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**EXPRESSION OF ACC OXIDASE GENES IN WHITE
CLOVER (*Trifolium repens* L.) ROOTS IN RESPONSE
TO PHOSPHATE SUPPLY**

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MARISSA B. ROLDAN

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ABSTRACT

The differential expression of members of the *Trifolium repens* ACC oxidase (*TR-ACO*) gene family and accumulation of TR-ACO proteins in white clover roots, and the temporal *TR-ACO* gene expression and TR-ACO protein accumulation in response to phosphate (Pi) stress has been investigated. Four-node stolon cuttings of wild type and transgenic white clover (designated *TR-ACOp::GUS* and *TR-ACO1p::mGFP5-ER*) plants were rooted and acclimatised in Hoagland's solution, and then subjected to either a Pi sufficiency (1 mM Pi) treatment or a Pi depletion (10 μ M Pi) treatment over a designated time course.

Using semi quantitative Reverse Transcriptase-Polymerase Chain Reaction (sqRT-PCR) and gene-specific primers it has been determined that the *TR-ACO* genes are differentially expressed in the roots of white clover. The *TR-ACO1* transcript abundance was greater in the lateral roots when compared to the main roots. By immunodetection analysis using antibodies raised against TR-ACO1, recognition of a protein of expected size (*ca.* 36 kDa) was also greater in the lateral roots. The tissue-specific localisation of *TR-ACO1* promoter activity was investigated first by light microscopy using a single genetic line of white clover transformed with a *TR-ACO1p::GUS* gene construct, and results then confirmed by confocal microscopy using several genetically independent lines of transgenic plants transformed with a *TR-ACO1p::mGFP5 ER* gene construct. In these lines, the *TR-ACO1* promoter activity was primarily located in the meristem of the main and lateral roots, lateral root primordia as well as in the pericycle of the root with nodes of expression in the emerging lateral roots, suggesting a role for ethylene in the development of young tissues where cells are actively dividing.

In terms of *TR-ACO2*, greater transcript abundance and protein accumulation of *TR-ACO2* were also observed in the lateral roots when compared to the main roots.

Histochemical GUS staining of roots of a single genetically-independent line transformed with a *TR-ACO2p::GUS* construct showed predominant promoter activity in the mature tissues of both the main and lateral roots but not in the meristematic tissues. In contrast, *TR-ACO3* showed greater transcript abundance in the main roots relative to the lateral roots, and the promoter activity, as determined using a single genetically-independent line of *TR-ACO3p::GUS* transformed plants was predominantly in the mature tissues of the main roots

In response to Pi depletion, the members of *TR-ACO* gene family were temporally expressed in the white clover roots. Using sqRT-PCR, the *TR-ACO1* transcript abundance was greater in Pi depleted roots at 12 h and 24 h after Pi depletion in both wild type plants and in the one genetically-independent line of white clover transformed with the *TR-ACO1p::mGFP5-ER* construct examined. Similarly, by western analysis using both α -*TR-ACO1* and commercially available α -GFP antibodies (for the transformed line), a greater accumulation of proteins was consistently observed in Pi depleted roots from the first up to the seventh day after Pi depletion. By confocal microscopy, it was determined for several genetically-independent line of white clover transformed with *TR-ACO1p::mGFP5-ER* that under Pi depletion more intense GFP fluorescence over a time course of 1 d, 4 d, and 7 d was observed, when compared to plants grown under Pi sufficiency.

For *TR-ACO2*, there was no significant difference in transcript accumulation and protein accumulation in response to short term Pi depletion of up to seven days.

However, at 15 d and 21 d after Pi depletion there was a greater protein accumulation in

the roots of Pi depleted plants relative to the Pi sufficient roots. Further, when main and lateral roots were compared, a greater protein accumulation occurred in the lateral roots. For *TR-ACO3*, there was no consistent trend of transcript accumulation in response to Pi depletion over a 24 h period. While a marked reduction in transcript accumulation was noted in Pi depleted roots at 1h, 12 h, 24 h, there was an increase in transcript accumulation at 6 h and 18 h after Pi depletion, indicating that factors other than Pi supply may be affecting gene regulation.

Root morphological studies revealed an increase in the main root length and lateral root production in white clover in response to Pi depletion with a greatest growth rate noted between the sixth and ninth day after Pi depletion, and this period overlapped with accumulation of *TR-ACO1* protein suggesting a role for ethylene in the Pi stress induced lateral root production in white clover. The differential regulation of the three *TR-ACO* genes in white clover roots in response to Pi depletion further suggests the divergence in terms of regulation of the ethylene biosynthetic pathway, which may play an important role in fine tuning the responses of plants to particular environmental cues.

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List of Abbreviations

| | |
|--------------------|--|
| 1°Ab | Primary antibody |
| 2°Ab | Secondary antibody |
| A ₂₆₀ | Absorbance at 260 nm |
| A ₅₉₅ | Absorbance at 595 nm |
| ACC | 1- aminocyclopropane-1-carboxylic acid |
| ACO | ACC oxidase |
| ACS | ACC synthase |
| Adomet | S-adenosyl-L-methionine |
| AHK | <i>Arabidopsis</i> histidine kinase |
| AM | Apical meristem |
| Amp ¹⁰⁰ | Ampicillin (100 mg.ml ⁻¹) |
| APS | Ammonium persulfate |
| APase | Acid phosphatase |
| ARR | Amplex red reagent |
| Au | Arbitrary unit |
| AVG | Aminoethoxyvinylglycine |
| 6-BAP | 6-Benzylamino purine |
| BCIP | 5 bromo-4-chloro-3-indolyl phosphate |
| bp | Base pair |
| BME | 2-β-Mercaptoethanol |
| BSA | Bovine serum albumin |
| °C | Degrees Celsius |
| ca | Approximately |
| CaMV | Cauliflower mosaic virus |
| Cef ³⁰⁰ | Cefotaxime (300mg.ml ⁻¹) |
| cm | Centimetre |
| CTR | Constitutive triple response |
| dATP | 2' deoxyadenosine 5'-triphosphate |
| dCTP | 2'deoxycytidine 5'-triphosphate |
| dGTP | 2'deoxyguanosine 5'-triphosphate |
| DEPC | Diethyl pyrocarbonate |
| DMF | Dimethylformamide |

| | |
|--------------------|---|
| DMSO | Dimethyl sulfoxide |
| DNase | Deoxyribonuclease |
| dNTP | Deoxynucleotide triphosphate |
| DPX | Dibutyl phthalate xylene |
| DTT | Dithiothreitol |
| DW | Dry weight |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| EDTA | Ethylenediaminetetraacetic acid |
| EFE | Ethylene forming enzyme |
| EIN | Ethylene insensitive |
| ELISA | Enzyme-linked immunosorbent assay |
| ETOH | Ethanol |
| ETR | Ethylene triple response |
| EtBr | Ethidium bromide |
| FAA | Formalin acetic acid |
| FU | Fluorescence unit |
| FW | Fresh weight |
| g | g force |
| GFP | Green fluorescent protein |
| GMO | Genetically modified organism |
| GUS | <i>E. coli</i> β -Glucuronidase |
| h | Hour |
| HCl | Hydrochloric acid |
| HRP | Horse radish peroxidase |
| IAA | Indole-3-acetic acid |
| IPTG | Isopropyl- β -D – thiogalactopyranoside |
| Kan ²⁰⁰ | Kanamycin (200 mg.ml ⁻¹) |
| Kb | Kilo basepair |
| kDa | Kilo daltons |
| K _M | Substrate concentration at half maximum reaction rate |
| kPa | kilo Pascal |
| L | Litre |
| LB | Luria-Bertani (media or broth) |
| LR | Lateral root |

| | |
|-------------------|--|
| LRP | Lateral root primordium |
| M | Molar, moles per litre |
| MACC | 1-(malonylamino) cyclopropane-1-carboxylate |
| MADS | The conserved domain of <u>M</u> CMI, <u>A</u> GAMOUS, <u>D</u> EFICIENS and <u>S</u> RF |
| MAPK | Mitogen activated protein kinase |
| 1-MCP | 1-methylcyclopropene |
| MDH | malate dehydrogenase |
| mg | milligram |
| Milli-Q water | water purified by a Milli-purification system |
| min | Minute |
| mL | Millilitre |
| Mr | Relative molecular mass (g/mol) |
| MR | Main root |
| MS | Murashige and Skoog base media |
| MUG | 4-methyl umbelliferyl glucuronide |
| n | Number of replicates |
| NAA | 1-naphthaleneacetic acid |
| NAD | Nicotinamide adenine dinucleotide |
| NaOAc | Sodium acetate |
| NBT | Nitrotetrazolium blue chloride |
| NCBI | National Center for Biotechnology Information |
| nL | Nanolitre |
| ng | Nanogram |
| nmol | Nanomole |
| NOS | Nopaline synthase |
| <i>npt</i> II | Neomycin phosphotransferase II |
| OD ₆₀₀ | Optical density 600 nm |
| ORF | Open reading frame |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline-Tween 20 |
| PCR | Polymerase chain reaction |
| PEPC | Phosphoenolpyruvate carboxylase |

| | |
|----------|--|
| PGU | Plant Growth Unit |
| pH | -Log [H ⁺] |
| Pi | Inorganic phosphate, orthophosphate |
| PI | Propidium iodide |
| ppm | Parts per million |
| PVDF | Polyvinylidene difluoride |
| RACE | 3'- rapid amplification of cDNA ends |
| RH | Relative humidity |
| Rnase | Ribonuclease |
| RO | Reverse osmosis |
| rpm | Revolutions per minute |
| RT-PCR | Reverse transcriptase- polymerase chain reaction |
| SAM | Shoot apical meristem |
| SAP | Shrimp alkaline phosphatase |
| SDS | Sodium dodecyl sulphate |
| s.e. | Standard error of the mean |
| SSC | Saline sodium citrate |
| SSPE | Saline sodium phosphate EDTA buffer |
| sqRT-PCR | Semi-quantitative RT-PCR |
| TAE | Tris-Acetate-EDTA |
| TBA | Tertiary butyl alcohol |
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| TR-ACO | <i>Trifolium repens</i> ACC oxidase |
| TR-ACS | <i>Trifolium repens</i> ACC synthase |
| Tris | Tris(hydroxymethyl)aminomethane |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| µm | Micrometer |
| UTR | Untranslated region |
| UV | Ultraviolet light |
| v/v | Volume per volume |
| WT | wild type |

| | |
|--------|---|
| w/v | Weight per volume |
| w/w | Weight per weight |
| X-Gal | 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside |
| X-Gluc | 5-Bromo-4-chloro-3-indolyl β -D-glucuronide cyclohexylamine salt |
| YM | Yeast Mannitol |

Chapter 1

Introduction

1.1 Overview

Ethylene is a gaseous plant hormone produced in different parts of the plant and is involved in the regulation of various plant developmental processes including seed germination, root elongation, leaf senescence and abscission, as well as fruit ripening. Ethylene is also produced in response to various forms of stress Abeles *et al.*, (1992). These multi-faceted roles indicate the complexity of the mechanisms involved in the synthesis and action of ethylene.

Briefly, ethylene biosynthesis starts from the amino acid methionine which is converted to *S*-adenosyl methionine (SAM) then to 1-aminocyclopropane-1-carboxylic acid (ACC) and finally to ethylene (Adams and Yang, 1979). Within each step, intricate mechanisms are involved which regulate biosynthesis particularly the last two steps involving the two key enzymes, the ACC synthase (ACS) (E.C.4.4.1.14) which is involved in the conversion of SAM to ACC, and the ACC oxidase (ACO) (E.C.1.4.3) which mediates the conversion of ACC to ethylene. Evidence has accumulated that, in many plants, expression of members of the *ACS* and *ACO* gene families are spatially and temporally regulated. For instance, in white clover, both the *ACS* and *ACO* gene families are differentially regulated during leaf developmental stages and in response to environmental cues (Hunter *et al.*, 1999; Gong and McManus, 2000; Murray and McManus, 2005; Chen and McManus, 2006).

The involvement of ethylene in plant responses to changes in phosphorus availability, specifically in the plant roots, has also been investigated in a number of plants (Lynch and Brown, 1997; Borch *et al.*, 1999; Ma *et al.*, 2003; Zhang *et al.*, 2003; Franco-