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-ACS1 (Trifolium repens ACC synthase 1)*, *TR-ACS2* and *TR-ACS3*. *TR-ACS1* 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-ACS1* 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-ACS1* 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_{260} nm	absorbance in a 1 cm light path at 260 nm
ACC	1-aminocyclopropane-1-carboxylic acid
Amp^{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