoplasts isolated from wild-type plants were divided into two identical portions; one was treated with 70 nM FB1 and the other with 0.001% methanol. The protoplasts were incubated in the light as described above, and samples were withdrawn at 6, 12, and 20 hr after treatment. A mixture of these three samples was used to

prepare total cellular RNA with the RNeasy kit (Qiagen, Valencia, CA), using the protocol for animal cells. The RNA then was treated with DNase I (amplification grade; Gibco BRL), and 1 μg of this DNase-treated RNA was used for cDNA synthesis with the SMART polymerase chain reaction (PCR) cDNA Synthesis kit (Clontech, Palo Alto, CA). To construct a cDNA library representing FB1-induced genes, we mixed cDNA molecules derived from FB1-treated protoplasts with excess cDNA from methanol-treated protoplasts, and subtraction was performed by using a PCR-based technique with the PCR-Select cDNA Subtraction kit (Clontech) as instructed by the manufacturer. Unsubtracted cDNA molecules then were ligated to a vector plasmid (pT-Adv; Clontech) and introduced into *Escherichia coli* cells by electroporation. The transformants were picked individually and used to amplify inserted cDNA molecules by PCR. (The PCR primers were provided in the above-described subtraction kit.) The amplified cDNA molecules were arrayed identically onto two nylon membranes with a slot blot manifold (PR648; Hoefer Scientific Instruments, San Francisco, CA); differential expression of these cDNA clones in FB1-treated protoplasts was confirmed by using the PCR-Select Differential Screening kit (Clontech). DNA fragments containing a part of the *UBQ10* coding sequence also were arrayed and used for an internal control. The fragments were amplified by PCR with the following primers: 5'-GGAGGTGGA-GAGTTCTGACACCATT-3' and 5'-CCTTGACGTTGCAATGGTGT-CGGA-3'. Identities of FB1-induced cDNA clones then were obtained by DNA sequence analysis and the advanced BLAST program (Altschul et al., 1997).

Protoplast Transient Expression Assays

The DNA regions (GenBank accession number AC006248) immediately upstream from the translation start sites of the glucosyltransferase (GenBank accession number AAD17393) and *PAL1* genes were amplified by PCR from Arabidopsis (Col-0) genomic DNA with the following oligonucleotide primers: glucosyltransferase, 5'-GGA-AAAGCTTAATGGTAGAAAGTAGAGG-3' and 5'-GGCCATGGTTAA-GAGTGGAAATCATG-3'; and *PAL1*, 5'-CGGGATCCGGAGGCTTTTGCC-TGCTAGT-3' and 5'-CCGTTAATGTCGACTTAGACTTTTGATC-3'. The sizes of the amplified fragments were 1400 and 1832 bp, respectively. The fragments were cloned in a *LUC* plasmid vector, pJD301 (Luehrsen et al., 1992), between the BamHI and the Sall sites. Protoplasts (2×10^5 cells) were transfected with the resulting plasmids (20 μg) by a polyethylene glycol procedure (Kovtun et al., 2000) and incubated in Murashige and Skoog complete medium containing 0.4 M glucose. LUC activity was determined as described by Luehrsen et al. (1992). To normalize the LUC activity for variation in transformation efficiency and cell viability, we cotransfected the protoplasts with a control plasmid (30 μg) carrying the constitutive *UBQ1* promoter (Holtorf et al., 1995) fused to the *GUS* reporter gene (Kovtun et al., 2000). The *UBQ1* promoter fragment (2312 bp) was amplified by PCR with the following primers: 5'-GTACCTCTCTGTGTT-CCAAACCATGG-3' and 5'-CTTTGGGATCCGTCTTCTCTCAC-GTAG-3'. *GUS* activities were determined as described previously (Jefferson, 1987).

Phenylalanine Ammonia-Lyase Enzyme Activity

Phenylalanine ammonia-lyase (PAL) enzyme activity was determined as described previously (Edwards and Kessmann, 1992).

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