Possible involvement of protein phosphorylation in the wound-responsive expression of *Arabidopsis* plastid ω-3 fatty acid desaturase gene

Hiroaki Kodama a,*, Takumi Nishiuchi a,1, Shigemi Seo b,c, Yuko Ohashi b,c, Koh Iba a

a Department of Biology, Kyushu University, Fukuoka 812-8581, Japan
b Department of Molecular Genetics, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan
c Core Research of Science and Technology (CREST), Tiyoda-ku, Tokyo 101-0062, Japan

Received 3 September 1999; received in revised form 21 January 2000; accepted 21 January 2000

Abstract

The plastid ω-3 fatty acid desaturase (FAD7) catalyzes the conversion of linoleic acid to linolenic acid. Wounding enhances the expression of the FAD7 gene in leaves and induces its expression in stems and roots. The wound-induced expression of the FAD7 promoter was investigated in transgenic tobacco plants carrying the −825 *Arabidopsis* FAD7 promoter:β-glucuronidase (GUS) fusion gene. The protein kinase inhibitor, staurosporine, and the protein phosphatase inhibitor, calyculin A, suppressed the wound induction of the FAD7 gene in stems. A tobacco mitogen-activated protein kinase (WIPK) was rapidly activated upon wounding not only in leaves but also in stems and roots, indicating that WIPK probably mediates the wound signals in most vegetative organs. The FAD7 promoter:GUS fusion gene was introduced into the transgenic tobacco plants in which the wipk gene was expressed constitutively at a high level or into the transgenic plants in which the wipk gene was suppressed possibly due to the transgene-induced gene silencing. The wound-induced expression of the FAD7 gene in stems was enhanced in the former transgenic tobacco plants and suppressed in the latter plants. These results suggest that the wound activation of the FAD7 promoter depends on both protein phosphorylation and dephosphorylation events especially in stems, and also that WIPK is involved in such signaling cascades. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Arabidopsis* plastid ω-3 fatty acid desaturase; calyculin A; jasmonic acid; staurosporine; wounding; wound-induced protein kinase

1. Introduction

Plants confront herbivore feeding by activating the defense systems that include the transcriptional activation of defensive genes, such as genes for proteinase inhibitor II (PI-II). Jasmonic acid (JA) is one of the signaling molecules for mechanical wounding (reviewed in [1]). Accumulation of JA in response to wounding causes a de novo induction of defensive genes and, therefore, is essential in plant defenses against insects [2] and pathogen attacks [3]. JA is synthesized from linolenic acid through a lipoxygenase-dependent process called the octadecanoid pathway [4,5]. The ω-3 fatty acid desaturases convert linoleic acid to linolenic acid in lipids and thus play a role in supplying a substrate for JA synthesis [6,7]. They are both localized in the microsome membranes and in plastid envelopes as two distinct isoforms (reviewed in [8]).

The FAD7 gene encodes the plastid ω-3 fatty acid desaturase [9]. Wounding enhances the accu-
mulation of FAD7 mRNA in leaf tissues of tobacco [10] and Arabidopsis thaliana [11]. In unwounded plants, the FAD7 gene is preferentially expressed in chloroplast-containing tissues. However, induction of the FAD7 gene by wounding was evident not only in leaf tissues but also in stem and root tissues. These changes in the spatial expression pattern of the FAD7 gene by wounding is thought to be mediated through different signal transduction pathways [11]. In leaf and stem tissues, the transcriptional activation of the FAD7 gene by wounding appears to occur independently of the wound-triggered JA accumulation. On the other hand, the wound activation of the FAD7 gene in root tissues is considered to be mediated by a JA-dependent signaling pathway (reviewed in [12]). In potato leaves, accumulation of transcripts by a JA-dependent signaling pathway (reviewed in [11]). In potato leaves, accumulation of transcripts of the plastid ω-3 desaturase gene preceded the wound-responsive expression of the PI-II gene. JA treatment had no effect on the level of potato plastid ω-3 desaturase mRNA, suggesting that wound-induced expression of this gene is also regulated by a JA-independent signaling pathway in leaves [7].

Protein phosphorylation cascades often constitute the intracellular signaling pathways that are activated in response to external signals. Several reports suggest that mitogen-activated protein (MAP) kinases are involved in the wound-activated signal transduction. In tobacco plants, several protein kinases with properties of MAP kinases have been identified [13–18]. Wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK) were demonstrated to be both wound-activated MAP kinases using antibodies specific for these protein kinases [19,20]. The role of WIPK in the wound-signaling cascade has been suggested by the analysis of transgenic tobacco plants in which the WIPK activity was modulated. When the wipk cDNA was introduced into tobacco plants under the control of a constitutively expressed promoter, two independent plant lines with distinct properties were isolated. In one transgenic line (TMP1S-13), the endogenous wipk gene was inactivated possibly by transgene-induced gene silencing and the accumulation of both JA and mRNAs for JA-inducible genes were suppressed after wounding [13]. Another line (35S::wipk-8) conferred a constitutive activation of WIPK without wounding and showed 3–4-fold higher levels of JA than did nontransformed tobacco plants. In the latter transgenic plant, the PI-II mRNA accumulated in unwounded leaves [20]. These results suggest that the level of activated WIPK is closely related to the JA level.

To delineate the signal transduction pathways that ultimately lead to the wound-responsive expression of the FAD7 gene, we investigated here the possible involvement of protein phosphorylation in the wound activation of the FAD7 promoter in presence of inhibitors for both protein kinases and protein phosphatases. We also investigated the wound responsiveness of the FAD7 promoter in the transgenic tobacco plants in which WIPK activity is modulated.

2. Materials and methods

2.1. Plasmid construction

The Ti-plasmid, pfD82, contains the -825 Arabidopsis FAD7 promoter::β-glucuronidase (GUS) reporter fusion gene (FAD7 pro::GUS, [21]). The 2-kb EcoRI fragment of the pYL3 [22] contains promoter sequence from the nopaline synthase gene, the hygromycin phosphotransferase gene and the terminator sequence from the nopaline synthase gene. This fragment was cloned into the EcoRI site of the pfD82 to generate plasmid, pfD82h.

2.2. Plant materials

Tobacco (Nicotiana tabacum cv W38) plants transformed with pfD82 were generated as previously described [21]. The WIPK-suppressed line (TMP1S-13) and another transgenic line in which the WIPK was constitutively active (35S::wipk-8) were generated by transformation of N. tabacum cv Samsun NN with the cauliflower mosaic virus (CaMV) 35S promoter::wipk cDNA construct (35S::wipk) as described [13,20]. In this paper, both the TMP1S-13 and the 35S::wipk-8 plants are mentioned as 35S::wipk plants. The N. tabacum cv Samsun NN plants into which the T-DNA of pfD82h was introduced were produced by Agrobacterium-mediated transformation. The R0 plants transformed with pfD82h were crossed with the TMP1S-13 or 35S::wipk-8 plants as pollen donors. The resulting R1 seeds were aseptically germinated on the Mu-

rashige–Skoog medium supplemented with 20 μg/ml hygromycin B. The parental plants, that were transformed with the 35S::wipk, are resistant to kanamycin but sensitive to hygromycin. Therefore, the R₁ seedlings that are hygromycin-resistant must carry the T-DNA of the pfD82h. The hygromycin-resistant R₁ plants were transferred to soil, grown at 26°C under continuous light illumination (2000 lux) and subjected to analyses of the segregation of 35S::wipk transgene and of their wound responsiveness.

All experiments used 2–3-month-old tobacco plants.

2.3. Segregation of the 35S::wipk transgene

To investigate the inheritance of the 35S::wipk, total DNA was prepared from leaves of each hygromycin-resistant R₁ plant according to the method of Edwards et al. [23]. The 35S::wipk transgene in the genome was PCR-amplified using the CaMV 35S promoter-specific primer, 35Sp1 (5’-ACTATCCTCGCAAGACCT-3’), and the wipk gene-specific primer, WIPK-N1 (5’-GGTGAAGATCAGTATG-3’). The 474-bp fragment with a portion of wipk was amplified from total DNA prepared from the hygromycin-resistant R₁ plants harboring the 35S::wipk transgene but the gene was not amplified from hygromycin-resistant R₁ plants in which the 35S::wipk transgene was segregated out.

2.4. Wound treatments with or without inhibitors

Tobacco leaves were cut into discs 7.5 mm in diameter with a cork borer. Stems were cut into about 1-mm-thick slices with a razor blade and roots were cut into about 1-cm-long sections as previously described [11]. Soluble proteins of an aliquot from each sample were prepared immediately after the preparation of tissue segments and subjected to the measurement of their GUS activity as unwounded tissues. Other tissue fragments were floated on 50 mM sodium phosphate buffer, pH 7.0, with or without dimethyl sulfoxide (DMSO), staurosporine, or calyculin A at indicated concentrations. Inhibitors were dissolved in DMSO, and final concentration of DMSO was 1% (v/v) or less in these wound treatments. The above solutions were vacuum-infiltrated into intracellular spaces of each organ tissue as described by Conrath et al. [24]. Leaf and stem fragments were incubated at 26°C under light, and root segments were placed in the dark for 6 h. After wounding, soluble proteins were prepared from leaves, stems, and roots and the GUS activities in soluble proteins were fluorometrically determined as previously described [21].

2.5. Assay of myelin basic protein kinase activity of immune-complex proteins

The wild-type tobacco plants (N. tabacum cv Samsun NN) grown in a greenhouse were subjected to an immune-complex kinase assay. Wounding were performed by cutting each organ with a pair of scissors. The soluble proteins are prepared from each organ as previously described [20]. Immunoprecipitation was performed with anti-WIPK antibody and myelin basic protein kinase activities of resultant immune-complexes were determined by the kinase assay as described [20].

3. Results

3.1. Effects of inhibitors of protein phosphorylation and dephosphorylation on the wound activation of the FAD7 promoter

A diarrhetic shellfish poison, calyculin A, inhibits protein phosphatases including protein phosphatases 1 and 2A [25]. Staurosporine is a natural broad range inhibitor of serine/threonine protein kinases [26]. To examine whether protein phosphorylation and dephosphorylation steps are involved in the signaling cascade leading to wound activation of the FAD7 promoter, we analyzed the wound-responsive expression of the GUS reporter gene in tobacco plants (N. tabacum cv W38, [21]) harboring the –825 FAD7 pro::GUS fusion gene in the presence of each inhibitor (Fig. 1). Local wounding treatments were performed by incubation of leaf discs, stem slices and root segments in sodium phosphate buffer with or without inhibitors. The solvent, DMSO, had slightly promotive effects on the induction of GUS expression by wounding in all organs tested. In the presence of 5 μM staurosporine or 1 μM calyculin A, wound-induced expression of GUS was still evident in leaf tissues. In stems, the presence of 1 μM stau-
Fig. 1. Effects of staurosporine and calyculin A on the wound induction of GUS expression driven by the FAD7 promoter. GUS activities in each organ of healthy plants transformed with the FAD7 pro::GUS gene were assayed in unwounded tissues (open boxes). Aliquot samples of each organ were incubated in sodium phosphate buffer for 6 h (hatched boxes). Alternatively samples were incubated for 6 h in sodium phosphate buffer supplemented with a solvent only (+ DMSO), staurosporine (+ Sta) or calyculin A (+ Cal) at the concentrations indicated in this figure (closed boxes). GUS activities were then assayed in wounded tissues. GUS activity was expressed as nmole of 4-methylumbelliferone (MU) generated per min per mg protein. (A) Promoter activity in leaves. (B) Promoter activity in stems. (C) Promoter activity in roots. Values are means ± S.E.M. (n = 5).

Staurosporine did not affect the induction of GUS expression significantly. However, the wound-induced elevation of GUS activity reduced to about 18% of controls by incubation of stems in 5 μM staurosporine. Incubation of stem tissues in calyculin A resulted in remarkable inhibitory effects on the induction of GUS expression by wounding. In the presence of 0.2 and 1 μM calyculin A, the wound-induced elevation of GUS activity decreased to about 55 and 6% of controls, respectively. In roots, 1 μM staurosporine or 0.2 μM calyculin A did not affect an increase in GUS activity in response to wounding. However, wound induction of GUS expression was partly inhibited when root tissues were treated with 5 μM staurosporine or 1 μM calyculin A. These results suggest that the wound-induced expression of the GUS gene under control of the FAD7 promoter is mediated by a signal transduction pathway involving the phosphorylation and dephosphorylation of unidentified proteins. These phosphorylation and dephosphorylation events seem to be relevant for FAD7 expression mainly in stems, partially in roots, and not in leaves.

3.2. Wound-induced WIPK activation in stem and root tissues

Possible candidates for wound-signal mediating protein kinases are MAP kinases [19,20]. In these studies, leaf tissues alone had been subjected to the kinase assays. Therefore, we assessed the activation of WIPK in other vegetative organs of N. tabacum cv Samsun NN plants. Total soluble proteins from leaves, stems and roots were immunoprecipitated with the WIPK-specific antibody, and the resultant immune-complexes were subjected to the kinase assay. Myelin basic protein kinase activity of WIPK drastically increased within 5 min after wounding in stems and roots, similar to leaf tissues (Fig. 2). This result suggests that protein kinases such as the WIPK participate in wound-induced signal transduction not only in leaves, but also in the stems and roots.

Fig. 2. Activation of WIPK by wounding in leaves, stems and roots of tobacco plants (N. tabacum cv Samsun NN). Leaves, stems and roots of the wild-type plants were cut with a pair of scissors, and harvested at indicated time points. Soluble proteins were subjected to an immune-complex kinase assay with myelin basic protein (MBP) as a substrate. An arrowhead indicates the phosphorylated MBP.
Several hygromycin-resistant R₀ plants were selected in which significant GUS activities in chlorophyllous tissues were observed as previously reported [21]. Among them, the transgenic lines that showed a segregation of T-DNA as single functional T-DNA insertional plants were subjected to the cross with the TMP1S-13 or 35S::wipk-8 plants. The resultant R₁ seeds were aseptically germinated on hygromycin-containing medium. Then, hygromycin-resistant R₁ plants were classified into two groups based on the PCR-amplification of a portion of the wipk gene (Section 2); one group had the 35S::wipk transgene (FAD7 pro::GUS/35S::wipk), and in another group the 35S::wipk transgene was crossed out (FAD7 pro::GUS/null). These two groups were provided from a common seed population that had been produced by crossing a R₀ FAD7 pro::GUS transformant with the 35S::wipk plants, and thus shared the same genetic backbone except for the inheritance of the 35S::wipk transgene. It has not been confirmed whether the 35S::wipk transgene confers the same phenotype to the FAD7 pro::GUS plants when compared to the previously published phenotypes for the 35S::wipk plants [13,20]. However the expression of the PI-II gene in the progeny plants produced by self-pollination of the TMP1S-13 and 35S::wipk-8 plants was nearly the same as those of the parental plants (Seo et al., unpublished results).

The GUS activities of these FAD7 pro::GUS transgenic plants in unwounded and wounded conditions are shown in Fig. 3. When the FAD7 pro::GUS plants were crossed with the 35S::wipk-8 plants or TMP1S-13, the resulting progenies showed markedly higher GUS activity in unwounded leaves than those in unwounded stems and roots (Fig. 3A and C). There was no significant difference in the GUS activity between plants with or without the 35S::wipk transgene in unwounded condition.

In a tobacco cultivar, W38, we repeatedly observed the wound-responsive elevation of GUS activity in leaves as seen in the control data in Fig. 1 [11]. However in the FAD7 pro::GUS/null plants, activation of the FAD7 promoter upon wounding was not obvious (Fig. 3B and D), and similar results were observed in another FAD7 pro::GUS transgenic line that had been generated by using N. tabacum cv Samsun NN plants (data not shown). The reason for such different wound
responses of the FAD7 promoter between W38 and Samsun NN is not clear at present. The progenies of TMP1S-13 plants containing the 35S::wipk transgene showed no apparent wound induction of GUS activity in leaves, and those of 35S::wipk-8 plants showed only a barely significant elevation of GUS activity (Fig. 3B and D).

In stems, wounding induced an increase in the GUS activity (about 4-fold) in the FAD7 pro::GUS/null plants. In the progenies of 35S::wipk-8 plants, the FAD7 pro::GUS/35S::wipk plants exhibited higher GUS activity in wounded stems than those of corresponding null plants. By contrast, the wound induction of the FAD7 promoter was weaker in the presence of the 35S::wipk transgene in the progenies of TMP1S-13 plants compared to the null plants (Fig. 3B and D).

There were only slight differences in the GUS activity of wounded roots between 35S::wipk and the corresponding null plants. However, the ratio of GUS activity of wounded roots to that of unwounded roots decreased by the 35S::wipk transgene in the TMP1S-13 progenies and increased significantly in the 35S::wipk-8 progenies (Fig. 3B and D).

4. Discussion

In this study, we showed that the staurosporine-sensitive protein kinase(s) might play a role in the signal transduction cascade that leads to wound-induced activation of the Arabidopsis FAD7 promoter (Fig. 1). Staurosporine exhibited the most pronounced effects in stem tissues. In tobacco plants, several MAP kinases have been found to be activated upon wounding [13,15,19,20]. Although in most previous studies, the wound activation of MAP kinases had been demonstrated only in leaf tissues, we found that WIPK was also activated in stem and root tissues by wounding (Fig. 2). Therefore, WIPK is one of the possible candidates for the protein kinases responsible for the wound induction of the FAD7 gene in stems.

Analysis of the phenotypes of FAD7 pro::GUS/35S::wipk plants also suggested that WIPK mediates the wound signal to induce the expression of the FAD7 gene (Fig. 3). In unwounded tissues, the expression levels of GUS gene driven by the FAD7 promoter were not much different between the progenies of the TMP1S-13 and 35S::wipk-8. Although the responsiveness of transcriptional activity of the FAD7 gene to wounding was lower and higher in all tissues examined of the FAD7pro::GUS/35S::wipk progenies of TMP1S-13 and 35S::wipk-8, respectively, when compared with the corresponding FAD7 pro::GUS/null plants (see the ratios shown under the Fig. 3B and D), the only significant difference of the FAD7 promoter activity between the two transgenic lines was observed when stem tissues were wounded (Fig. 3B and D). These results suggest that WIPK might phosphorylate certain proteins that function as positive regulators for transcriptional activation of the FAD7 promoter in stem tissues. In stems, a nuclear protein was detected that interacted with the wound-responsive region (−242 to −223) of the FAD7 promoter, and formation of this nuclear protein–DNA complex was stimulated significantly by wounding [27]. Interestingly, nuclear extracts treated with alkaline phosphatase lost the ability to form this complex, suggesting that a phosphorylated nuclear protein binds to this wound-responsive region (Nishiuchi et al., unpublished data). Therefore, this nuclear protein is a possible candidate for the target protein of the WIPK or its downstream protein kinases, although further study is required to clarify the identity and role of this phosphorylated nuclear factor in the wound-inducible expression of the FAD7 gene. In this context, it is implied that WIPK is involved not only in the regulation of the wound-induced responses via JA biosynthesis [13,20] but also in JA-independent wound signaling cascade at least in stem tissues.

In root tissues, it was suggested that the FAD7 promoter activity is modulated by JA accumulation in response to wounding [11]. Although the 35S::wipk-8 plants can be expected to contain increased JA levels not only in unwounded leaves [20] but also in roots, the activity of the FAD7 promoter was almost the same in both TMP1S-13 and 35S::wipk-8 progenies (Fig. 3A and C). The elucidation of involvement of JA in the regulation of transcriptional activity of FAD7 gene in the root tissue of 35S::wipk plants requires more detailed analyses since JA levels in root tissues of these parental transformants has not been determined.

In this report, we showed that protein kinases and phosphatases possibly take part in the wound-induced signal transduction that leads to transcriptional activation of the FAD7 gene. In
Arabidopsis, it has been suggested that protein kinases positively regulate the wound-responsive expression of several genes, such as WR3 and choline kinase genes, and that the activity of protein phosphatases is also required for the activation of several other wound-responsive genes [28]. In this case, it was shown that protein kinases and phosphatases may play opposite roles in the activation of wound-responsive genes. By contrast, our results suggest a unique mode of regulation of activation of wound-responsive genes. By contrast, it was shown that protein kinases and phosphatases may play opposite roles in the activation of wound-responsive pathways leading to protein kinases and phosphatases may function in wound signal transduction pathways leading to expression of the FAD7 gene.

Acknowledgements

This research was supported in part by Grant RFTF96L00602 from the Japan Society for Promotion of Science to KI.

References


