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Constitutive expression of the oat *arginine decarboxylase* cDNA in transgenic wheat plants does not influence expression of its orthologue

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SUMMARY – The Polyamines (PAs) putrescine (Put), spermidine (Spm) and spermine (Spm) play vital roles in the growth and development of all living organisms. Elevated amounts of PAs, especially Put, appear to be important in the physiological response of plants exposed to various stresses such as potassium deficiency, acidic soils, environmental pollutants, temperature and drought. We reported previously, that over-expression of the *Datura stramonium arginine decarboxylase* (*adc)* cDNA conferred tolerance in transgenic rice plants when these were subjected to drought stress. We subsequently generated a wheat population expressing the oat *adc* cDNA. We investigated spatial and temporal expression of the transgene and the endogenous gene at the mRNA level. Transcript and polyamine profiles were correlated. This homozygous transgenic wheat population is currently being used for further abiotic stress experiments.

Introduction

The PA biosynthetic pathway has been of high interest as a model in metabolic pathway engineering to test hypotheses and answer fundamental questions on the manipulation of such pathways in plants.

It is a relatively short pathway in terms of the number of enzymes involved and the pathway affects crucial physiological, developmental and regulatory processes where PAs are involved. All genes have been cloned (Kakkar and Sawhney, 2002) and the enzymes encoded by these genes have been characterized in detail. The dissection of the PA pathway through the use of molecular genetics has revealed the importance and influence of the different molecular and biochemical components that need to be studied in some detail to allow effective modulation of complex pathways in plants. In order to develop better strategies to manipulate the PA in important cereal crops we focussed on transgenic wheat expressing the oat /adc/ cDNA driven by the strong constitutive maize /Ubi-1/ promoter. In order to investigate potential interactions between the transgenic and endogenous PA pathways in wheat, we cloned a putative wheat /adc/ gene. In this communication we describe and discuss our latest results and we put forward a hypothesis that links Pas and abiotic stress response in wheat.

Material and methods

Plasmids, transformation and plant regeneration

The 2.124 kb oat *adc* cDNA (Bell and Malberg, 1990) was excised as an *Eco*RI fragment from pAMC2 (Burtin and Michael, 1997) and subcloned into the *Eco*RI site of pAL76 (Christensen and Quail, 1996), which contains the maize *Ubi-1* promoter and first intron and an *Agrobacterium tumefaciens nos* transcriptional terminator. This plasmid was subsequently referred to as *Ubi:Oadc* (Fig. 1A). Wheat transformation, selection and regeneration procedures were performed as described in detail (Stoger *et al*., 1998). Gold particles for bombardment were coated with a mixture of *Ubi:Oadc* and pAHC25 (plasmid containing screenable and bar selectable marker; Christensen and Quail, 1996) at a molar ratio of 3:1. Wheat plants were grown in growth rooms and greenhouse as described in Stoger *et al*. (1998).

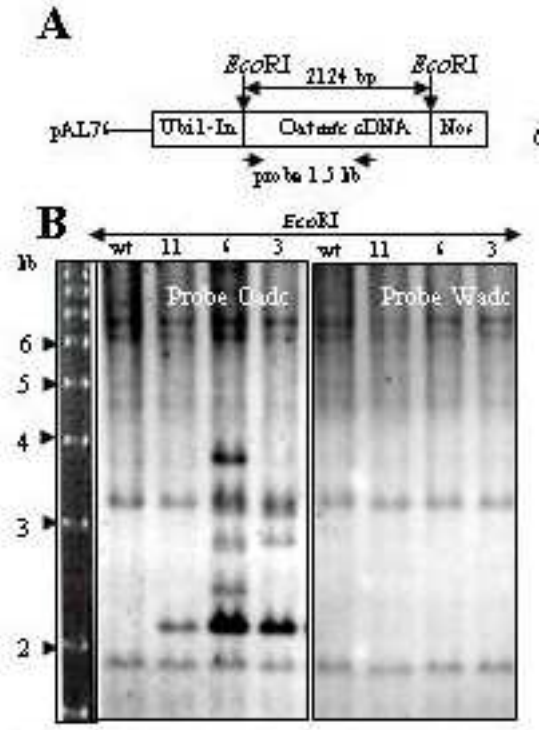

Fig. 1. A. Map of *Ubi:Cadc* showing transcription unit and relevant restriction sites. The cDna es 2124 kb in size. *EcoRI* has two restriction sites. Nos: nopaline synthase. B. Genomic DNA (25 μg) was digested with *EcoRI*. DNA was probed with oat *adc* and re-probed with wheat *adc*. Exposure time was 30 and 50 minutes; wt: wild-type; numbers represent putative transgenic plants; L: molecular size marker.

Cloning of a partial wheat adc sequence

The oat *adc* cDNA (X56802) was used as query sequence to search against the TIGR wheat database (http://www.tigr.org/) using the BLAST program (http://tigrblast.tigr.org/tgi/). One sequence TC241040 with 82% homology to the 5'-end of the oat *adc* sequence was identify and selected for further studies. ClustalW (http:/www.ebi.ac.uk/clustalw/) was used for alignment of nucleotides and amino acid deduced sequences of oat *adc* and TC241040. Total RNA was extracted using the RNeasy Plant Mini Kit from wheat leaf tissue. Five micrograms of total RNA were reversely transcribed (RT) into cDNA using the QIAGEN OMNISCRIPT RT. A primer combination was designed to amplify a putative wheat *adc*. Primer sequences: forward tc24Ra, 5'-GCAACGGCTACAAGGACCTCGAG-3' and reverse, tc24Rb 5'-CTGCACTTCCACATTGGCTCCCA-3' giving a fragment of 315 bp. The PCR product was used as a wheat *adc* probe.

DNA and RNA gel blot analysis

Genomic DNA was extracted from leaf tissue according to the method of Edwards *et al*., (1991). Following *Eco*RI digestion and electrophoresis on a 0.8 % TBE agarose gel (Sambrook *et al*., 1989), DNA (25 μg) was transferred to a positively charged nylon membrane. Nucleic acids were fixed by UV crosslinking. Labeling of the oat and wheat *adc* probes, hybridization and washes were carried out as described in detail in Bassie *et al*., (2000). Chemiluminescence detection was carried out according to the manufacturer's instructions. Re-probing of the membranes with the wheat *adc* probe was performed as described in Lahaye *et al*., (1996). Total RNA for RNA gel blot analysis, was extracted from leaves from one month-old plants using Trizol®. Denatured RNA (30 μg) was subjected to electhophoresis on 1.2% agarose-formaldehyde gel using 1X MOPS buffer (Sambrook *et al*., 1989). Hybridization of the RNA gel blots with the oat and wheat *adc* probes was as described earlier.

Determination of ADC and ODC activities and polyamine analysis

Enzyme activity analyses for both enzymes were carried out as described in detail in Bassie *et al*., (2000). One nKat of ADC or ODC activity was defined as the amount (μmol) of $^{14}CO_2$ released per min and per mg protein. Crude extracts from leaves were dansylated and separated by TLC (Thin Layer Chromatography) and quantified as described earlier (Lepri *et al*., 2002). Results were expressed as nmol g^{-1} fw.

Results and discussion

Molecular characterization of transgenic wheat plants

We analyzed 14 independent transgenic wheat plants and we confirmed integration of the *Ubi:Oadc* by genomic DNA gel blot analysis. Digestion was carried out using *Eco*RI which releases a 2.1 kb diagnostic fragment comprising the entire coding sequence of the oat *adc* cDNA (Fig. 1B). This digestion demonstrated that most of the lines contained the intact *adc* coding sequence (Fig. 1B). Re-probing of the membrane with the putative wheat *adc* confirmed background bands as the wheat *adc* ortholog (Fig. 1B). Transgenic plants where steady-state oat *adc* mRNA was detected were selected for further analysis (Fig. 2A). Significant increases in ADC activity (Fig. 2B) resulted in a increase in the content of Put, Spd and Spm in leaf tissue (Fig. 2D). No significant variation was detected in ODC activity (Fig. 2C). A specific line, clone 6, was selected for further analysis. Transgene expression in this line (Fig. 3A) did no affect endogenous *adc* expression (Fig. 3B). We have taken this population to homozygocity and is currently being tested for drought stress response.

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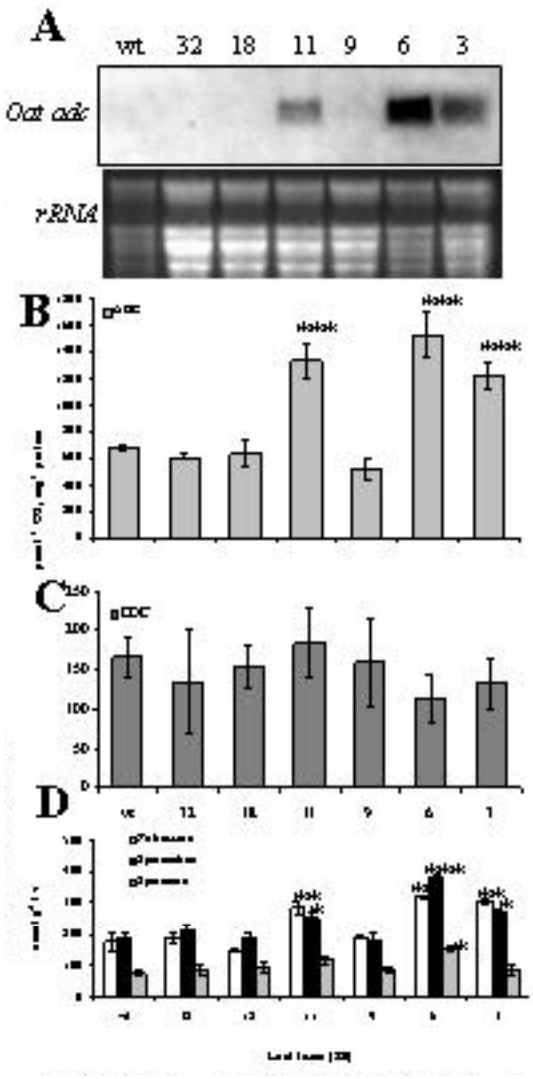

Fig. 2. A. RNA gel blot analysis of transgenic wheat. B. ADC enzyme activity. C. ODC enzyme activity. D. PAs levels in leaf tissue. ***P>0.001; **0.01>p>0.001; *0.05>P>0.01.

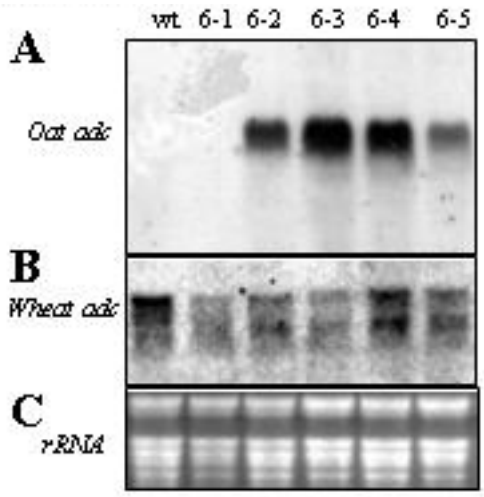

Fig. 3. RNA gel blot analysis of progeny plants from clone 6. A. Oat *adc* steady-state mRNA; B. Putative wheat *adc* steady-state mRNA; C. EtBr picture of total mRNA showing equal loading.