Arabidopsis knockout mutation of *ADC2* gene reveals inducibility by osmotic stress

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Abstract We isolated an *Arabidopsis thaliana* mutant line carrying an insertion of the *En-1* transposable element at the *ADC2* locus. The insertion causes a knockout of the arginine decarboxylase 2 gene. We demonstrated that *ADC2* is the gene responsible for induction of the polyamine biosynthetic pathway by osmotic stress. No induction of ADC activity by the osmolite sorbitol could be observed in the homozygous mutant, indicating a predominant role of *ADC2* in stress response. ADC activity is reduced in the mutant by 44% under non-stressed conditions and the mutant shows no obvious phenotype. This is the first report of a genetically mapped mutation in the polyamine biosynthetic pathway in plants.

Key words: Arginine decarboxylase; Osmotic stress; Polyamine; *Arabidopsis thaliana En-1*

1. Introduction

Arginine decarboxylase is the first enzyme in the biosynthesis of polyamines from the amino acid arginine. A parallel pathway using ornithine is initiated by ornithine decarboxylase (ODC). This second route seems to be the only polyamine biosynthetic pathway in animals and humans [1], but in most plants, ODC appears to be restricted to certain organs like apical bud [2], flower buds or fruits [3] and shows only low activity in the vegetative tissues [4–7].

The arginine pathway, present also in bacteria, starts by decarboxylation of arginine yielding agmatine. This is converted to *N*-carbamoylputrescine and finally to putrescine. The other most abundant polyamines, spermidine and spermine, are synthesized by addition of an aminopropyl-group donated by decarboxylated *S*-adenosylmethionine, which is catalyzed by the enzymes spermidine synthase and spermine synthase, respectively [1,8].

Polyamines have been implicated in several cellular processes, e.g. DNA replication, cell division, protein synthesis [9] and plant responses to abiotic stress [8]. It is supposed that ODC and ADC have different physiological roles during plant growth and development. ODC seems more involved in regulation of cell cycle and rapid cell division, while ADC has been linked to stress responses [10,11].

Feirer et al. [7] showed that ADC is the enzyme primarily responsible for biosynthesis of putrescine in osmotically stressed *Arabidopsis thaliana*, but it is unclear which of the two ADC genes present in *Arabidopsis* is involved in this process.

Approaches to study polyamine function made use of suicide inhibitors [12] but the effects of difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA) on ODC and ADC, respectively, in different plant systems are extremely variable, e.g. ranging from inhibition to no effect to stimulation of root growth, and are a function of concentration and of the particular plant system tested [13].

Overexpression as well as antisense inhibition of biosynthetic enzymes has been employed to study polyamine function. This made use either of the constitutive 35S promoter [14–17] or inducible promoters [18–20]. Less is known about mutants affecting polyamine metabolism in plants. Mutants with high level of putrescine and high levels of ADC activity have been identified because of their abnormal floral morphology [21] but the basis of the mutation is still not known.

Screening for resistance to the *S*-adenosylmethionine decarboxylase inhibitor methylglyoxal-bis(guanylhydrazone) [22–24] or inhibitory concentrations of spermine [25], yielded mutants that showed reduced sensitivity to the respective agent, but these mutants have not been exploited for the analysis of polyamine function.

Recently, Watson et al. [26] isolated EMS mutants of *A. thaliana* that are reduced in ADC activity. The mutants fall into two complementation groups, *spe1* and *spe2*, and the strongest alleles within each group showed a reduction of ADC activity down to 23 and 36%, respectively. The double mutant *spe1-1 spe2-1* had lower ADC activity than each single mutant but still about 20% of the wild-type activity remained. Because two gene copies encoding ADC, *ADC1* and *ADC2*, are found in all members of the Brassicaceae studied to date except the basal genus *Aethionema* [27], the authors suggest that *spe1* and *spe2* might correspond to both ADC genes. The mutations have not been mapped and therefore it cannot be excluded that other functions, e.g. regulatory elements, are affected.

Here we describe the first genetically mapped mutant of a polyamine synthesizing enzyme in plants. The mutant EN9 was obtained by PCR-screening of an *En-1*-mutagenized *A. thaliana* population [28] for insertions at the *ADC2* locus.

En-1 is a transposable element of maize and is able to transpose in the heterologous host *A. thaliana* (ecotype Columbia) [29]. The 8.2 kb large transposon causes gene disruption or a frame shift when leaving a footprint during excision [30,31]. In contrast to the mutants described by Watson et al. [26], EN9 should therefore be regarded as a complete loss-of-function or knockout mutation.

The mutant line EN9 shows no obvious phenotype under normal growth conditions but is completely devoid of ADC induction by osmotic stress as studied by incubating leaf disks

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in 0.6 M sorbitol. As *ADC1* gene expression is not affected in the mutant, we conclude that *ADC2* is the gene responsible for induction of arginine decarboxylase and polyamine biosynthesis under osmotic stress.

2. Materials and methods

2.1. Plants

The *A. thaliana En-1* mutant collection [28,32] was made available by the Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829 Cologne, Germany. Plants were grown in soil individually in 6 cm pots in a greenhouse at 16 h light periods with additional fluorescent light (total light intensity approximately 100–200 μmol photons s^{-1} m^{-2}) at 22°C (day) and 15°C (night). Tissue for DNA, RNA, protein and polyamine extraction was frozen in liquid nitrogen and stored at −80°C.

2.2. Reverse genetic screen

DNA extracted from 3000 *En-1* mutagenized lines was pooled in a three-dimensional grid as described by Baumann [28] and screened by PCR using the primers addII (5′-TGAAGCATCCGGTGATTTG-CAG-3′), addIII (5′-TTCAAGCATAAACTGATGATCAGTTAGG-GG-3′), homologous to ADC2 and the *En-1* homologous primers EN205 and EN8130 described in [28].

2.3. Mapping the En-1 insertion

Sequences of *En-1* insertion sites were obtained by PCR-amplifying the transposon/plant-DNA transition and direct sequencing of the PCR fragments. Fragments were amplified using the primers: aad1 (5′-GCGGTACCATGCCTGCTTTAGCTTGCGTTG-3′) and *En-1* specific primer EN8130 [28].

2.4. RNA and DNA extraction

Northern and Southern blot analysis was performed as described in Heyer and Gatz [33]. Fragments were labeled using the Rediprime DNA labeling system from Amersham (Braunschweig, Germany). Filters were washed two times in 2×SSC, 0.5% SDS, 65°C and one time in 1×SSC, 0.5% SDS at 65°C.

2.5. Osmotic stress and assay of ADC activity

The osmotic stress induction and the ADC activity measurements were performed according to the methods described by Feirer et al. [7]. N_2-frozen tissue samples were ball-milled in a Retsch MM2000 (Retsch, Haan, Germany) to a fine powder and incubated in 50 mmol HEPES/NaOH (pH 8.5), containing 2 mmol dithiothreitol (DTT), 1 mmol ethylenediaminetetraacetic acid (EDTA), 0.5 mmol pyridoxal 5-phosphate (PLP) for 1 h on ice. After centrifugation for 5 min at top speed in an Eppendorf microfuge at 4°C, the supernatant was loaded on a NAD-5 column (Pharmacia) and eluted with the same buffer. The eluates were used for analysis of enzyme activity. The reaction mixture contained 110 μl of extract and 1 μl 0.05 μCi L-[U-^{14}C]arginine monohydrochloride (Amersham). After 45 min at 37°C the reaction was stopped by adding 200 μl of 10% trichloroacetic acid. Protein concentration was determined by the method of Bradford [34] using the Bio-Rad dye reagent.

3. Results and discussion

3.1. Identification of an ADC2 mutant

The *En-1*-mutant collection of *A. thaliana*, ecotype Columbia, generated by Baumann et al. [28], is described to cover about 50% of the genome of *Arabidopsis*, providing knock-out mutants for nearly 15 000 structural genes of this plant.

DNA preparations of 3000 lines, each carrying about 10 transposon integrations, have been pooled in a three-dimensional scheme of rows, columns and trays that allows identification of mutant plants for the gene of interest within three consecutive rounds of 112 PCR reactions [28]. We screened these pools with two primers for the *ADC2* gene and two *En-1* primers described in Section 2 and we isolated a line that carried an *En-1* insertion at the *ADC2* [35] locus. This plant, being a putative knockout mutant of *ADC2*, was named EN9.

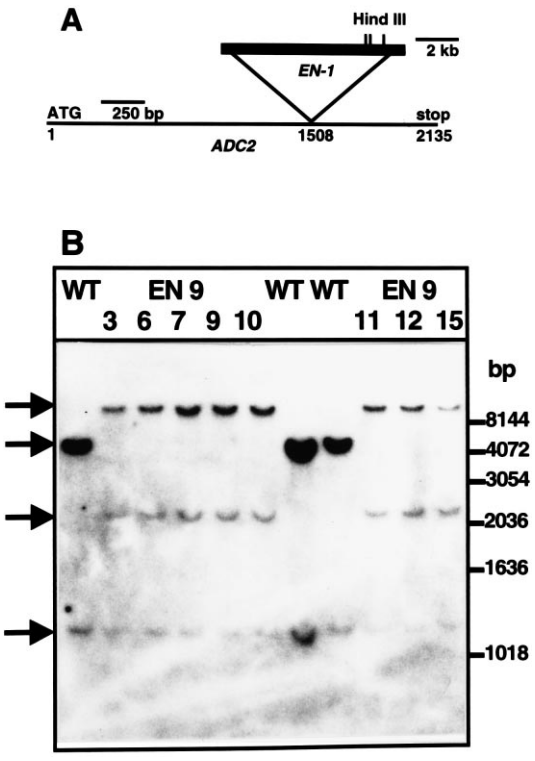

Fig. 1. Physical analysis of the *En-1* insertion at the *ADC2* locus of mutant line EN9. A: Restriction map and sequence of the *ADC2* gene at the *ADC2*/*En-1* border. Sequence data are derived from sequencing PCR products obtained with *En-1*- and *ADC2*-specific primers. B: Southern blot analysis of *Hin*dIII digested DNA from wild-type and individual EN9 progeny plants. The blot was hybridized with the complete *ADC2* gene. Lane WT: wild-type; EN9-1 to EN9-15: progeny lines of the original mutant EN9. DNA prepared from 50–100 mg of leaf tissue was loaded without prior quantification.

A PCR product obtained for DNA of EN9 with primers aad1 and En8130 was sequenced directly using primer En8130. The *En-1* insertion maps at nucleotide (nt) 1508 of the coding region of the intron-less *ADC2* gene (Fig. 1A). This was further confirmed by sequencing PCR products obtained with the same primers using DNA preparations of progeny plants of EN9. The insertion causes a rupture of the coding region at amino acid Ser-503 and introduces a stop codon 18 amino acids downstream. The transposable element is integrated in antisense orientation with respect to the coding region of the mosaic protein [30].

Verification of homozygosity of the mutation in individual descendants of EN9 was performed by Southern blot analysis of genomic DNA of 26 independent lines. An exemplary re-

Table 1
Effect of osmotic stress on ADC activity

	Wild-type	EN9
Control, fresh leaf	3.9 ± 1.3	2.2 ± 0.6
Buffer, 7 h	5.8 ± 2.1	2.7 ± 0.6
Buffer+0.6 M sorbitol, 7 h	102.8 ± 14.2	5.3 ± 0.4

Enzyme activity is expressed in pmol CO_2 released h^{-1} mg $protein^{-1}$. The values represent the means of at least five replicates ± S.D.

sult of the analysis is given for eight lines in Fig. 1B. Genomic DNA was prepared from 50 to 100 mg of leaf material and digested with the restriction endonuclease *Hin*dIII. After digestion, the DNA was directly loaded on an agarose gel and subjected to electrophoresis. The gel was blotted and hybridized with the complete *ADC2* coding region. As demonstrated in Fig. 1B, the wild-type *Hin*dIII band of about 4 kb, which is indicated by an arrow (middle), was absent in all lines. Instead, two other bands appeared, one at about 9 kb (top arrow) and the other at about 2 kb (lower arrow). This latter band derives from a *Hin*dIII restriction site of *En-1* at nt 898 and another one downstream of the *ADC2* stop codon, whereas the 9 kb band derives from the *Hin*dIII restriction site of *En-1* at nt 1794 and one upstream of the *ADC2* start codon.

Appearance of these two bands instead of the 4 kb wild-type band in all progeny plants analyzed, proves that they are homozygous for the *ADC2*::*En-1* insertion and indicates that already the original EN9 plant was homozygous for the mutated *ADC2* locus.

An additional weak band at 1.2 kb is visible in wild-type and all mutant lines. As hybridization was done under rather stringent conditions (50% formamide, 42°C), and as *ADC1* shows about 80% identity to *ADC2* at the nucleotide level, we conclude that this band most likely represents the *ADC1* gene, which contains one *Hin*dIII restriction site at nt 709 and another at nt 1904 of the coding region, giving a fragment of the observed size. No additional signal was detectable, suggesting that ADC is a two or low-copy number nuclear gene. This is in accordance with the report of Galloway et al. [27], who indicated that only two ADC genes exist in *Arabidopsis*.

In contrast, Watson et al. [26] discuss the possibility of at least three genes coding for ADC. This would explain the high residual activity in the double mutant *spe1-1 spe2-1* that is probably affected at both, the *ADC1* and *ADC2*, loci. There is currently no evidence for a third ADC gene and the *spe1* and *spe2* mutations described by Watson et al. [26] are not yet mapped.

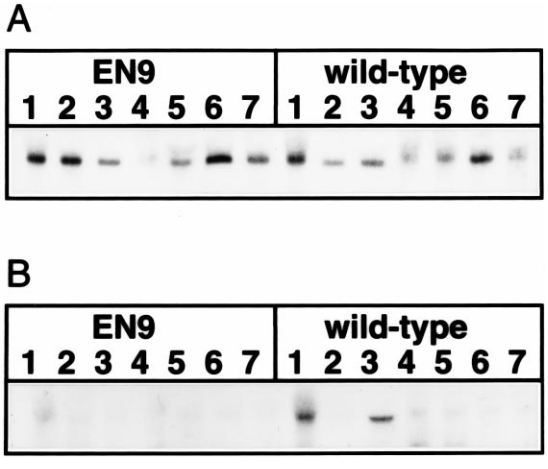

Fig. 2. Analysis of ADC gene expression in EN9. Total RNA (20 μg) from wild-type and EN9 were loaded in each lane and hybridized to an *ADC1* specific probe (A) and a *ADC2* specific probe (B), respectively. The tissues analyzed were: (1) siliques, (2) flowers, (3) cauline leaves, (4) upper inflorescence, (5) lower inflorescence, (6) rosette leaves, (7) roots.

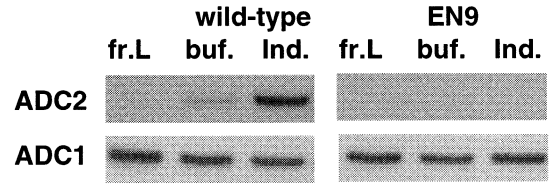

Fig. 3. Induction of *ADC* expression by osmotic stress. Total RNA (15 μg) from leaves of wild-type plant and EN9 plants were loaded for each lane. fr.L, fresh leaf; buf., buffer alone, incubation time 7 h; Ind., buffer+0.6 M sorbitol, incubation time 7 h. The membrane was first hybridized to an *ADC2* specific probe (ADC2) and afterwards with an *ADC1* specific probe (ADC1).

3.2. Molecular and biochemical analysis of the ADC2::En-1 mutant EN9

Plants homozygous for the *ADC2*::*En-1* insertion were further characterized by RNA gel blot analysis to assess the effect of the *En-1* insertion on transcription of the two known ADC genes. Expression of both genes was analyzed for different organs of *Arabidopsis* plants by RNA gel blot experiments. Twenty micrograms of total RNA were used for denaturing agarose gel electrophoresis and blotted on nylon membrane. The 3′ regions of *ADC1* and *ADC2*, which show only low homology and should therefore yield gene-specific probes, were amplified by PCR and directly used for hybridization. The primers used for the PCR reactions annealed at nt 2287 and nt 2679 in the case of *ADC1* [36] and in case of *ADC2* at nt 2001 and nt 2446 [35].

As expected, no *ADC2* transcript of the correct size was detectable in all tissues of EN9 plants analyzed (Fig. 2B). Because we could not find an *ADC2* specific signal at a lower or higher molecular weight, we believe that a putative transcript of the mutated gene was unstable or of a very large size and insufficiently transferred to the nylon membrane.

Expression of *ADC1* in the wild-type plants was detectable in all organs analyzed, being strongest in siliques and leaves, weaker in flowers and cauline leaves, and barely detectable in the upper and lower part of the stem and in roots (Fig. 2A). Preferential expression of an ADC gene in the younger tissues (e.g. siliques, cauline leaves) has also been reported for pea [37] but characteristic differences occur: whereas no ADC transcript could be detected in fully expanded leaves of pea, *ADC1* of *Arabidopsis* gave a strong signal in this tissue (lane 6 in Fig. 2A).

As shown in Fig. 2A, the expression pattern for *ADC1* is not altered in the mutant and there is no obvious difference in expression level, except for flowers, where the transcript level seems to be higher in EN9. Whether the difference in *ADC1* gene expression in flowers is an effect of the *ADC2*::*En-1* insertion or just reflects developmental differences of the plants, has to be investigated. The age of the plants tested was the same, i.e. 6 weeks from sowing, and no developmental differences between mutant and wild-type were visible. However, we cannot exclude consequences of the mutation on developmental processes affecting *ADC1* gene expression in flowers. Several groups report that regulation at the transcript level is not important in determining ADC activity [37–40], but Pérez-Amador et al. demonstrated transcriptional regulation of ADC in reproductive tissue in pea [37].

While *ADC1* was found to be constitutively transcribed, a more restricted expression pattern was observed for *ADC2* in the wild-type. Transcript accumulated to high levels in siliques

and cauline leaves, as was the case for *ADC1*. But *ADC2* transcript was only barely detectable in stem, leaves, roots and flowers. The difference in expression pattern might indicate that *ADC1* and *ADC2* serve different functions. Tiburcio et al. [12] assign ADC a physiological role on the one hand in non-dividing tissue and on the other hand in response to environmental stresses. Judged on the basis of low transcript abundance under normal growth conditions, *ADC2* would come into question as the stress responsive form.

3.3. Physiological analysis of ADC2::En1 mutants

ADC activity was compared in leaves of EN9 and wild-type plants. Because it is not possible to discriminate between the *ADC1* and *ADC2* gene products based on measurements of enzyme activity, we measured total ADC activity using ^{14}C labeled arginine and quantified CO_2 release in vitro (Table 1). In 6 weeks old plants, ADC activity in leaves is slightly reduced in the EN9 line. The mean value of ADC activity of the mutant is 56% of that for the wild-type under normal growth conditions. As differences in the physiological status of the plants influence the ADC activity, the variation is very high. It is therefore not possible to conclude a 50% contribution of the *ADC2* gene to total ADC activity from these data. Watson et al. [26] obtained a similar reduction for mutants identified in an in vivo screen for reduced arginine decarboxylase activity. The authors defined two complementation groups, *spe1* and *spe2*, of mutants each leading to a reduction of ADC activity down to between 50 and 23%. Interestingly, in both mutant groups plants could be identified that were reduced to less then 40%, indicating that there might be epistatic interaction of the *spe* loci. From our analysis of transcript profiles for *ADC1* and *ADC2* we have no evidence for such an epistatic effect of the *ADC2* mutation. When plants were grown on soil, the mutant displayed no obvious phenotype, indicating that ADC activity might not be limiting in leaf.

A lack of an obvious phenotype was also described by Watson et al. for the EMS mutants *spe1-1* and *spe2-1*. Only the double mutant *spe1-1 spe2-1* exhibited a phenotype. It had strongly kinked roots and narrower leaves, sepals and petals [26]. This double mutant still retained about 20% of ADC activity, what could either be explained by the existence of at least one other ADC gene (see above), non-null alleles at both loci, or the mutations affecting other than ADC genes.

3.4. Response to osmotic stress of ADC2::En1 mutants

As we concluded from our data that EN9 is most probably a knockout mutation of *ADC2*, we wanted to define conditions under which this gene would significantly contribute to total ADC activity.

Because ADC has been implicated in stress responses of different plant species [41,42], we tested the reaction of the EN9 mutant to osmotic stress. According to the method described by Feirer et al. [7], detached leaves of the mutant and wild-type plants were incubated in sodium phosphate buffer containing 0.6 M sorbitol. After incubation for 7 h, we measured ADC activity in the detached leaves. The data are represented in Table 1. Each value given represents two independent experiments with five independent samples. Incubation of leaves in isotonic buffer caused an induction of ADC in the wild-type. This was also reported by Feirer and co-workers, who ascribed this induction to a wound response. In our studies, however, this response was not as dramatic as demonstrated by Feirer and co-workers. We suggest that differences in the plant material might be responsible for this discrepancy, because we do not know the age of the plants used in the experiments reported by Feirer et al. In independent experiments with different sets of plants, we observed the highest scattering of ADC activity for leaves incubated in isotonic buffer, whereas the values for freshly sampled leaf material and stress induced leaves did not vary as much.

In wild-type plants, osmotic stress leads to an 18-fold increase in total ADC activity as compared to the isotonic incubation, being 26-fold higher than in leaves immediately homogenized after removal from the plant. Feirer et al. [7] measured a difference of ADC activity of only two-fold after incubation of detached leaves in buffer with or without 0.6 M sorbitol, but compared to the untreated control, the ADC activity was already 10-fold higher after incubation in isotonic solution. In the mutant, ADC activity was almost unaffected by the treatment (Table 1). Compared to the wild-type, the mutant has a 20-fold lower total ADC activity under stress conditions. This result strongly suggests that ADC2, not ADC1, is the gene that is responsible for the increase of ADC activity under osmotic stress conditions in *Arabidopsis*.

To investigate the principle of regulation of *ADC2*, we performed RNA gel blot analysis of leaf RNA from wild-type and EN9 mutant plants subjected to the different treatments. As demonstrated in Fig. 3, *ADC2* expression in wild-type was again undetectable in untreated leaves (lane fr.L). Incubation in isotonic sodium phosphate buffer caused a weak induction of expression (lane buf.) and a strong increase in *ADC2* mRNA was observed after osmotic stress treatment (lane Ind.) of wild-type leaf. In the EN9 mutant, the *ADC2* mRNA was undetectable under all conditions. In leaves of EN9 as well as wild-type plants, *ADC1* expression was unaffected by the different treatments. The result obtained for *ADC2* expression in wild-type leaf is in perfect agreement with the data obtained for ADC activity, and therefore we conclude that transcriptional regulation of *ADC2* is at least in part responsible for the induction of ADC activity by osmotic stress.

Watson and Malmberg [36] suggested that there might be different mechanisms involved in regulating ADC during short-term acute osmotic stress and chronic potassium deficiency in whole plants at least in oat. The results shown here allow extension of the concept of differential regulation to *A. thaliana*: one of the two ADC genes present in *Arabidopsis*, the *ADC2* gene, is involved in the reaction of the plant to osmotic stress.

We are currently investigating physiological consequences of the *ADC2* loss of function and we are also aiming at defining the role of *ADC2* under non-stressed conditions.

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