Characterization of F_2 Progenies of Wound Minus *Arabidopsis* Mutant Crossed with Wild Type Plant

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To understand the signal transduction pathway that leads to the activation of the wound-inducible proteinase inhibitor II (*pin*2) promoter, F_2 progenies of wound (-) mutant crossed with wild-type *Arabidopsis* plants were biochemically and genetically characterized. Wound (-) mutant was derived from transgenic *Arabidopsis* plants containing bacterial cytosine deaminase gene under the control of *pin*2 promoter. The cytosine deaminase assays indicated that wound (-) mutant is a dominant inhibitor of wound-inducibility as only 3 of the 20 F_2 progenies showed cytosine deaminase (CDase) activity. To construct a structural map of the wound (-) mutant chromosomal regions, cleaved, amplified polymorphic sequences (CAPS) markers that cover all chromosomes were used. Chromosomal regions covered by three different CAPS markers could be candidates for further fine mapping of the location of the wound (-) mutation. g4026, RGA1, and ASA1, located at 84.9 on recombinant inbred (RI) map of chromosome I, at 1.75 on RI map of chromosome II, and 18.35 on RI map of chromosome V, respectively.

Key words: *cytosine deaminase, CAPS markers, wound-inducible, jasmonate.*

The biochemistry of wound-induction in plant involves extracellular signals that trigger physiological responses within cells. This process is mediated by alteration of lipid metabolism. Chloroplastic lipids are modified and subsequently metabolized into jasmonic acid. Jasmonate then triggers the induction of quiescent genes in the nucleus.[1,2] In spite of this knowledge on the induction of wound-inducible genes, much yet remains to be elucidated to fully understand the molecular details of the wound-inducible pathway. Specific details that are missing in our understanding of this signal transduction pathway include the nature of cellular receptors that mediate the response to the extracellular signals and the transfer of that information to the chloroplast where lipid metabolism is altered. Further mechanisms that lead to the induction of the wound-inducible genes by methyl jasmonate are completely unknown. These last steps are particularly important, because they are the intermediate events that precede gene activation. The wound-inducible potato proteinase inhibitor II (*pin*2) promoter[3] was used as a molecular tool for the study of these processes.

To understand the signal transduction pathway that leads to the activation of the *pin*2 promoter, an attention on the steps late in the induction process, specifically the events following jasmonate production. A unique method was established to produce directed mutations in plants. This method utilizes the antimetabolite, FC, with CDase.[4] FC by itself is not toxic. But once inside the cell, if the CDase is present, FC is converted into fluorouracil which is eventually metabolized into 5-fluorodeoxy UMP (FdUMP). This FdUMP is a suicide inhibitor of thymidylate synthase in both microbes and plants, resulting in a thymidine starvation that kills cells.[4] While most bacteria[5,6] and many fungi[7] produce CDase, plants do not.[8] Cells which do not express CDase are completely insensitive to FC as FC is not metabolized further in cells which lack CDase. Because wild-type plants are insensitive to FC, they provide the basis for a very strong negative selection system.[4] A chimeric construct that express CDase gene (*cod*A) under the regulation of wound-inducible *pin*2 promoter was engineered. *Arabidopsis* plants were transformed with *pin*2-*cod*A construct. Transgenic plants (Tr349) which contained CDase activity induced in the presence of methyl jasmonate and killed in the presence of FC were selected (Zhou and Thornburg, unpublished). Seeds of selected transgenic plants were mutagenized with the mutagen, ethyl methanesulfonate (EMS), to produce wound (-) mutants (Zhou and Thornburg, unpublished). Wound (-) mutant plants could be selected for plants that fail to express the *pin2-codA* transgenes in the presence of jasmonic acid. To evaluate the genetics of the wound (-) plants, crosses to plants with wound-inducible wild type were prepared. Selection of the F_1 plants on kanamycin insures that the progenies are of the desired cross because kanamycin resistant gene is present with *pin*2-*cod*A construct in the plant cell. In this report, F_2 plants which

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Abbreviations: CAPS, cleaved, amplified polymorphic sequences; CDase, cytosine deaminase; Col, *Columbia*; FC, 5-fluorocytosine, Ler, *Lansberg erecta;* PCR, polymerase chain reaction; RI, recombinant inbred; TLC, thin-layer chromatography.

were made by self-pollinating F_1 plants were biochemically and genetically examined.

Materials and Methods

Plant growth. F_2 seeds were germinated on the Murashige-Skoog (MS)[9] solid medium containing kanamycin (50 mg/l). After two weeks, small plants were transferred to soil and grown at 28°C under 3000 lux light for 12 h/day and 12 h/night for 2 or 3 weeks.

Induction of *pin*2 promoter. For the induction in leaves, a half leaf assay similar to the method of Kernan and Thornburg[10] was used. One half of *Arabidopsis* leaf was incubated in MS liquid media containing 25 mM methyl jasmonate under the fluorescent light for 18 H. The other half of leaf was stored at -70°C as an uninduced sample.

Cytosine deaminase assay. Cytosine deaminase (CDase), a critical test in this experiment, assay was conducted according to the method of Stougaard[4] as modified below. In the protocol of Stougaard, 10 μl plant extracts was used per assay. Though extracts containing 100 μg gave better results, it was difficult to load so much extract on to a TLC plate and also the extracts interfered TLC development. Thus, the protocol was modified by extracting reaction mix with 1-pentanol/ethyl acetate (v/v, 1:1) after enzyme reaction to eradicate other interfering materials from plant extract. This organic extraction recovered almost 100% of 2-^{14}C-cytosine and 2-^{14}C-uracil from the reaction which made loading and development easier.

Plant leaves were homogenized in extraction buffer [(0.2 M Tris-HCl, pH 7.8, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 0.1% Sarkosyl and 2 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenized juice was centrifuged at 10,000 g for 10 min, and the supernatant was used for protein concentration determination method of Bradford.[11] Ten microliter of assay buffer (0.2 M Tris-HCl pH 7.8, 1 mM DTT, 1 mM EDTA, 2 mM PMSF, 0.05 μCi 2-^{14}C-cytosine) was added to 100 μg protein extracts. The enzyme reaction was carried out at 37°C for 3 H. The reaction mixture was made up to 100 μl with dH_2O and extracted with 1 ml of 1-pentanol/ethyl acetate (v/v, 1:1). The organic phase was dried in vacuum. Twenty microliter of 1-pentanol/ ethyl acetate (v/v, 1:1) was added to each tube and spotted on a DC-Alufolien cellulose TLC plate (Alltech Associate, Inc.). The TLC plate was developed in 1-butanol/H_2O (v/v, 86:14) for 10 H. The plate was dried, wrapped, and autoradiographed. The position of uracil of cytosine on the TLC plate was determined by the locationg radiolabelled uracil and cytosine. For quantitation of cytosine deaminase activity, the spots containing 2-^{14}C-uracil and cytosine were counted in a scintillation counter. The enzyme activity was defined as the percent conversion of cytosine into uracil.

Polymerase chain reaction (PCR) primers for CAPS markers and PCR amplification of *Arabidopsis* genomic DNA. The fine structure map of the wound (-) mutant in *Arabidopsis* chromosomes can be developed by utilizing cleaved CAPS markers that cover all chromosomes.[12] PCR primers for CAPS markers were synthesized at Iowa State University Nucleic Acids Facility. Nucleotide sequences of PCR primers and location on chromosomes are shown in Table 1.

Table 1. Primers used for initial CAPS mapping.

Marker	Chromosome	Position[a]	Enzyme	Size of products in kb		Primers
				Columbia	Lansberg	
UFO	I	47.52	*Taq* I	0.983, 0.816	0.6, 0.383, 0.316	5'-GTGGCGGTTCAGACGGAGAGG-3' 5'-AAGGCATCATGACTGTGGTTTTT-3'
g4026	I	84.9	*Rsa* I	0.65	0.8	5'-GGGGTCAGTTACATTACTAGC-3' 5'-GTACGGTTCTTCTTCCCTTA-3'
RGA	II	1.75	*Rsa* I	0.143, 0.120	0.263	5'-GTTTAAGCAAGCGAGTATGC-3' 5'-TTCGATTCAGTTCGGTTTAG-3'
er	II	50.64	*Dde* I	0.5	0.36, 0.14	5'-CTAATGTAGTGATCTGCGAGGTAATC-3' 5'-GAGTTTATTCTGTGCCAAGTCCCTG-3'
ve017	II	69.14	*Pst* I	0.214, 0.143	0.357	5'-CTTGAAGCTTAAATCTCTCAGC-3' 5'-GAGCAATCCAGTAGAGGATA-3'
GAPC	III	8.41	*EcoR* V	0.735, 0.713	0.713, 0.39, 0.34	5'-CTGTTATCGTTAGGATTCGG-3' 5'-ACGGAAAGACATTCCAGTC-3'
GL1	III	48.45	*Taq* I	0.298, 0.1, 0.074, 0.047	0.372, 0.1, 0.0047	5'-ATATTGAGTACTGCCTTTAG-3' 5'-CCATGATCCGAAGAGACTAT-3'
GA1	IV	17.71	*BsaB* I	0.707, 0.527	1.196	5'-AAGCTTCGAACTCAAGGTTC-3' 5'-CCGGAGAATCGTACGGTAC-3'
g4539	IV	57.64	*Hind*III	0.6	0.48, 0.12	5'-GGTCATCCGTTCCCAGGTAAAG-3' 5'-GGACGTAGAATCTGAGAGCTC-3'
ASA1	V	18.35	*Bcl* I	1.042, 0.686	0.686, 0.553, 0.489	5'-CTTACTCCTGTTCTTGCTTAC-3' 5'-CCTCTAGCCTGAATAACAGAAC-3'
g2368	V	125.12	*Hind*III	1.4	1.35, 0.05	5'-AAGCTTTTGAATAGGACAGCATTG-3' 5'-CGTTTTCATTGGTCCACTGCATGG-3'

[a]at recombinant inbred (RI) map

Genomic DNA isolation and PCR reactions were performed according to the published method[12] with minor modification. Leaves of *Arabidopsis* were ground with a grinder (Con-Torque, Eberbach) in the presence of 500 µl extraction buffer (100 mM Tris, pH 8, 50 mM EDTA, 500 mM NaCl, and 10 mM β-mercaptoethanol), and 35 µl of 20 % SDS was added. The samples were incubated at 65°C for 10 min and 130 µl of 5 M potassium acetate was added. After 5 min incubation on ice, the precipitate was pelleted at 15,000 g for 10 min. The supernatant was transferred to a new tube containing 640 µl isopropyl alcohol and 60 µl of 3 M sodium acetate, mixed, and incubated at -20°C for 10 min. The precipitated DNA was centrifuged at 15,000 g for 15 min and redissolved in 200 µl of 50 mM Tris, pH 8.0, and 10 mM EDTA. This solution was centrifuged at 15,000 g for 5 min to remove insoluble material, and the supernatant was transferred to a tube containing 20 µl of 3 M sodium acetate and 440 µl ethanol. After incubation at -20°C for 10 min, DNA was pelleted through centrifugation at 15,000 g for 5 min, and washed with 70% ethanol. Pellets were dried and dissolved in 50 µl water.

PCR, agarose gel electophoresis, extraction of PCR products, and digestion with restriction enzymes were carried out according to the published protocols[13]. Conditions for the PCR amplification were as follows: denaturation at 94°C for 30 sec; annealing at 56°C for 30 sec; and polymerization at 72°C for 2 min. The cycle was repeated 50 times.

Results and Discussion

Wound (-) mutant is a dominant inhibitor of wound-inducibility. To evaluate the genetics of the wound (-) plants, we prepared crosses of plants with the wild-type, wound-inducible phenotype. The wild type plants were the *Lansberg erecta* CS2 and CS9 lines which we received from the ABRC (Arabidopsis Biological Resource Center).

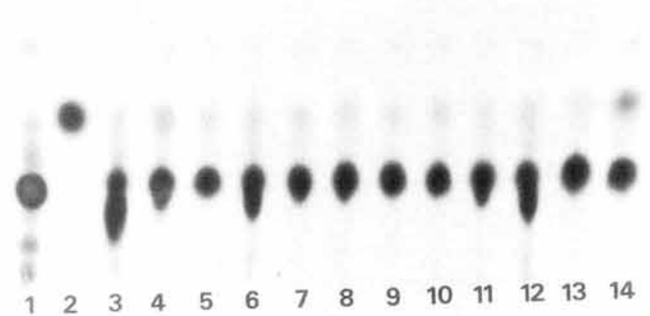

Fig. 1. Autoradiogram of cytosine deaminase assays of F_2 plants. Induction of *pin*2 promoter was carried out on half leaves of F_2 progenies of wound (-) mutant crossed with *Lansberg erecta* (Ler) CS9 in the presence of methyl jasmonate (25 mM). The assays were carried out at 37°C for 3 hrs in the presence of 0.05 µCi 2-^{14}C-cytosine. Solvent extraction of radiolabelled cytosine and uracil, TLC and autoradiography were carried out as described in Materials and Methods. Lane 1: 2-^{14}C-cytosine, Lane 2: 2-^{14}C-uracil, Lanes 3, 5, 7, 9 and 11: wound(-) x CS9 #1, #2, #3, #4 and #5 (uninduced), Lanes 4, 6, 8, 10 and 12: wound(-) x CS9 #1, #2, #3, #4 and #5 (methyl jasmonate-induced), Lane 13: Tr349 (uninduced), Lane 14: Tr349 (methyl jasmonate-induced).

Crosses were made using the wound (-) mutants as the male parent. Selection of the F_1 plants on MS medium containing kanamycin (50 µg/ml) insured that the progeny were of the desired cross. The F_1 plants were not sacrificed for biochemical analysis, but rather they were allowed to self-pollinate. The F_2 seed were germinated on kanamycin and grown for biochemical analysis. These plants were scored for the wound-inducible phenotype following treatment with methyl jasmonate. The leaves were assayed for CDase as described in Materials and Methods. Typical CDase assays are shown in Figure 1. Positive plants that converted cytosine into uracil were identified. Table 2 shows an analysis of the wound (-) mutants. *coi*1 mutant in the Table 2 is a coronatine-insensitive *Arabidopsis* mutant and was selected

Table 2. Expected genotypes of wound (-) mutant crossed with wild type and biochemical analysis of F_2 progenies.

	Allelic		Non-allelic	
F_0	wound(-) x *coi*1[a] wound(-) *coi*1		wound(-) (+) x (+) *coi*1 wound(-) (+) (+) *coi*1	
F_1	wound(-) *coi*1		wound(-), (+) (+), *coi*1	
F_2	wound(-)/wound(-) wound(-)/*coi*1 *coi*1/*coi*1	1[b] 2[b] 1[b]	wound(-)/(+) wound(-)/*coi*1 (+)/(+) (+)/*coi*1	9[b] 3[b] 3[b] 1[b]
F_2 progeny analysis	*pin*2[c] on *pin*2 off	0[d] 4[d]	*pin*2 on *pin*2 off	3[d] 13[d]
MJ/FC[e] survival (%)	100		81	
wound-inducible (%)	0		19	

[a]*coi*1: coronatine-insensitive, jasmonate-responsive
[b]expected ratio of segregation
[c]*pin*2: wound-inducible, jasmonate-inducible
[d]the number of plants
[e]MJ/FC: methyl jasmonate/5-fluorocytosine

using coronatine, a chlorosis-inducing phytotoxin.[14] The mutant is resistant to the phytotoxin and insensitive to methyl jasmonate inhibition of root growth. The *coi*1 protein has been known to control jasmonate perception or response. Three of 20 progenies showed *cod*A activity indicating *pin*2 promoter was induced by jasmonate in only 3 plants. Because wild type *Lansberg* plant should be jasmonate-responsive and wound-inducible in signal transduction pathway, this jasmonate-responsiveness seems to have been lost through crossing with the wound (-) mutant. Therefore, CDase assays indicated that wound (-) mutant was a dominant inhibitor of wound-inducibility. The wound (-) phenotype segregates independently from kanamycin resistant (the *pin*2-*cod*A loci) verifies that wound (-) is a

Table 3. Mapping of F_2 progenies of wound (-) mutant (Col) crossed with wild type (Ler CS2 or CS9).

Chromosome	Position	Marker	Progeny															
			(-) x CS2 #1	(-) x CS2 #2	(-) x CS2 #6	(-) x CS2 #8	(-) x CS2 #10	(-) x CS2 #14	(-) x CS2 #15	(-) x CS2 #17	(-) x CS2 #20	(-) x CS2 #21	(-) x CS2 #22	(-) x CS2 #1	(-) x CS2 #2	(-) x CS2 #3	(-) x CS2 #4	(-) x CS2 #5
	47.52	UFO	Ler/Col[a]	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	-[b]	-	-
	84.9	g4026	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col
	1.75	RGA1	Col	Col	Col	Col	Col	-	Col	Col	Col	-	Col	Col	Col	Col	Col	Col
	50.64	er	Col	Col (Ler)[c]	Col (Ler)	Col (Ler)	Col (Ler)	-	Col (Ler)	Col (Ler)	-	-	Col (Ler)	Col	Col (Ler)	Col	Col	Col
	69.14	ve017	-	Ler (Col)[d]	-	Ler (Col)	-	-	Ler (Col)	Ler (Col)	Ler (Col)	-	Ler (Col)	Ler (Col)	Ler (Col)	-	Ler (Col)	Ler (Col)
	8.41	GAPC	-	-	Ler (Col)	-	-	Ler (Col)	-	Ler	-	Ler	-	Ler	Ler (Col)	Ler (Col)	-	-
	48.45	GL1	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col	Ler/Col
	17.72	GA1	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)	Ler (Col)
	57.64	g4539	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)
	18.35	ASA1	Col	Col	Col	Col	Col	Col	Col	Col	Col	Col	-	-	-	Col	Col	-
	125.12	g2368	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	Col (Ler)	-	Col (Ler)	-	-	-

[a]Ler/Col: contains both characteristics
[b]-: no sample
[c]Col(Ler): contains both characteristics but Col preference
[d]Ler(Col): contains both characteristics but Ler preference

second-site mutation. This type of mutation is similar to the classical C1-1 allele in maize,[14,15] in which deletion of a C-terminal activation domain of the C1 transcription factor results in a dominant suppressive effect on anthocyanin expression[16]. However, we have no evidence that wound (-) mutant encodes a transcription factor.

Mapping the location of the wound (-) mutation. To obtain a structure map of the wound (-) mutant chromosomal regions, CAPS markers were utilized. CAPS markers covering all chromosomal locations are available at http://www.arabidopsis.org/aboutcaps.html. Two or three different CAPS markers per chromosome were chosen based upon the location in the chromosome and the availability of restriction enzymes. Genomic DNAs were isolated from the above-mentioned F_2 plants which were germinated in the presence of kanamycin. After PCR with primers for specific CAPS markers (Table 1), specific restriction enzymes were applied to the PCR products, which were then separated on 2 to 3% agarose gels. A typical photograph of agarose gel is shown in Figure 2. Most gel electrophoresis did not show clear-cut characteristics of parent, *Columbia* (Col) or *Lansberg erecta* (Ler), but showed both characteristics of parent. The Col contains *pin*2-*cod*A construct and wound (-) mutant phenotype is derived from transgenic Col while Ler is wild type. Three of 12 different CAPS markers produced characteristic of Col only (Table 3), indicating these CAPS markers might related to wound (-) phenotype because Col contains *pin*2-*cod*A construct. Therefore, the chromosomal regions covered by three CAPS markers, g4026, RGA1, and ASA1, located at 84.9 on recombinant inbred (RI) map of chromosome I, at 1.75 on RI map of chromosome II, and at 18.35 on RI map of chromosome V, respectively could be candidates for further fine mapping of the location of the wound (-) mutation, These mapping results and the above mentioned CDase assay indicated that some factor(s) suppress the wound-inducible expression of *pin*2 promoter and that the gene(s) encoding factor(s) may be located in chromosome I, II, and V.

The final goal was to determine whether these genes represent novel signal transduction intermediates. Once the gross chromosomal location is determined, this location will be refined by increasing the saturation of CAPS markers in the wound (-) mutation region. Furthermore, site specific CAPS markers can be generated from anchored BAC and YAC ends whose sequences are available at a variety of sites on the web (http://194.94.225.1/101/mpi_mp_map/bac.html, http://aims.cps.msu.edu/aims/). CAPS markers can be generated from previously sequenced chromosomal regions. Currently, about 30% of the *Arabidopsis* genome has been sequenced and these sequences are available at the web sites. These chromosomal regions will be identified from overlapping anchored YAC and BAC fragments (http://www.arabidopsis.org/maps.html). Any potential wound (-) genes will be PCR amplified using oligonucleotides derived from the BAC clone sequences. These amplified candidate genes will then be tested in transient transformation assay. If

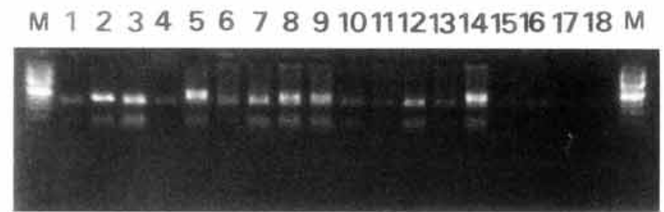

Fig. 2. Agarose gel electrophoresis of *Taq* I digests of PCR products. PCR products were obtained with GL1 primers and genomic DNAs of F_2 progenies of wound (-) mutant crossed with ***Lansberg erecta*** (Ler) CS2 or CS9. GL1 primers cover the region of 48.45 at RI map of *Arabidopsis* chromosome III. After digestion of PCR products with *Taq* I, digested reactions were separated on 2.5% agarose gel. M stands for DNA molecular weight standard (100bp DNA step ladder, Promega). Lane 1: Tr349, Lane 2: Ler, Lanes 3~13: wound(-) x CS2 #1, #2, #6, #8, #10, #14, #15, #17, #20, #21, and #22, respectively. Lanes 14~18: wound(-) x CS9 #1, #2, #3, #4, and #5, respectively.

the transient transformation assay identifies clones that compliment the wound (-) mutation, then stable transformants of the wound (-) mutant containing the potential wound (-) genes will be prepared to verify that the region responsible for the wound (-) mutation can be identified.

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