Induction of the Arginine Decarboxylase *ADC2* Gene Provides Evidence for the Involvement of Polyamines in the Wound Response in Arabidopsis[1]

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Polyamines are small ubiquitous molecules that have been involved in nearly all developmental processes, including the stress response. Nevertheless, no direct evidence of a role of polyamines in the wound response has been described. We have studied the expression of genes involved in polyamine biosynthesis in response to mechanical injury. An increase in the expression of the arginine decarboxylase 2 (*ADC2*) gene in response to mechanical wounding and methyl jasmonate (JA) treatment in Arabidopsis was detected by using DNA microarray and RNA gel-blot analysis. No induction was observed for the *ADC1* gene or other genes coding for spermidine and spermine synthases, suggesting that *ADC2* is the only gene of polyamine biosynthesis involved in the wounding response mediated by JA. A transient increase in the level of free putrescine followed the increase in the mRNA level for *ADC2*. A decrease in the level of free spermine, coincident with the increase in putrescine after wounding, was also observed. Abscisic acid effected a strong induction on *ADC2* expression and had no effect on *ADC1* expression. Wound-induction of *ADC2* mRNA was not prevented in the JA-insensitive *coi1* mutant. The different pattern of expression of *ADC2* gene in wild-type and *coi1* mutant might be due to the dual regulation of *ADC2* by abscisic acid and JA signaling pathways. This is the first direct evidence of a function of polyamines in the wound-response, and it opens a new aspect of polyamines in plant biology.

In contrast to animals, plants are unable to mobilize specialized cells devoted to wound healing after herbivore attach. Plant cells have evolved the capacity to activate defense responses that include wound healing and protection against further damage. Wound-activated repair/defense responses largely rely on the transcriptional activation of specific genes. These wound-inducible genes encode proteins implicated in activating wound signaling pathways, repairing damaged tissue, adjusting the metabolism for the production of toxins against the herbivore insect, and in the regulation of the internal nutritional demands (León et al., 2001). In Arabidopsis, two different wound signaling pathways have been characterized (Titarenko et al., 1997). Upon wounding, expression of several genes is mediated by jasmonic acid (JA), whereas expression of others is independent of JA, but is activated by oligogalacturonides (Rojo et al., 1999). Very recently, a new mechanism, independent of JA or oligogalacturonides, has been reported to control the expression of the S-like RNase gene *RNS1* (Taylor and Green, 1991) in systemically wounded tissue (LeBrasseur et al., 2002).

The diamine putrescine and polyamines spermidine and spermine are small, ubiquitous compounds, positively charged at cytosolic pH, so they can interact with anionic macromolecules such as DNA, RNA, phospholipids, and proteins. Polyamines have been implicated in a variety of plant growth and developmental processes involving cell proliferation and differentiation, morphogenesis, dormancy and germination, tuberization, flower induction and development, embryogenesis, fruit-set and growth, fruit ripening, and also in senescence and in the stress response (for review, see Kumar et al., 1997; Walden et al., 1997; Malmberg et al., 1998; Bouchereau et al., 1999; Martin-Tanguy, 2001). A protective role against stress has also been attributed to polyamines, particularly during mineral nutrient deficiency, and osmotic, salt, heat, chilling, and oxidative stresses (Richards and Coleman, 1952; Bouchereau et al., 1999). However, although polyamines play an essential role in wound healing responses in animals, mainly by regulating the expression of genes encoding cytoskeletal proteins (Kaminska et al., 1992) and by activating macrophages (Messina et al., 1992), there is no experimen-

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tal evidence for the involvement of polyamines in plant wound responses.

In addition to a different cellular mechanism of dealing with wound stress, plants have a different polyamine biosynthesis pathway. Thus, whereas in mammals and fungi, putrescine is exclusively synthesized from Orn, via Orn decarboxylase (ODC; EC 4.1.1.17) activity, plants and bacteria can also use Arg as a metabolic precursor, via Arg decarboxylase (ADC; EC 4.1.1.19) activity (Martin-Tanguy, 2001). The presence of alternative pathways has lead to the hypothesis of differential regulation of the expression of each gene and compartmentalization of the respective proteins. In addition, two *ADC* genes have been described in several plant species such as Arabidopsis (*ADC1* or *ARGdc*, At2g16500 and *ADC2* or *SPE2*, At4g34710; Watson and Malmberg, 1996; Watson et al., 1997; Galloway et al., 1998). *ADC1* has been found to be expressed in all tissues tested, whereas *ADC2* is mainly expressed in siliques and cauline leaves, and is induced upon osmotic stress (Soyka et al., 1999).

DNA microarray technology is changing the way we analyze gene expression. There are many examples in the literature showing the usefulness of this approach for unraveling complex plant responses and signal transduction processes (Schena et al., 1995; Schaffer et al., 2000). We constructed a DNA microarray that contained about 600 cDNAs and genomic DNAs, including mainly genes related to RNA metabolism but also genes related to pathogen attack, lipid metabolism, and wound response, among others. A complete list of clones included can be seen at http://www.bch.msu.edu/pamgreen/Perez-Amador_etal/600_list.htm (Pérez-Amador et al., 2001). This microarray was formerly used to test gene expression in a variety of tissues and conditions, including the *dst1* mutant (Johnson et al., 2000; Pérez-Amador et al., 2001).

In this paper, we report the use of the 600-element DNA microarray to examine gene expression in response to mechanical wounding and methyl-JA (MetJA) treatment. Our results indicate that the *ADC2* gene is induced by wounding, jasmonates, and ABA, suggesting the involvement of polyamines in the wound defense response.

RESULTS

DNA Microarray Analysis Revealed the Induction of *ADC2* Gene upon Wounding and MetJA Treatment

To study the expression of genes included in the 600-element DNA microarray during mechanical wounding, we carried out a comparison of wounded plants, locally and systemically, with unwounded control plants. For this purpose, rosette leaves were wounded with a forceps (local wounding), and were harvested along with unwounded rosette leaves from the same plants (systemic wounding) and leaves from unwounded plants (control), at 0, 2, and 24 h after wounding. For microarray analysis, only 2-h time points were used. Poly(A)$^+$ RNA was extracted and used as probe to hybridize the 600-element microarray. Two different arrays were used for each comparison, with direct (test plants labeled with Cy5-dUTP, and control plants labeled with Cy3-dUTP) and reverse (test plants labeled with Cy3-dUTP, and control plants labeled with Cy5-dUTP) labeling. Both arrays were analyzed, data were normalized, and a mean of mRNA ratios was obtained by inverting the ratio of the reverse labeling. Mean ratio above and below 1 corresponded to increased and decreased mRNA levels in the test plants versus control, respectively. Microarray data can be found at http:/www.bch.msu.edu/pamgreen/Perez-Amador_etal_2/wounding_600data.htm. Figure 1A shows a scatter plot of the mean ratio of DNA microarray analysis. Local and systemic wounding resulted in an increase in mRNA levels for a number of genes (Fig. 1A, top and middle panels, respectively). In both experiments, the highest increase was for the S-like RNase *RNS1* (At2g02990; Taylor and Green, 1991). Other genes induced upon wounding have also been previously described as wound-induced, such as a Tyr amino transferase-like protein (*TAT*; At2g24850, EST nos. N97159 and AA395846; Titarenko et al., 1997), the allene oxide synthase (*AOS*; At5g42650, EST nos. AA394958 and N65720; Laudert et al., 1996), and *Lox3* (At1g17420, EST no. AA585774; Stintzi et al., 2001). Furthermore, the *ADC* gene *ADC2* (*SPE2*; At4g34710, EST no. T46784; Watson et al., 1997), that has not been previously shown to be induced by wounding, appeared in both plots with elevated mRNA levels in local and systemic wounded leaves. *ADC2* mRNA level was elevated 2.9- and 1.8-fold upon local and systemic wounding, respectively (http://www.bch.msu.edu/pamgreen/Perez-Amador_etal_2/wounding_600data.htm).

As JA was implicated in the transduction of the wound signal (Sembdner and Parthier, 1993; León et al., 2001), we also studied changes in gene expression by DNA microarray analysis of MetJA-treated plants. Plants were sprayed with 0.01% (w/v) MetJA or buffer alone (control) and samples were collected at 0, 2, and 24 h after treatment. For microarray analysis, only 2-h time points were used. After microarray data analysis, mean ratios were plotted (Fig. 1A, bottom). *ADC2* mRNA level was increased 2.3-fold 2 h after MetJA treatment, along with mRNAs for *TAT*, *AOS*, and lipoxygenases, *Lox2* and *Lox3*. *RNS1* was not induced after treatment. The other gene coding for ADC present in Arabidopsis, *ARGdc* or *ADC1* (At2g16500), was not included in this microarray. The expression of *ADC2* in local and systemic wounding, and after MetJA treatment, suggests that *ADC2* is involved in wound-activated signaling mediated by JA or is an early target downstream of the signaling cascade.

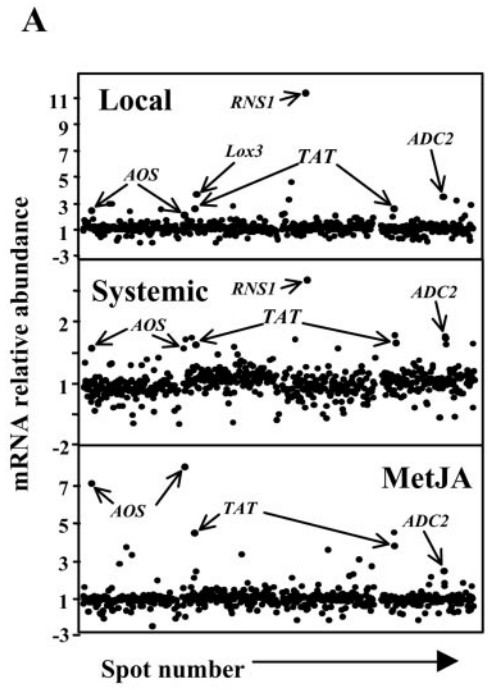

Figure 1. DNA microarray gene expression analysis during wounding and MetJA treatments in Arabidopsis. A, Scatter plot of mRNA ratios from DNA microarray analysis of local wounded plants (top), systemic wounded plants (middle), and MetJA-treated plants (bottom) compared with control unwounded or untreated plants. mRNA ratios (mRNA level treatment versus control) correspond to average ratio of two slides hybridized with direct and reverse labeling, as indicated in "Materials and Methods." *RNS1*, S-like RNase 1 At2g02990; *ADC2*, ADC2 At4g34710; *TAT*, Tyr amino transferase-like protein At2g24850; *AOS*, allene oxide synthase At5g42650; and *Lox3*, lipoxygenase 3 At1g17420. B, RNA gel analysis of genes induced during wounding and MetJA treatment according with the DNA microarray analysis. Lanes contain 10 μg of total RNA extracted from control leaves (C), local (L) and systemic (S) wounded leaves, or leaves treated with MetJA (J). Blots were hybridized sequentially with ^{32}P-labeled *RNS1* (At2g02990; Taylor and Green, 1991), and *AOS* (expressed sequence tag [EST] nos. AA394958 and At5g42650). Ethidium bromide staining was used to ensure equal loading of all lanes in the gel.

To confirm changes in gene expression observed in the microarray analysis, we carried out RNA-blot analysis. As expected, *RNS1* and *AOS* mRNAs were induced after wounding (Fig. 1B). In addition, expression of *AOS*, but not *RNS1*, was induced after MetJA treatment (Laudert and Weiler, 1998; Reymond et al., 2000; LeBrasseur et al., 2002; Fig. 1B).

The same RNA samples were used to analyze the expression of several genes involved in polyamine biosynthesis. This was intended to confirm the wound-induced expression of *ADC2* and also to test the expression pattern of *ADC1* gene, which was not included in the microarray. As shown in Figure 2, *ADC2* mRNA was induced after wounding and MetJA treatment. Little or no change was observed in mRNA levels for *ADC1*. No change was observed for the two spermidine synthases that are annotated in the genome of Arabidopsis (*SPDS1*, At1g70310 and *SPDS2*, At1g23820), and *S*-adenosyl Met decarboxylase (At3g02470). The only gene reported to encode a spermine synthase, *ACL5* (At5g19530; Hanzawa et al., 2000), did not show any hybridization signal (data not shown). These results indicate that, among

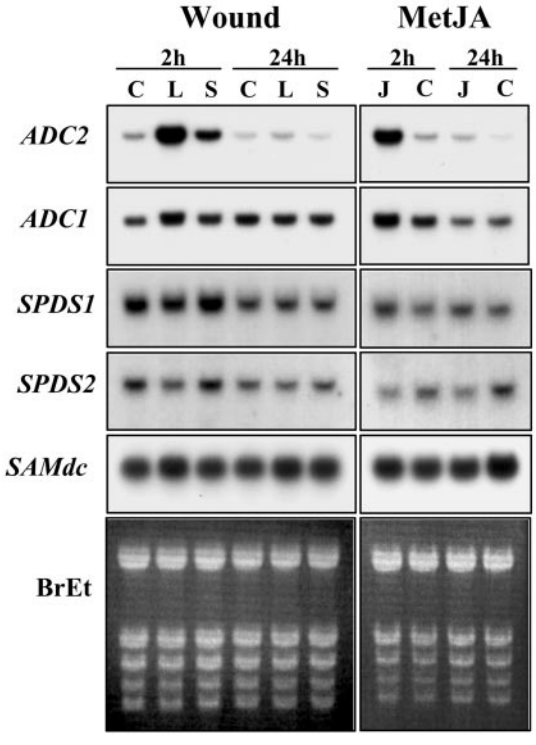

Figure 2. RNA gel analysis of the expression of genes involved in polyamine biosynthesis during wounding and MetJA treatment. Lanes contain 10 μg of total RNA extracted from control leaves (C), local (L) and systemic (S) wounded leaves, or leaves treated with MetJA (J). Blots were hybridized sequentially with ^{32}P-labeled *ADC2* (EST nos. T46784 and At4g34710), *ADC1* (EST nos. H36915 and At2g16500), *SPDS1* (EST nos. AA597626 and At1g70310)l *SPDS2* (EST nos. T20920 and At1g23820), and *S*-adenosyl Met decarboxylase (EST nos. AA395848 and At3g02470). Ethidium bromide staining was used to ensure equal loading of all lanes in the gel.

of all genes tested, *ADC2* is the only gene involved in polyamine biosynthesis that seems to be part of the wounding response.

ADC2 Is Transiently Induced upon Wounding, Local and Systemically

To extend the analysis of *ADC* gene expression during wounding, we carried out an independent wounding experiment in which we harvested local and systemically wounded leaves at different time points and measured *ADC* mRNA levels and polyamine content. As shown in Figure 3, *ADC2* mRNA level increased as early as 15 min after damage in the locally wounded leaves, reaching a maximum between 1 and 2 h. By 24 h, *ADC2* mRNA levels were similar in wounded and control leaves (Fig. 3A; see Fig. 4A for quantification of *ADC2* mRNA levels). *ADC2* was also induced systemically upon wounding, with a maximum after 6 h (Fig. 3B). The magnitude of this increase was lower than in the damaged leaves. Little or no induction was observed for *ADC1* in local or systemically wounded leaves.

To investigate whether the changes in *ADC2* expression could result in an increase in putrescine level, we determined the concentration of free polyamines using samples from wounded leaf tissue harvested along with those for RNA-blot analysis from the previous experiment. We observed a transient increase in the level of free putrescine after the increase in the mRNA level for *ADC2* (Fig. 4). Putrescine reached the maximum level 6 to 10 h after damaging in locally wounded leaves, and decreased to basal levels 24 h after wounding. A similar variation in putrescine level was detected in systemic leaves (data not shown). We also observed a transient decrease in the level of free spermine, which is coincident with the increase in putrescine after wounding (Fig. 4).

ADC2 Is Induced in the *coi1* Mutant and by ABA

Although jasmonates seem to play an important role in wound signaling pathways, other molecules such as ABA, and physical processes such as water stress and electrical impulses have been related to the wound defense response (for review, see León et al., 2001). Moreover, recent data suggest that wound signaling proceeds through different complex interactive pathways instead of through a single linear pathway. Therefore, we have used *ADC2* expression to look for interactions between upstream signaling networks.

We analyzed the expression of *ADC2* after JA treatment in wild-type Arabidopsis compared with the JA-insensitive *coi1* mutant (Fig. 5). In wild-type plants, *ADC2* mRNA transiently increased with maximum 2 to 6 h after JA treatment. This expression pattern is similar to that after wounding or MetJA treatment shown in Figures 2 and 3 (Fig. 5A). In contrast, the wound-induced expression of *ADC2* in the *coi1* mutant background followed a different temporal pattern, with an increase that started between 2 and 6 h and continued 24 h after wounding (Fig. 5B).

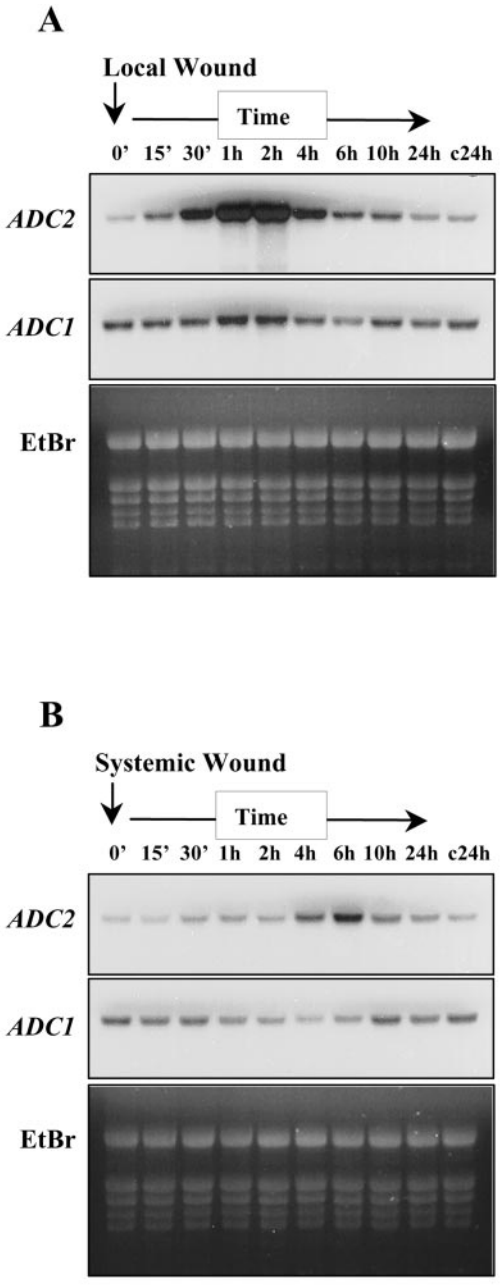

Figure 3. RNA gel analysis of local (A) and systemic (B) wounded plants. One-half of the rosette leaves were wounded and harvested (A, local wounding) at different time points along with unwounded leaves from the same plants (B, systemic wounding) or leaves from unwounded plants (control). Lanes contain 10 μg of total RNA. Blots were hybridized sequentially with ^{32}P-labeled *ADC2* (EST nos. T46784 and At4g34710) and *ADC1* (EST nos. H36915 and At2g16500). Ethidium bromide staining was used to ensure equal loading of all lanes in the gel.

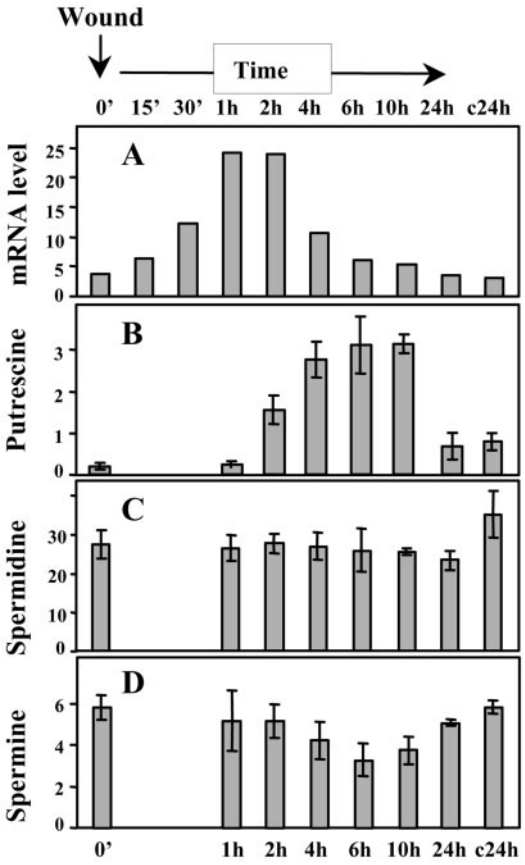

Figure 4. Changes in polyamine levels after local wounding. Samples from locally wounded leaves were collected at different time points and polyamines were analyzed as indicated in the "Materials and Methods." A, Quantification of mRNA levels for *ADC2* (from Fig. 3A; relative units); B, putrescine level (μg g^{-1} fresh weight); C, spermidine level (μg g^{-1} fresh weight); D, spermine level (μg g^{-1} fresh weight). B, C, and D correspond to the mean of three independent samples ± SE.

The consistent decrease in *ADC1* mRNA level observed 6 h after wounding in treated and control wild-type and *coi1* mutant plants suggests that other regulatory mechanisms not related to wound responses are involved in *ADC1* regulation.

Beside JA, ABA has also been implicated in wound signaling in Solanaceae (Peña-Cortés et al., 1995, 1996), although the precise role of this molecule in the wound response remains controversial (Dammann et al., 1997; Birkenmeier and Ryan, 1998; Carrera and Prat, 1998). To test if ABA also induces the expression of *ADC2*, we treated liquid-cultured plants with ABA and studied *ADC2* and *ADC1* mRNA levels by RNA gel blot in a time course experiment. *ADC2* mRNA levels increased 2- to 3-fold after wounding in a similar way as in wounded or MetJA- and JA-treated soil-grown plants (Fig. 6A compared with Figs. 2 and 5A). It was remarkable that ABA showed a stronger effect on *ADC2* expression than any other treatment. Fifty micromolar ABA induced a 10-fold increase in *ADC2* mRNA levels, and had no effect in *ADC1* expression. In addition, ABA treatment resulted in a 2- to 4-fold increase in free putrescine (data not shown). *ADC2* and *ADC1* were not found to be affected by putrescine (Fig. 6A).

α-DL-Difluoromethyl-Arg (DFMA), an irreversible inhibitor of ADC activity, causes a subtle increase in *ADC2* mRNA level similar in its kinetic to that caused by mechanical wounding (Fig. 6B). The addition of 1 μM DFMA to wounded plantlets caused a 4- to 5-fold increase in *ADC2* mRNA level, suggesting a cooperative inducing effect. Again, no changes were observed for *ADC1* mRNA abundance.

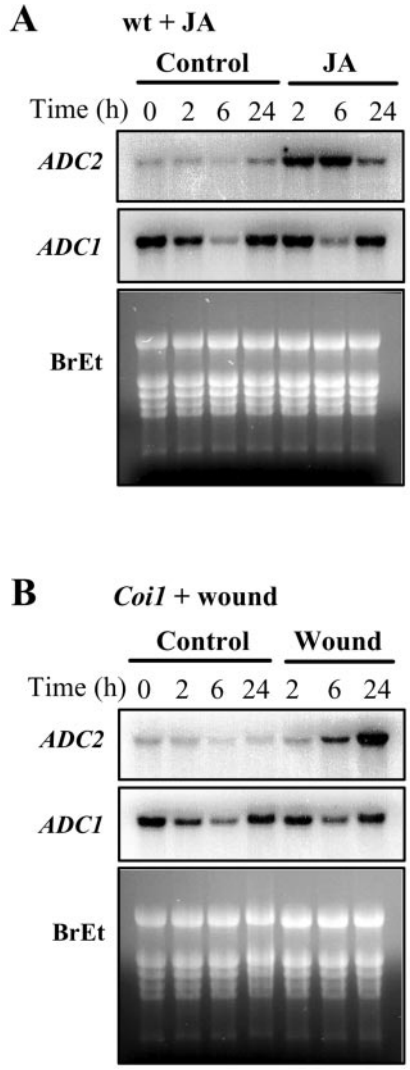

Figure 5. RNA gel analysis of JA-treated wild-type plants (A) and locally wounded *coi1* mutant (B). Lanes contain 10 μg of total RNA extracted from wild-type plants treated with 50 μM JA (JA) or untreated control plants, or *coi1* mutant wounded or unwounded, and harvested at different time points. Blots were hybridized sequentially with ^{32}P-labeled *ADC2* (EST nos. T46784 and At4g34710) and *ADC1* (EST nos. H36915 and At2g16500). Ethidium bromide staining was used to ensure equal loading of all lanes in the gel.

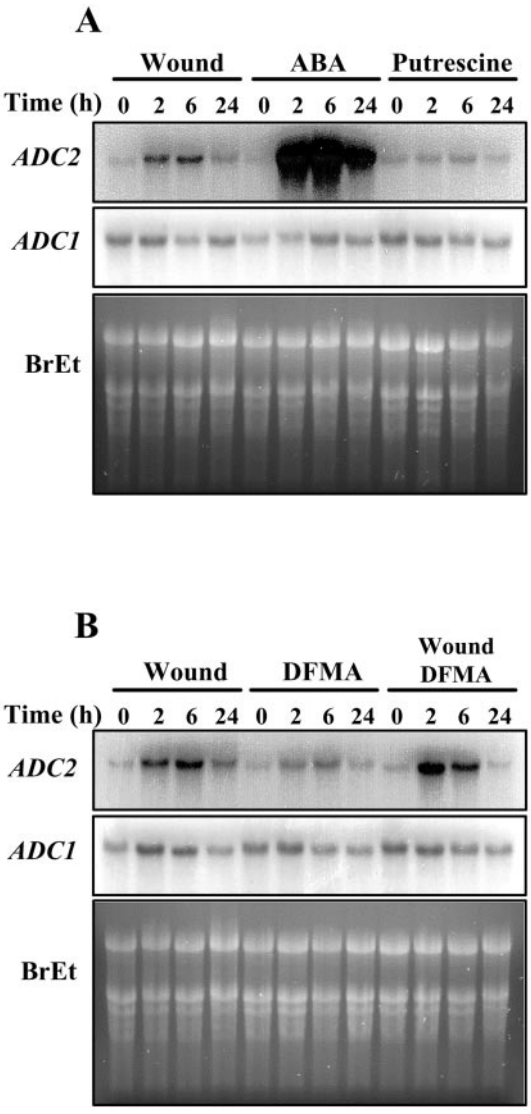

Figure 6. RNA gel analysis of wounded, 50 μM ABA, or 50 μM putrescine (A) and wounded, 1 μM DFMA-treated, or wounded and DFMA-treated (B) liquid-cultured plants. Lanes contain 10 μg of total RNA extracted from seedlings grown in liquid as described in the "Materials and Methods." Blots were hybridized sequentially with ^{32}P-labeled *ADC2* (EST nos. T46784 and At4g34710) and *ADC1* (EST nos. H36915 and At2g16500). Ethidium bromide staining was used to ensure equal loading of all lanes in the gel.

DISCUSSION

Use of DNA Microarray to Discover Novel Gene Functions

DNA microarray analysis of gene expression in leaves of wounded plants showed a set of genes that were not previously related with local and systemic wounding, as well as others previously described as wound inducible. We obtained the first experimental evidence showing that the expression of the *ADC2* gene is altered in response to wounding, providing an excellent example of the use of DNA microarrays to assign new functions to known genes. *RNS1*, which is known to be induced by wounding locally and systemically, but not by JA, was also induced, defining a JA-independent systemic signaling pathway (Reymond et al., 2000; LeBrasseur et al., 2002). Very recently, a macroarray analysis intended to identify JA-responsive genes revealed the induction of *ADC2* mRNA upon MetJA treatment in Arabidopsis, whereas *ADC1* mRNA was not altered (Sasaki et al., 2001; http://www.kazusa.or.jp/en/pub_data/sasaki_et_al_2001/). In barley (*Hordeum vulgare*), DNA microarray analysis has been used to reveal the induction of *ADC* genes during drought stress (Ozturk et al., 2002).

Specificity of *ADC2* versus *ADC1* Expression

In addition to ADC, putrescine in plants can be also synthesized by ODC activity. *ODC*s have been isolated and characterized from several plant species such as thorn-apple (*Datura stramonium*; Michael et al., 1996), tomato (*Lycopersicon esculentum*; Alabadí and Carbonell, 1998), and tobacco (*Nicotiana tabacum*; Malik et al., 1996). In contrast, Arabidopsis does not contain any genomic sequence homologous to known *ODC* genes and lacks ODC activity (Hanfrey et al., 2001), despite earlier evidence about the presence of this activity (Feirer et al., 1998; Tassoni et al., 2000).

Several plants, mainly Brassicacea, contain two genes coding for ADC proteins (Galloway et al., 1998). In Arabidopsis, two different genes, *ADC1* and *ADC2*, have been described to encode ADC activity. Our results showed that only *ADC2* is induced by wounding, suggesting that *ADC2*, but not *ADC1*, is involved in wounding response. Consistent with this, a transient increase in putrescine was observed. Induction of *ADC2* was also observed when plants were treated with JA and MetJA. Tobacco Bright Yellow-2 cells treated with MetJA showed a strong increase in the activity of several enzymes involved in the biosynthesis of nicotine, including *ODC*, that led to an increase in the levels of putrescine and nicotine, but not those of spermidine and spermine (Imanishi et al., 1998). Induction of Arabidopsis *ADC2* and *ODC* tobacco by jasmonates may suggests that these genes play a similar role.

ADC1 and ADC2 proteins are almost identical in sequence, showing 80% of identity. It is interesting that the differences in sequence between the two *ADC* genes are concentrated in the amino-terminus of the proteins. Subcellular localization of ADC proteins according to TargetP and PSort software results in contradictory prediction. ADC1 seems to be targeted to the chloroplast according to the TargetP program (Emanuelsson et al., 2000). In contrast, PSort reveals that ADC2 has a higher possibility to be a chloroplastic protein. Borrell et al. (1995) previously reported that oat (*Avena sativa*) ADC is targeted to the chloroplast. If one of the Arabidopsis ADC proteins

is a chloroplast localized-protein, it may indicate that it could be equivalent to the oat ADC. In contrast, the other ADC could remain in the cytoplasm, playing a different role. Different localization of ADC1 and ADC2 proteins in Arabidopsis may add another level of control besides the differential regulation of the cognate genes.

Participation of Polyamines in the Stress Response

Several studies have identified ADC as one of the enzymes involved in the stress response in plants (for review, see Bouchereau et al., 1999). In Arabidopsis, this evidence came initially from studies on potassium deficiency and osmotic stress. ADC activity and putrescine levels increased 10- and 20-fold, respectively, as a response of the plant to potassium starvation (Watson and Malmberg, 1996). During osmotic stress, ADC activity dramatically increases as a consequence of the expression of the *ADC2* gene (Feirer et al., 1998; Soyka and Heyer, 1999).

Other evidence for a role of polyamines during stress in plants comes from the observation that *ODC* and other polyamine metabolism (synthesis, oxidation, and conjugation) and nicotine biosynthesis genes are induced after MetJA treatments in tobacco thin layers and cell cultures (Biondi et al., 2001; Imanishi et al., 1998). Whether this induction is specific to JA or is also produced by other molecular signaling network related to the mechanical wounding has to be demonstrated.

In Arabidopsis, paraquat treatment, which induces an oxidative stress response, led to an increase in putrescine, with no significant changes in spermidine and spermine levels (Kurepa et al., 1998). Feirer et al. (1998) reported an increase in ADC activity in leaves that were incubated in buffer for 7 h. In contrast, no differences in ADC activity were reported in an almost identical experiment by Soyka and Heyer (1999). However, polyamines play an essential role in the wound healing responses in animals, mainly by regulating the expression of genes encoding cytoskeletal proteins (Banan et al., 1998) and by activating macrophages (Messina et al., 1992).

Response of Polyamines to Plant Hormones

In our experiments, we show that the expression of Arabidopsis *ADC2* is strongly induced by ABA treatment. Two additional pieces of evidence agree with a regulation of *ADC2* expression by ABA. First, analysis of the expression of *ADC2* in microarray experiments through the Stanford Microarray Database (Sherlock et al., 2001) revealed an induction of *ADC2* mRNA by ABA that was abolished by the *abi1* mutation (http://genome-www5.stanford.edu/cgi-bin/SMD/spotHistory.pl?state=parameters&suid=142991). Second, sequence analysis of both *ADC* promoters revealed the presence of an ABA response element core sequence C*ACGT*G (Busk and Pages, 1998) in *ADC2*, but not in *ADC1*, promoter (data not shown).

Wound-induction of *ADC2* mRNA is not prevented in the JA-insensitive *coi1* mutant. However, the time course of the induction is different from that in wild-type plants. This different behavior may be due to the dual regulation of *ADC2* by ABA and JA signaling pathways. In wild-type plants, both pathways would be active in inducing *ADC2*, whereas only the ABA pathway would be active in the *coi1* mutant, resulting in a different kinetics in the induction.

Extensive work has been done in the past that shows the effect of the different plant hormones in altering polyamine levels and polyamine-related enzymatic activities (for review, see Rastogi and Davies, 1991). Here, we have shown the induction of *ADC2* gene expression by ABA. To our knowledge, our data is one of the few, if not the first, reports on the regulation of a gene involved in putrescine biosynthesis by a plant hormone. Indirect evidence came, for example, from studies of the role of polyamines in fruit set and early fruit development. In pea (*Pisum sativum*) and tomato, *ADC* and *ODC* mRNA levels and enzymatic activity increased transiently during fruit set and early parthenocarpic fruit growth mediated by gibberellins, auxins, or cytokinins (Pérez-Amador and Carbonell, 1995; Pérez-Amador et al., 1995; Alabadí et al., 1996; Alabadí and Carbonell, 1998). In Arabidopsis, the spermine synthase gene *ACL5* is specifically induced by indole-3-acetic acid, but not by benzoic acid, ABA, brassinolide, or gibberellic acid (Hanzawa et al., 2000). A better understanding of the regulation of polyamine levels in plants will come from a more systematic analysis of the effect that the different plant hormones and growth regulators have in the expression of the polyamine metabolism genes. In Arabidopsis, isolation of almost all genes implicated in putrescine and polyamine biosynthesis along with the availability of phytohormone-related mutants will now allow such studies.

Role of Putrescine in Wound Healing

As mentioned above, an increase in polyamines and nicotine content in tobacco treated with MetJA has been reported (Imanishi et al., 1998; Biondi et al., 2001). In Arabidopsis, we have detected a transient increase of putrescine levels, coincident with a decrease in spermine levels, after wounding. Putrescine produced by the stress-induced ADC2 activity could be used as a substrate for spermidine and spermine biosynthesis. The transient decrease in the spermine level observed upon wounding could be the result of a higher rate of spermine catabolism, conjugation, or transport, than spermine biosynthesis. The spermine or any other compound that is derived from spermine (i.e. a conjugate of spermine) could play a role in wound-induced responses.

Putrescine can also be conjugated to soluble and insoluble compounds. Little is known about the conjugation of polyamines in Arabidopsis. Conjugated putrescine has been detected in most tissues of the plant, with maximum levels in cotyledons and flowering stalks (Tassoni et al., 2000). Upon wounding, freshly synthesized putrescine could be conjugated to small phenolic molecules such as hydroxycinnamic acids.

Putrescine synthesized upon wounding can be use in the synthesis of γ-aminobutyric acid (GABA) via putrescine catabolism (Flores and Filner, 1985). GABA has been reported to be accumulated in response to mechanical manipulation in soybean (*Glycine max*; Wallace et al., 1984). Although the main pathway for GABA synthesis is thought to be the decarboxylation of Glu by Glu decarboxylases, GABA can also be generated from putrescine via putrescine oxidase and pyrroline dehydrogenase activities (Flores and Filner, 1985). Arabidopsis possesses at least two Glu decarboxylase genes (*GAD1* and *GAD2*; Turano and Fang, 1998; Zik et al., 1998). Expression of these two genes has still to be determined to understand their contribution to GABA synthesis in stress conditions.

Our work opens a new aspect of polyamines in plants: Putrescine, or another polyamine or a putrescine conjugated compound, may have a role in the wound response. Future work needs to be done to determine the physiological relevance of the regulation of the putrescine levels upon wounding, and the mechanism of regulation of *ADC1* and *ADC2* expression by wounding and plant hormones.

MATERIALS AND METHODS

Plant Material

Wild-type Arabidopsis plants (Colombia-0) were grown in growth chambers under a 12-h light:12-h dark regime and 80% relative humidity at 22°C. *Coi1* plants, provided by Dr. Turner (School of Biological Science, University of East Anglia, Norwich, UK), were selected in Murashige and Skoog medium containing 1 μM coronatine, as previously described in Benedetti et al. (1995). *coi1* plants were transferred to soil and were kept under a 16-h light:8-h dark regime, 80% relative humidity, and 20°C for 4 weeks. Liquid-grown plants were as described in Rojo et al. (1998). Wound lesions in whole 5-week-old plants were generated with forceps by squeezing two to three times each leaf of one-half of the rosette. Systemic-wounded leaves were those on the opposite side of the rosette of the wounded plant. For the MetJA treatments, plants were sprayed with 0.01% (w/v) MetJA in 0.1% (v/v) ethanol and 0.01% (v/v) Tween 20 as a surfactant, and they were kept in a sealed plastic box during the experiment. Control plants were treated with 0.1% (v/v) ethanol and 0.01% (v/v) Tween 20 in a different plastic box, but were kept in the same growth chamber as the MetJA-treated plants. For the JA treatment experiment, plants were sprayed with a 50 μM JA solution or water alone for controls. Wounding or treatments of liquid-cultured plants with 50 μM ABA, 50 μM putrescine, or 1 μM DFMA were as in Rojo et al. (1999). Tissue samples were harvested, weighted, and immediately frozen in liquid N_2.

Total RNA Extraction, Poly(A)$^+$ RNA Purification, and RNA-Blot Hybridization

Total RNA from leaf samples was extracted as previously described (Newman et al., 1993). Poly(A)$^+$ RNA was purified from 200 μg of total RNA using the Oligotex mRNA kit (Qiagen, Valencia, CA). Total RNA (10 μg) was analyzed by electrophoresis on 2% (v/v) formaldehyde/1.2% (w/v) agarose gels and was blotted onto a nylon membrane (Nytran Plus; Schleicher & Schuell, Keene, NH). DNA probes were labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983) or by using Ready to Go, a DNA-labeling kit (Amersham Bioscience, Piscataway, NJ), and were purified from unincorporated nucleotides using probe purification columns (NucTrap; Stratagene, La Jolla, CA or Quick Spin; Roche Molecular Biochemicals, Summerville, NJ). The RNA blots were hybridized as described in Taylor and Green (1991) for Figures 1 and 2, or according to the protocol described by Church and Gilbert (1984) for Figures 3, 5, and 6 using the indicated ^{32}P-labeled probes. The ethidium bromide staining of the RNA gel was used as a control for equal loading of all lanes. Blots were stripped between hybridizations in 0.1% (w/v) SDS at 90°C to 95°C for 1 h. Quantification of hybridization signals was achieved using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or FujiBass (Fuji, Tokyo).

DNA Microarray Construction, Hybridization, and Analysis

EST clones were selected based on sequence similarity by BLAST analysis (Altschul et al., 1997) and were obtained from Dr. Thomas Newman's EST collection (Newman et al., 1994). PCR products from these clones were deposited in duplicate onto glass slides as previously described (Pérez-Amador et al., 2001). A complete list of the genes included can be found at http://www.bch.msu.edu/pamgreen/Perez-Amador_etal/600_list.htm.

Probe labeling was as described in Pérez-Amador et al. (2001). In brief, 1 μg of poly(A)$^+$ RNA was used for each probe in a retrotranscription reaction using Superscript II (Invitrogen, Carlsbad, CA). Second-strand DNA synthesis was carried out from one-half of the first-strand reaction using one of each Cy3- and Cy5-dUTP (Amersham Biosciences) and Klenow (Invitrogen). For a single DNA microarray, Cy3- and Cy5-labeled probes were mixed, denatured, and hybridized to the microarray under a glass coverslip in a microarray hybridization chamber (ArrayIt Hybridization Cassette; TeleChem, Sunnyvale, CA). After hybridization in a water bath at 65°C between 12 and 20 h, the microarray was washed and dried by centrifugation. The slide was scanned once in the ScanArray 3000 (GSI Lumonics, Billerica, MA) for channels 1 and 2 (corresponding with Cy3-labeled and Cy5-labeled probes, respectively) with 10 μm resolution. The image files obtained were analyzed with ScanAnalyze software (v. 2.32; M. Eisen, Stanford University, http://genome-www5.stanford.edu/MicroArray/SMD/restech.html). To ensure that only spots of high quality were used in the analysis, spots with GTB2 values lower than 0.50 for both channels were removed and not considered for further analysis.

Data from each channel was transformed to the natural logarithm, and a Z-score was calculated to normalize the channel values to account for variation in RNA labeling as described in Pérez-Amador et al. (2001). For each comparison, we generated two slides, with direct and reverse labeling. Ratios from reverse labeling were reversed to be compared with the ratios from direct labeling: Ratios above 1 and below 1 indicated elevated and decreased mRNA levels in the test samples versus control, respectively. The mean of the two ratios was determined and used as final mean ratio of mRNA levels.

Gene expression data for *ADC2* by DNA microarray analysis was retrieved from the Stanford Microarray Database at http://genome-www5.stanford.edu/cgi-bin/SMD/spotHistory.pl?state=parameters&suid=142991.

Polyamine Determination

Leaf samples were homogenized in a cold mortar with a pestle in 1 to 2 volumes of cold 0.2 N perchloric acid and 0.25 or 0.5 volumes of 0.130 μg mL^{-1} 1,6-diaminohexane solution in perchloric acid. Homogenates were clarified by centrifugation in a microfuge at 12,000 rpm for 10 min. One hundred microliters was used in a dansylation reaction, and dansyl-polyamines were separated and quantified by HPLC using 1,6-diaminohexane as internal standard as previously described (Carbonell and Navarro, 1989).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial research purposes.

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