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CHARACTERISATION OF ACC SYNTHASE DURING LEAF DEVELOPMENT IN WHITE CLOVER

(Trifolium repens L.)

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Abstract

ACC synthase catalyses the rate limiting step in the ethylene biosynthetic pathway, and in all plants studied has been shown to be encoded by a highly divergent gene family. These different ACC synthase genes are differentially regulated in response to a variety of developmental and environmental stimuli. In this thesis, ACC synthase gene expression during leaf ontogeny in white clover (Trifolium repens L.) has been studied. This study utilises the stoloniferous growth pattern of white clover, which provides leaf tissue at different developmental stages, ranging from initiation at the apex, through mature green to senescent, and then finally necrotic.

RT-PCR, using degenerate primers to conserved regions of ACC synthase genes in the database, was used to amplify putative ACC synthase sequences from mRNA isolated from white clover leaf tissue. Sequencing and GenBank database alignment of the PCR products revealed that ACC synthase sequences comprising approximately 670 bp of the reading frame were amplified. Sequence alignments indicate that the sequences from white clover represent three distinct ACC synthases, and these were designated TR-ACSl (Trifolium repens ACC synthase 1), TR-ACS2 and TR-ACS3. TR-ACSl is 62 % and 71 % homologous to TR-ACS2 and TR-ACS3 respectively, and TR-ACS2 and TR-ACS3 are 63 % homologous, in terms of nucleotide sequence. Genomic Southern analysis, using the amplified reading frame of each gene as a probe, confirmed that the sequences are encoded for by distinct genes.

In a GenBank database search, TR-ACSl shows highest homology to an ACC synthase sequence from IAA-treated apical hooks of pea, and TR-ACS2 shows highest homology to an ACC synthase isolated from etiolated hypocotyls of mungbean. An ACC synthase isolated from white lupin, which was found to have increased expression during germination and in response to IAA and wounding, was most similar to TR-ACS3. Phylogenetic analysis determined that the three white clover ACC synthase genes are highly divergent. Phylogenetic analysis also determined that TR-ACSl groups with ACC synthase sequences isolated from IAA-treated apical hooks of etiolated pea seedlings and IAA-treated mung bean hypocotyls. TR-ACS2 was found to group with
ACC synthases isolated from etiolated hypocotyls of mung bean and TR-ACS3 was closest to a cDNA clone isolated from Citrus parapdisi.

Northern analysis has shown that two of these genes are expressed differentially during leaf ontogeny. TR-ACS1 is expressed in mature green leaves and TR-ACS3 is expressed in senescent leaf tissue. The expression of TR-ACS2 was unable to be determined by northern analysis, and so the more sensitive method of RT-PCR was used. This procedure determined that TR-ACS2 is expressed predominantly in the apex, newly initiated and mature green leaves, and again at the onset of senescence.

Sequence analysis of TR-ACS3 revealed that the coded protein is missing the active site of the enzyme. Using a primer specific for the conserved active site of ACC synthase, a sequence which was similar to TR-ACS3, but not completely homologous, was amplified by RT-PCR and designated TR-ACS3A. This sequence included the region encoding the active site of the enzyme, but contained an additional four nucleotides in the sequence. Thus the sequence may also encode a non-functional protein, and the possible roles of such non-functional proteins are discussed.

The pattern of TR-ACS gene expression observed during leaf ontogeny suggests that these genes are under precise developmental control. Further, an indication as to the nature of the stimuli that may regulate the expression of the ACC synthase genes was provided by the phylogenetic analysis. To learn more of these physiological stimuli, white clover leaves were treated with two of the primary stimuli of ACC synthase gene expression, IAA and wounding; factors that were also shown to regulate the expression of sequences phylogenetically related to each TR-ACS gene. While the results of this experiment do not appear to be definitive, TR-ACS1 was the only gene that hybridised to timepoints in the IAA-treated tissue, and TR-ACS3 was the only gene to hybridise to the wound-induced tissues. The significance of ACC synthase gene expression during leaf ontogeny in white clover and its regulation is discussed.

Antibodies were raised to the gene product of TR-ACS1 expressed in E. coli. Using western analysis, the antibodies were shown to recognise the gene products of all three TR-ACS genes and a protein of 55 KD with highest intensity in mature green leaf extracts. Minor recognition of proteins of 29, 34, 37, 69 and 82 KD was also observed.
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Abbreviations

A\textsubscript{260 nm} absorbance in a 1 cm light path at 260 nm
ACC 1-aminocyclopropane-1-carboxylic acid
Amp\textsuperscript{100} ampicillin (100 mg/ml)
APS ammonium persulphate
BCIP 5-bromo-4-chloro-3-indoyl phosphate
BSA bovine serum albumin
DNA deoxyribonucleic acid
DNase deoxyribonuclease
DTT dithiothreitol
EDTA ethylenediaminetetraacetic acid
EIN ethylene insensitive
FW fresh weight
h hour
IAA indole-3-acetic acid
Kb kilo-bases
kD kilo-dalton
LB Luria-Bertani media
MACC 1-(malonylamino)cyclopropane-1-carboxylate
1-MCP 1-methylcyclopropane
min minute
NaOAc sodium acetate
NBT p-nitro blue tetrazolium chloride
Ni-NTA nickel-nitrilotriacetic acid
PAG photosynthesis-associated gene
PAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
pI isoelectric-point
RNase ribonuclease
RO reverse osmosis
RT-PCR reverse transcriptase-dependent PCR
SAG senescence associated gene
SAM S-adenosylmethionine
SA-PMP  streptavidin magne-sphere particles
SDS    sodium dodecyl sulphate
TEMED  \(N,N,N',N'-\)tetramethylethylenediamine
Tris   tris (hydroxymethyl)aminomethane
U      units
UV     ultra violet
V      volt