IDENTIFICATION OF A PLANT-ENCODED ACC DEAMINASE

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The Faculty of Graduate Studies

of

Carleton University

by

Lisa McDonnell

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ABSTRACT

In most plants, 1-aminocyclopropane-1-carboxylic acid (ACC) is converted to ethylene by ACC oxidase. In some bacteria ACC can be broken down into ammonia and α -ketobutyrate by ACC deaminase. In the poplar stem, differential expression of ACC oxidase and of an ACC deaminase-like sequence suggested that there may be an ACC deaminase in plants and that it might be responsible for regulating ethylene biosynthesis across developing vascular tissues. I have shown that a putative ACC deaminase gene from *Arabidopsis thaliana* encodes for a bona fide ACC deaminase. The enzyme activity has been correlated with many plant developmental processes such as seed development and germination, to development of highly vascular tissues of *Arabidopsis*, tomato fruit ripening, and during phloem formation in poplar. This is the first indication of a functional plant-encoded ACC deaminase and its role in plants offers a new mechanism for regulation of ethylene levels.

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"He that plants trees loves others besides himself."
Thomas Fuller

And for those of you that know me well; "Whatever, I'll do what I want!"

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List of Abbreviations

ACC – 1-aminocyclopropane-1-carboxylic acid
CDS – coding sequence
DF – Dworkin and Foster
DNTB – 5,5'-dithiobis(2-nitrobenzoic acid))
DTT – Dithiothreitol
EST – expressed sequenced tag
GACC - 1-(γ -L-glutamyl-amino) cyclopropane-1-carboxylic acid
IAA – indole acetic acid
MACC - malonyl ACC
MTA – 5'methylthioadenosine
PCD – programmed cell death
PGPB – plant growth promoting bacteria
SAM – S-adenosylmethionine

1 INTRODUCTION

1.1 The plant hormone ethylene

Ethylene was first recognized to have effects on plant growth in the late 1800's. Ethylene emitted from street lamps was observed to cause trees to lose their leaves and prevent carnation flower buds from opening (Crocker and Knight, 1908). Evidence that ethylene may have been an endogenous hormone was reported in 1910 and since then it has been shown to affect plant growth during most stages of development and in response to stress (Abeles *et. al.*, 1992). Although this hormone has been studied for nearly a century, the mechanisms by which ethylene affects plant development and the mechanisms that regulate when, where, and how much hormone is produced require investigation.

1.1.1 Ethylene biosynthesis

Ethylene biosynthesis occurs in almost all tissues of most plants, but the rate and amount produced varies depending on the tissue and developmental stage. In seed-bearing plants ethylene is formed from the precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The amino acid methionine is the first substrate in the ethylene biosynthetic pathway. Methionine adenosyl transferase converts methionine to S-adenosylmethionine (SAM) at the cost of one ATP per methionine molecule. SAM is used to create ACC by ACC synthase. A by-product of this reaction is 5'-methylthioadenosine (MTA). MTA is converted back into methionine by a salvage pathway which prevents ethylene

biosynthesis from putting a strain on methionine pools (Wang *et. al.*, 2002). ACC is converted into ethylene by ACC oxidase which also results in the formation of carbon dioxide and hydrogen cyanide. Efficient detoxification pathways prevent poisoning by hydrogen cyanide (Abeles *et. al.*, 1992). The ethylene biosynthesis pathway is illustrated in Figure 1.

The action of ACC synthase to produce ACC is reported by many as the rate limiting step in ethylene biosynthesis. This is because the enzyme is quite labile, produced at low levels, and regulated by a variety of factors (Wang *et. al.*, 2002). ACC synthase is part of a multigene family in most plants. In *Arabidopsis*, seven ACC synthase genes have been characterized (Wang *et. al.*, 2002). Two have been reported as non-functional (Woeste *et. al.*, 1999). Active ACC synthases are differentially expressed during various plant developments and in response to environmental changes (Liang *et. al.*, 1992). ACC synthase production and activity is regulated by a variety of factors such as ethylene, auxin, and many environmental stresses (Wang *et. al.*, 2002; Liang *et. al.*, 1992). Due to the labile nature of ACC synthase, *in vivo* and *in vitro*, and a low concentration of the enzyme in total protein, studying its activity is difficult (Abeles *et. al.*, 1992). It is believed, however, that post-translational modifications are responsible for regulating ACC synthase activity (Woeste *et. al.*, 1999; Wang *et. al.*, 2002).

ACC oxidase is also part of a multigene family (Johnson and Ecker, 1998). Three ACC oxidase genes in tomato are reported to be differentially expressed in response to plant developments and environmental stimuli (Barry *et. al.*, 1996).

Figure 1: Ethylene biosynthesis. Synthesis begins with the conversion of the amino acid methionine to SAM. SAM can be shuttled into ethylene production but is also used as a methyl donor for other cellular molecules such as nucleic acids, proteins, and lipids. The conversion of SAM to ACC, by ACC synthase, also creates MTA (5'methylthioadenosine) which can be recycled back into methionine. The action of ACC oxidase to produce ethylene is oxygen dependent. By-products of ethylene production include carbon dioxide and cyanide. These by products can be shuttled into argentine biosynthesis to prevent toxicity to the plant.

Adapted from Wang *et. al.* (2002) and Abeles *et. al.* (1992)

Positive feedback loops seem to regulate activity in which ethylene promotes the increase of both ACC oxidase mRNA and ACC oxidase activity (Johnson and Ecker, 1998). ACC oxidase is not generally considered a rate limiting step in ethylene biosynthesis. In tomato fruit, it was reported that ACC oxidase mRNA was present before the climacteric ethylene burst (Nakatsuka *et. al.*, 1998). ACC synthase, however, appeared to be up-regulated and then accumulated just prior to ethylene production. This may not be the case in all developmental processes. For example, during tension wood formation in poplar trees ACC was available at stable levels, but it was ACC oxidase which was induced and differentially expressed (Andersson-Gunneras *et. al.*, 2003).

ACC oxidase is believed to be the evolutionary step that lead to the development of ethylene production from ACC in higher plants. Ferns and other non-flowering plants do not synthesize ethylene from ACC, however some contain ACC synthase and accumulate ACC (John, 1997). The flood tolerant monocot *Potamogeton pectinatus* accumulates ACC in response to auxin but does not produce ethylene via ACC (Johnson and Ecker, 1998). The evolution of ACC oxidase to utilize ACC for ethylene production is thought to have occurred in higher plants, providing the above mentioned ethylene biosynthesis pathway.

Ethylene is perceived by a variety of ethylene receptor proteins. In *Arabidopsis*, there are five receptors comprising the ethylene receptor family. In an over-simplification, they generally contain a histidine kinase sensor domain (receptor) and a response regulator that receives the autophosphorylation from the histidine kinase (Johnson and Ecker, 1998). An overview of the ethylene

response pathway is illustrated in Figure 2. Ethylene has an incredibly diverse and complex array of effects on plant growth and development, some of these will be described in the sections following.

1.2 Ethylene and plant development – from seed to seed

Ethylene is associated with many plant developments such as seed germination, root and shoot elongation, senescence, abscission of flowers and leaves, responses to stress, and wood formation. Within these developments ethylene can have a variety of influences from stimulation to inhibition. Of interest in this thesis was the involvement of ethylene in seed dormancy and germination, formation of tissues such as hypocotyls, leaves, and stems, during fruit ripening, and during xylem formation.

1.2.1 Seed dormancy and germination

Ethylene has been linked to regulating dormancy and germination potential. Recently, the roles of different ethylene receptors have been implicated in regulating dormancy and germination (Yee, 2003). Dormancy can be described as a temporary arrest in embryo growth (Raven *et. al.*, 1999). Dormancy can be imposed by the seed coat barrier or by the embryo, or both. Entrance into dormancy occurs when seeds are developing on the plant and prevents them from germinating before development is complete. In *Arabidopsis*, dormancy is embryo-induced and seed-coat-induced and can be broken by stratification (treatment of darkness, cold, and wetness over time)

Figure 2: The ethylene response pathway. The multigene families of ACC synthase and ACC oxidase are differentially regulated by a variety of factors to produce ethylene. Other hormones, such as auxin and cytokinin, act as inducers in response to an event, such as wounding. Ethylene binds to the membrane-spanning regions of the sensing domains of ethylene receptors. Binding of ethylene to a receptor can stimulate phosphorylation of a receiver domain on other ethylene receptors or of a receiver domain on a response regulator protein. This regulates the activity of a Raf-like kinase which stimulates a MAP kinase (MAPK) response pathway. The MAPK responses transmit the ethylene signal. It is thought that receptors of MAPK signals induce ethylene responses by activating transcription factors (Txn). This activates genes that are involved in the phenotypic responses elicited by stimuli such as (a) flooding, (b) pathogen response, (c) wounding, (d) fruit ripening, (e) senescence, (f) triple response.

Adapted from Wang *et. al.* (2002).

which reduces the seed-coat barrier and activates developmental processes within the embryo (Yee, 2003). Seed development has been broken up into different developmental stages among which dormancy and germination potential vary. These stages are illustrated in Figure 3.

Dormant seeds from many species can be stimulated to germinate by the application of ethylene. Naturally, dormant seeds produce low levels of ethylene (Abeles *et. al.*, 1992) and it has been suggested that low levels of ethylene

detected by the ethylene receptor ERS1 causes seeds to enter dormancy (Yee, 2003). Elevated ethylene levels detected by the ethylene receptor ETR1 are thought to stimulate an exit from dormancy (Yee, 2003). The rate of ethylene production increases in non-dormant seeds and during germination and early seedling growth (Abeles *et. al.*, 1992). Inhibiting ethylene synthesis and perception has been shown to decrease germination potential (Abeles *et. al.*, 1992). At germination, embryos resume growth and seedlings begin to form. This signals the end of dormancy.

1.2.2 Cell elongation and expansion – roots to stems

Cell division, elongation, and expansion are involved in the development of all tissues. Ethylene reversibly inhibits and promotes cell growth in different plant species and different cell types (Abeles *et. al.*, 1992). The effects of ethylene on cellular growth influence root elongation, hypocotyl elongation, and leaf expansion, for example. The mechanisms by which ethylene alters cell

Figure 3: Seed development in *Arabidopsis*. Seeds begin as pre-mature and gradually mature on the mother plant. Maturation can be observed by changes in silique colouration and size. Stage 1 (pre-mature) seeds are unable to germinate because they have not completed development to survive separation from the mother plant. Stage 2 (precocious germination) are fully developed and have low germination potential. Somewhere at the end of stage 2 and early stage 3 seeds enter dormancy. Seeds in stages 3 and 4 are dormant and will not germinate even under favourable environmental conditions. Stage 5 and 6 (germination ready) are mature seeds that are capable of exiting dormancy.

Adapted from Yee (2003).

growth are not completely understood, but may be linked to microtubule organization and cell wall structures and properties, which are both involved in the potential of a cell to elongate or expand.

Expansins are proteins found in plant cells that can induce extension and loosening of cell walls, which is necessary for cell expansion (McQueen-Mason *et. al.*, 1992). Many expansins are thought to alter the pH in cell walls which induces cell-wall-loosening during cell expansion. In tomato fruit, it was found that expansin genes were differentially expressed during ripening and that ethylene-enhanced accumulation of an expansin protein occurred (Rose *et. al.*, 2000). Accumulation of this expansin was not observed in the *Never ripe* tomato mutant which has reduced ethylene biosynthesis (Rose *et. al.*, 2000). Ethylene-regulated expansins have also been identified during the ripening of pear fruit (Hiwasa *et. al.*, 2003).

During root elongation, ethylene can inhibit longitudinal cell expansion, but increases radial cell expansion, resulting in short and wide roots (Dolan, 1997). It is thought that developmentally regulated ethylene biosynthesis and sensitivity to ethylene are involved in the ethylene-induced spatial regulation of cell differentiation and growth in roots (Dolan, 1997). The inhibition of cell elongation can be observed when seeds are germinated and grown in the dark (etiolated) with elevated levels of ethylene, or its precursor ACC. The etiolated seedlings exhibit the triple response characterized by short roots, short and wide hypocotyls, and an exaggerated apical hook. All of these effects are attributed to the cell elongation inhibition effects of ethylene (Wang *et. al.*, 2002).

In the dark, ethylene inhibits cell elongation, as illustrated by the triple response. In the light, however, it has been shown that ethylene can promote longitudinal cell expansion in hypocotyls of *Arabidopsis* seedlings (Smalle *et. al.*, 1997; Lehman *et. al.*, 1996). Ethylene-regulated inhibition of cell elongation in the dark and promotion in the light are thought to be controlled by the same ethylene transduction pathway. It was suggested, however, that the upstream or downstream effects of ethylene might be regulated by light, thus causing the opposite effects. The mechanism by which ethylene increased longitudinal cell expansion was not identified but it could be due to affects of ethylene on the extensibility of the cell wall. Other studies have also found that enhanced ethylene alone, or in combination with gibberellins, causes an increase in elongation and expansion of cortical cells in hypocotyls of *Arabidopsis* seedlings (Saibo *et. al.*, 2003). The number of cells in a cortical cell file also increased when ACC was added to growth media suggesting that ethylene might be involved in stimulating cell division.

A gene involved in ethylene-induced promotion of cell elongation was identified by Lehman *et. al.* (1996). An ethylene responsive gene, *HOOKLESS1* (*HLS1*) promoted differential elongation of cells in the apical hook of *Arabidopsis* seedlings. Investigation suggested that ethylene might be involved in regulating the transport of auxin during cell elongation which further regulates differential elongation of cells in the apical hook. This information also suggests that during various stages of development ethylene does have cell elongation-promotion functions.

It is crucial to note, however, that the promotion of cell elongation by ethylene has only been noted in seedling tissues. In mature hypocotyl tissue it is evident that ethylene reduces cell elongation. This is evidenced by the phenotype of full grown *ctr1* mutants of *Arabidopsis*. *ctr1* (constitutive triple response 1) mutants are unable to down regulate the ethylene response, hence they constitutively exhibit triple response characteristics (Kieber *et. al.*, 1993). Fully grown *ctr1* mutants are short, with wide hypocotyls and small leaves. These responses are due to ethylene induced inhibition of cell elongation.

It appears that ethylene is involved in cell division, elongation, and expansion in various tissues during plant growth. The ability to promote and inhibit cell elongation seems to be tissue and developmental specific. Cross-talk of ethylene with other hormones such as auxin and gibberellins may also be involved in these developments. It is obvious that the role of ethylene in regulating cell growth is very complex.

1.2.3 Fruit ripening

A variety of physical changes occur during tomato fruit ripening such as fruit enlargement (involving changes in cell expansion), changes in colour, texture, and aroma. Fruits begin as green, enter a mature green stage, obtain the first spot of visible colour (breaker stage), and fruits become softer as their colour changes to a deep red. Around the breaker stage an increase in ACC synthase, ACC oxidase, ethylene production, and respiration occurs (Alexander and Grierson, 2002). As ripening progresses ethylene levels decrease, but only

to moderate levels in comparison to the very low levels present in immature fruits. Tomato ripening is climacteric because of the requirement for a burst of ethylene and an increase in respiration (Alexander and Grierson, 2002). Tomato mutants that have reduced levels of ethylene production show delayed and incomplete ripening due the lack of a climacteric ethylene burst (Nakatsuka, *et. al.*, 1998; Klee *et. al.*, 1991).

It has been suggested that a positive feedback loop regulates the production of ethylene at the breaker stage in which an initial increase in ACC oxidase elevates ethylene levels which in turn induces ACC synthase to generate more ACC for ethylene production (Jiang and Fu, 2000). The exact downstream effects initiated by ethylene that stimulate ripening are not completely understood. However, it is understood that specific differential regulation of ethylene biosynthesis is a requirement for proper and complete tomato fruit ripening.

1.2.4 Wood formation

The vascular cambium is a region formed by cells that can differentiate into the vascular tissues, xylem and phloem. Xylem and phloem systems facilitate the transport of water and nutrients throughout the plant, respectively. From the cambium, xylem is produced towards the centre of the stem and phloem is produced outwards. During secondary growth, mature xylem are dead cells with lignified, thick secondary cell walls; these comprise the wood tissue of a plant. Mature, secondary phloem are non-lignified. The cortex and bark is

produced by the cork cambium and is deposited outside the layer of living phloem.

The formation of wood is often referred to as xylogenesis. Xylogenesis begins with the initial differentiation of a cell from the vascular cambium into a xylem which then elongates and radially enlarges. Cells develop thick secondary cell walls which become heavily lignified and then undergo programmed cell death finally becoming mature secondary xylem (Mellerowicz *et. al.*, 2001).

Vascular tissues at the vascular cambium of a poplar stem are depicted along with an illustration of xylogenesis in Figure 4a and 4b.

The amount of wood produced in a plant is dependent on the number of xylem initials that are formed at the vascular cambium (Mellerowicz *et. al.*, 2001). Auxin has been identified as a regulator of vascular cambium activity in the stem of poplar, and therefore regulates the amount of xylem formation (Uggla *et. al.*, 1996; Tuominen *et. al.*, 1997). A specific gradient of auxin across the vascular cambium region, in which a peak of auxin occurs at the edge of the cambial/phloem region, appears to be a positional signal of vascular tissue differentiation. Ethylene and auxin are frequently linked in plant development, and it is no surprise that they are both involved in vascular tissue formation. Ethylene, however, has been implicated more specifically in xylem and wood-specific formation.

Figure 4: Vascular tissues of the poplar stem. (A) A cross section of a young poplar stem reveals an abundance of xylem (towards the centre of the stem). Vessels transport water, fibres support the structure, and rays act as storage centres. Vessels and fibres are dead at maturity with lignified secondary cell walls. Rays are living cells with primary cell walls. Phloem develops outwards from the vascular cambium. Phloem elements that transport nutrients are alive at maturity and have primary cell walls. Fibres are visible as dark bundles and they are dead at maturity with lignified secondary cell walls. Beyond the phloem are the cork and bark tissues. Wood tissue is comprised of mature xylem. **(B)** Xylogenesis is the process by which xylem differentiate from the vascular cambium and undergo a series of developments to become wood tissue. Deposition of a secondary cell wall, lignification, and programmed cell death (PCD) are markers of this process.

Cross section photograph courtesy of Vijaya Sharma, Carleton University.
Illustration of xylogenesis adapted from Kozela and Regan (2003).

Reaction wood is often used to study xylem formation in trees.

Compression wood forms on the underside of a bent stem of gymnosperm trees. In angiosperms, tension wood forms on the upper side of the bent stem. Both forms are considered reaction wood and are a response to maintain an upright stem. Compression and tension wood involves a rapid increase of xylem production from the vascular cambium. Compression wood contains more lignin and less cellulose than regular wood (Raven *et. al.*, 1999). Tension wood, in contrast, contains gelatinous fibers within the secondary cell wall and has little to no lignification (Raven *et. al.*, 1999). Therefore, reaction wood is useful to study certain processes of wood formation, but is not identical to normal wood tissues. It is important to note, however, that ethylene has been implicated in both compression and tension wood formation.

An increase in ACC oxidase activity, and therefore ethylene levels, was found to be spatially restricted to tension wood and not on the phloem side of the vascular cambium in poplar (Andersson-Gunneras *et. al.*, 2003). Increases in ACC oxidase in xylem tissues when secondary cell walls were forming was also observed. This could suggest that ethylene stimulates xylem formation from the vascular cambium and is involved in the maturation of wood tissue.

Compression wood formation in balsam fir also induces enhanced ethylene production and the abundance of xylem tissue increases (Little and Eklund, 1999).

Flood-induced ethylene has also been shown to affect xylem and wood formation. When flooded, an increase in the number of xylem, the density of

xylem tissue, and thickness of secondary cell walls was observed in *Pinus halepensis* seedlings (Yamamoto and Kozlowski, 1987a; Yamamoto and Kozlowski, 1987b). Ethylene has also been shown to increase xylem differentiation in lettuce pith (Miller and Roberts, 1984) showing the conservative presence of ethylene during xylem formation.

In addition to an increase in the volume of xylem produced in the presence of elevated ethylene levels, the hormone is also believed to affect specific stages of xylem maturation such as secondary cell wall formation and programmed cell death (PCD). Some genes and enzymes involved in lignification and PCD are ethylene-responsive and changes in ethylene levels have been shown to alter secondary cell wall biosynthesis and the rate of PCD (Greenberg, 1996; Sitbon *et. al.*, 1999).

It is apparent that ethylene, perhaps along with auxin, plays a significant role in the differentiation of xylem from the vascular cambium and may promote stages of xylem maturation into wood tissues. These functions may also be true in xylem formation of non-woody tissues.

1.3 Regulation of ethylene biosynthesis

Regulation of ethylene biosynthesis in plant tissues can occur at a variety of levels from biosynthesis, perception, to downstream reactions. However, most research has focused on the regulation of ethylene at the level of biosynthesis. The production of ACC is achieved by a ring-closing displacement of methylthioadenosine from SAM, catalyzed by ACC synthase (Abeles *et. al.*,

1992). ACC was not initially identified as a component of ethylene biosynthesis, it was only known as a component of fruit juices. It was identified as the precursor to ethylene by Adams and Yang in 1979 when they reported that ethylene in apple tissue was derived from ACC. The production of ACC and the use of ACC to produce ethylene are both steps in biosynthesis where regulation can occur. Differential regulation of ACC synthase and ACC oxidase in tissue and developmentally specific patterns combined with post-translational modifications seem a likely method to regulate the levels of ACC and ethylene synthesis (Johnson and Ecker, 1998).

Biosynthesis is dependent on the amount of available precursor. ACC can be conjugated to malonyl-ACC (MACC) via ACC malonyl transferase (Martin and Saftner, 1995) and 1-(γ -L-glutamyl-amino) cyclopropane-1-carboxylic acid (GACC) via γ -glutamyltranspeptidase (Martin *et. al.*, 1995). Conjugation to MACC and GACC has been suggested as a possible means of regulating ACC levels in apples, tomatoes, and spruce (Tan and Bangerth, 2000; Martin and Saftner, 1995; Martin *et. al.*, 1995; Wilksch *et. al.*, 1998). Conjugation of ACC is thought to be irreversible however this is still under debate. Also, it is not clear if ACC-conjugates have a distinct function in regulation of ethylene biosynthesis.

1.4 ACC deaminase

ACC deaminase is an enzyme that breaks down ACC. Specifically, ACC deaminase cleaves the cyclopropane ring of ACC and removes an amino group (deamination) to produce α -ketobutyrate and ammonia (Penrose and Glick, 2001). To date, however, the enzyme has only been found in microorganisms. ACC deaminase from yeast (*Hansenula satumus*), and especially plant growth promoting bacteria have been widely studied. The ACC degrading activity is conserved across all microorganisms studied however the enzyme structure can vary. For example, in most bacteria ACC deaminase subunits form a trimer but in yeast they form a dimer and final enzymes have molecular weights ranging between 68 and 130 kDa (Penrose and Glick, 2001).

ACC deaminase does not have a very high affinity for ACC, especially in comparison to ACC oxidases. The K_m values for ACC deaminases from a variety of species range between 1.5 and 17.4 millimoles (Penrose and Glick, 2001) whereas those for ACC oxidase have been reported between 66 and 170 micromoles (Abeles *et. al.*, 1992). Optimum pH and temperature for activity *in vitro* is 8.0 and 30°C respectively (Honma and Shimomura, 1978; Jacobson *et. al.*, 1994; Penrose and Glick, 2001). ACC deaminase requires pyridoxal phosphate as a cofactor which puts it in the aminotransferase family of enzymes. It is also reactive with other D-amino acids but is competitively inhibited by L-serine (Penrose and Glick, 1997). ACC deaminase activity can be quantified using colourimetric assays that measure the amount of α -ketobutyrate or

ammonia produced in the presence of substrate (Honma and Shimomura, 1978; Penrose and Glick, 2001).

It has been suggested that ACC-degrading microorganisms, in particular soil microorganisms, contain ACC deaminase as a result of an abundance of ACC and malonyl-ACC in the soil from dead and decaying plants (Klee *et al.*, 1991). In plant growth promoting bacteria (PGPB), ACC deaminase activity appears to affect plant growth by reducing plant endogenous ethylene levels (Hall *et al.*, 1996). It is thought that PGPB stimulate plants, to which they have formed a microrhizal association, to produce extra ACC (Hall *et al.*, 1996; Penrose and Glick, 2001). PGPB release indoleacetic acid (IAA) which is absorbed by the seed. This may stimulate an increase in synthesis of ACC synthase causing an increase in production of ACC. It is thought that ACC is exuded from the plant and taken up by the PGRP where it is converted to ammonia and α -ketobutyrate. This process lowers the amount of ethylene precursor therefore reducing the amount of ethylene produced within the root and the whole plant. Figure 5 illustrates the mechanism of ACC deaminase-induced reduction of plant ACC. ACC deaminase-reduced ethylene promotes elongation of roots and can increase a plant's tolerance to water and salt stress (Glick *et al.*, 1997; Shah *et al.*, 1998; Mayak *et al.*, 2004). This role of ACC deaminase in PGPB is supported by the fact that non-ACC deaminase bacteria, or those with ACC deaminase mutations, do not have root elongation properties (Jacobson *et al.*, 1994).

Figure 5: Activity of ACC deaminase within plant growth promoting bacteria. Bacteria bound to a seed or roots release indole acetic acid (IAA) from bacteria bound to a seed or root which is absorbed by the plant. Bacterial IAA along with plant endogenous IAA is thought to stimulate ACC synthase to up-regulate ACC production. ACC is exuded from the plant roots, along with other molecules, and absorbed by the associated bacteria. Within the bacterium, ACC deaminase cleaves and deaminates ACC into ammonia and α -ketobutyrate. This provides the bacteria with a unique source of nitrogen. Consequently, plant endogenous ACC levels decrease resulting in reduced ethylene production. This relieves the inhibition of root elongation normally imposed by ethylene. As a result, plants that have microrhizal associations associated with ACC deaminase-containing plant growth promoting bacteria often exhibit elongated roots compared to non-associated plants.

Illustration adapted from Hall *et. al.* (1996).

1.5 An ACC deaminase in poplar?

A cDNA library was previously created by Dr. S. Regan and colleagues to analyze gene expression during vascular tissue formation in poplar. Annotation of this library revealed the presence of genes involved in ethylene biosynthesis, including ACC oxidase and ACC synthase, and another sequence with similarity to bacterial ACC deaminases. Expression of ACC oxidase, ACC synthase, and the ACC deaminase-like EST were measured within tissues across the poplar stem including xylem, cambium/phloem (because cambial tissue is not easily separated from the phloem), and cortex (Figure 6). ACC synthase was measured at low and stable levels across all tissues. ACC oxidase was expressed at high levels in the developing xylem tissues and less in the cambium where phloem tissue was developing. The ACC deaminase-like EST sequence was expressed at dramatically higher levels in the phloem tissue and not at all in xylem. The differential expression of these genes suggests that ethylene is being synthesized where xylem were developing and that ACC may be diverted from ethylene biosynthesis in the phloem by a putative ACC deaminase.

This was the first indication of a plant-encoded ACC deaminase. Differential expression of an ACC deaminase may be a mechanism for regulation of ethylene in poplar during phloem development and perhaps in other plants during growth and development. It was of great interest to further examine the potential for a functional plant-encoded ACC deaminase.

Figure 6: Expression of ethylene genes in the poplar stem. Northern blot analysis (Bjorn Sundberg, Sara Andersson-Gunneras, Sharon Regan, unpublished) revealed differential expression across the poplar stem of genes coding for enzymes involved in ethylene biosynthesis and an ACC deaminase-like EST. ACC synthase, which produces the precursor of ethylene (ACC) from S-adenosylmethionine, was expressed at relatively low and constant levels across all tissues. ACC oxidase, responsible for producing ethylene, was highly expressed in xylem tissue but low in the phloem. In contrast, an ACC deaminase-like sequence was highly expressed in the phloem but not in the xylem. This suggests that ethylene biosynthesis may be regulated by ACC oxidase and an ACC deaminase in vascular tissues of the poplar stem. This was the first identification of a plant-encoded putative ACC deaminase.

Specifically, in this thesis *Arabidopsis* was used to elucidate an ACC deaminase and examine its role in ethylene regulation. The partial EST from poplar that had similarity to bacterial ACC deaminases was used to isolate an ACC deaminase-like sequence from the *Arabidopsis* genome. ACC deaminase activity was then measured in various tissues of *Arabidopsis*, tomato fruit, and poplar vascular tissues to ascertain its presence within plants.

2 MATERIALS AND METHODS

2.1 Extraction of tissues from the poplar stem

Phloem/cambium, xylem, and cortex tissues were extracted from young poplar (hybrid poplar, *Populus tremula* x *P. alba*) stems. Trees were grown in the Carleton University greenhouse under natural light conditions. Tissue was extracted between April and October of 2003. A small branch (diameter of approximately 1 cm) was cut from the tree using clippers. Using a scalpel blade a line was lightly cut longitudinally down the surface of the stem. Once cut, the top layer consisting of the cortex and phloem/cambium tissues is easily peeled away. The developing xylem tissue is the outside layer of the inner section. The separation of tissues is depicted in Figure 7. Phloem/cambium tissue was removed by lightly scraping the top layer from the inside of the peeled-off section. The phloem/cambium tissue is light green and wet. The remaining tissue of this section was scraped off and represented cortex tissues. The outer layer of tissue from the inner piece of the stem (pale beige in colour) was lightly scraped (longitudinally) to remove the developing xylem layer. This tissue is also wet but is waxy compared to the soft phloem/cambium tissues. Tissues were either used immediately for protein extraction or frozen with liquid nitrogen and stored at -80°C to be used at a later date.

Figure 7: Separation of tissues from the poplar stem. In order to measure ACC deaminase activity in different tissues of the poplar stem, xylem, cambium/phloem, and cortex tissues were isolated. From a section of stem, a longitudinal cut made down one side of the stem allows the outer layer, consisting of the bark, cortex, and phloem/cambium (from outside to inside) to be peeled away. The remaining inner piece consists of xylem and pith (from outside to inside) from the inner section (xylem, pith). From the separated layers, xylem, and cambium/phloem, and cortex can be scraped off.

2.2 Plant protein extraction

Total protein was extracted from tissues of wild-type *A. thaliana*, cherry tomatoes (*Lycopersicon esculentum*), and from poplar in order to measure ACC deaminase activity. From *Arabidopsis*, leaves, stem, hypocotyls, and siliques and their seeds (across six stages of development) were separated for protein extraction. *Arabidopsis* plants of the Columbia ecotype were provided by Graham Thurston (Carleton University) and had been grown under long day conditions (16 hours light, 8 hours dark) in growth chambers at approximately 22°C. From tomatoes, fruits were harvested at immature green, mature green, breaker, orange, and ripe red stages. Stages were based on colouration and texture. Cherry tomato plants, kindly donated by Dr. J. Cheetham (Carleton University), were grown under natural light conditions in the green house during the summer months. From poplar, xylem, phloem/cambium, and cortex tissues were used (tissue isolation described in section 2.1).

The protein extraction method was adapted from Beardmore *et. al.* (2002). About 100 mg of tissue was frozen with liquid nitrogen and ground, vigorously, in a mortar and pestle. Samples were kept frozen by adding liquid nitrogen when needed. Grinding was performed until the tissue was a fine powder. One millilitre of basic protein extraction buffer (25 mM KH_2PO_4 , 1 M NaCl, using deionized water and adjust pH to 7) was added and the samples were ground again until a thick paste had formed. The thick liquid was transferred to a chilled microcentrifuge tube and vortexed for one minute to further disrupt the cells. The mixture was spun for 30 minutes at 14,000 r.p.m. at 4°C. If, after this spin, there

were floating pieces of tissue within the supernatant, the samples were spun again at the same settings. Supernatants were carefully removed using sterile Pasteur pipettes and placed in another sterile, chilled microcentrifuge tube. Samples were immediately used for ACC deaminase activity assays. Protein concentration was measured against a standard curve of BSA (bovine serum albumin) using the Bradford method. The Bradford assay of protein concentration was performed as follows: 50 μ l of the protein extraction was placed into two clean glass tube (1.3 x 15 cm) and mixed with 3 ml of 1x Quick Start BioRad Bradford Dye Reagent (BioRad, Hercules, CA, USA). Blanks were set up using protein extraction buffer instead of extract. The extract-dye solution was mixed by inverting and incubated at room temperature for 5 minutes. After incubation the absorbance of the solution at 595 nm was measured. Protein concentration was calculated using a BSA standard curve, created by performing the assay on dilutions of BSA (Sigma, Oakville, Ontario) from 0 to 1 mg/ml.

2.3 ACC deaminase assay

ACC deaminase activity was measured by the amount of α -ketobutyrate produced in the presence of ACC as described by Penrose and Glick (2003). Before the assay a stock solution of 0.5 M ACC (Sigma) was prepared. ACC was dissolved in sterile water, filtered through a 0.2 μ m syringe filter, and aliquots were frozen at -20°C. Just before use, ACC stock solutions were thawed. Two-hundred microlitres of protein extract was placed into a microcentrifuge tube. To this tube, 20 μ l of 0.5 M ACC (test) or water (blank) was added. All reactions

were made in duplicate. The mixtures were vortexed briefly to mix, and incubated at 30°C for 15 minutes. After incubation, 1 ml of 0.56 M HCl was added, the mixture was vortexed and then centrifuged for 5 minutes at 16 000 xg at room temperature. To a clean glass tube (1.3 x 15 cm), 1 ml of supernatant and 800 µl of 0.56 M HCl were added and vortexed together. To this solution, 300 µl of 0.2% (w/v in 2 M HCl) 2,4-dinitrophenylhydrazine (2,4,-D) were added and vortexed. The mixture was incubated for 30 minutes at 30°C. During this incubation 2,4-D reacts with α -ketobutyrate, amino ketones, and keto-acids to produce hydrazones (Friedman and Haugen, 1943). Following incubation, 2 ml of 2 N NaOH were added and the absorbance of the solution was measured at 540 nm. The change in pH causes the hydrazones to produce a yellow/brown colour (Friedman and Haugen, 1943). The absorbance of the assay reagents, including the substrate (ACC) and the protein extract were subtracted from the values recorded for the reaction containing ACC (test) of the taken into account and the final values were used to calculate the amount of α -ketobutyrate produced by the action of ACC deaminase. ACC deaminase activity was calculated as nanomoles of α -ketobutyrate produced per milligram of protein per hour.

A standard curve of absorbance at 540 nm of α -ketobutyrate was produced to make such measurements. A stock solution of 10 mM α -ketobutyrate (Sigma) made in 0.1 M Tris-Cl, pH 8.5, was made the day of use and stored in the fridge. From this a 100 µM stock solution was made, this would be used to prepare dilutions for the standard curve. Dilutions were made in 0.1

M Tris-HCl, pH 8.5, from 0 to 1.0 μ mol, right before use. Two hundred microlitres of each dilution was transferred to separate clean glass tubes and mixed with 300 μ l of 0.2% 2,4-D solution. Duplicates of each dilution were made. The mixture was vortexed and incubated for 30 minutes at 30°C. Afterwards, 2 ml of 2 N NaOH was added and the absorbance at 540 nm was measured.

Absorbance at 540 nm versus the concentration of α -ketobutyrate was plotted to create a standard curve. All absorbance readings were made on a Pharmacia Biotech Novaspec II spectrophotometer.

2.4 Germination on L-serine

To assess the affects on growth of an ACC deaminase inhibitor, seeds were germinated on media containing various concentrations of L-serine. Seeds from wild-type *Arabidopsis thaliana* (Columbia ecotype) and *ers-1* mutants of *A. thaliana* were used in these experiments. Seeds were collected and kindly provided by Donna Yee and Graham Thurston (Carleton University). Seeds were surface-sterilized by incubating in 70% (v/v) ethanol for 2 minutes followed by an 8 minute incubation in 30% bleach/0.2% Triton X-100 (v/v) solution. Sterilization solutions were removed, using sterilized Pasteur pipettes, and seeds were thoroughly washed with sterile, distilled water (at least 10 times). Seeds were left in the last water rinse, the tubes were covered with foil, and then stored at 4°C for four days for stratification (treatment of cold with wetness and darkness).

Surface-sterilized and stratified seeds were plated (50 to 75 seeds per plate) on Murashige and Skoog (M&S) basal salt mixture (Sigma) buffered to pH 5.7-5.8 with 1 M KOH, solidified with 0.8% (w/v) agar. Plates were supplemented with L-serine (Sigma) at final concentrations of 0 to 5 mM. A 0.5 M stock solution of L-serine was prepared in sterile, distilled water just before use. The L-serine was not added until autoclaved media had cooled to about 55°C. Plates inoculated with seeds were incubated on their side (to allow for easy observation of hypocotyl and root length) in the dark (to allow for any triple response characteristics to develop) for 4 to 7 days. As a control, surface-sterilized and stratified seeds from *Arabidopsis ers-1* mutants were plated (approximately 20 to 25 seeds) on each plate. *ers-1* mutants are insensitive to ethylene, thus if ethylene levels are changed by the presence of L-serine the *ers-1* should continue to grow normally. After incubation, the length of seedling hypocotyls and roots were measured. The percentage of seeds germinated was also recorded. It was important to take measurements in a timely manner as triple response characteristics can be quickly affected by exposure to light.

2.5 ACC deaminase gene sequence in *Arabidopsis thaliana*

A partial EST sequence from poplar provided by Dr. S. Regan was used to perform searches in the NCBI databases using the BLAST facility (www.ncbi.nlm.nih.gov/blast). BLASTN, BLASTX, and TBLASTX searches against the entire database revealed the best match (highest degree of sequence similarity) to an *Arabidopsis thaliana* gene on chromosome 1 (At1g48420). The

database provided a complete mRNA sequence of 1245 nucleotides which was used to design primers for gene amplification via the polymerase chain reaction.

2.6 Gene amplification and isolation for cloning

Total RNA was extracted from whole *A. thaliana* plants (Columbia ecotype) to be used for cDNA synthesis. The putative ACC deaminase gene was amplified using PCR and the used for cloning.

2.6.1 RNA extraction

RNA was isolated from *A. thaliana* plants, of the Columbia ecotype, to be used for cDNA synthesis. Whole plants were used for extractions. Mortars and pestles used to grind tissue for RNA isolation were soaked in 0.2% (v/v) diethylpyrocarbonate (DEPC) overnight and then autoclaved prior to use to minimize the amount of RNase present in the extraction environment. RNA was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada). All buffers, solutions, and columns were provided in the kit. One hundred milligrams of tissue was frozen in liquid nitrogen and ground to a fine powder using the sterilized mortar and pestle. The powder was kept frozen with liquid nitrogen and placed into a pre-chilled, sterile, 1.5 ml microcentrifuge tube. Immediately after transfer 450 µl of Buffer RLT mixture was vortexed to disrupt and lyse the cells. Buffer RLT is a detergent that will aid in lysing cells and RNase protects the RNA from degradation. The lysate was then transferred to a QIAshredder spin column in a 2 ml collection tube and spun for 2 minutes at

maximum speed in a microcentrifuge (approximately 13,000 r.p.m.) at room temperature. The shredder column removes insoluble material from the lysate. The supernatant, containing RNA, was transferred to a new, sterile, 1.5 ml centrifuge tube. Care was taken to avoid disrupting the pellet containing cellular debris. To the supernatant, 225 μ l of 100% ethanol was added and the solution was mixed by pipetting and transferred to an RNeasy Mini Spin Column sitting in a 2 ml collection tube. Ethanol in the RNA-containing solution enhances binding of RNA to the RNeasy silica-gel membrane. The sample was spun for 15 seconds at 11,000 r.p.m. in a microcentrifuge at room temperature. The flow-through was discarded and the column was placed back in the same 2 ml collection tube. To wash the column membrane of contaminants, 700 μ l of Buffer RW1 was added and the column was spun for 15 seconds at 11,000 r.p.m. at room temperature. The column was then placed in a new collection tube. To ensure all contaminants were eluted from the column membrane before the final RNA elution two washes with Buffer RPE were performed. To the column, 500 μ l of Buffer was added and it was spun for 15 seconds at 11,000 r.p.m. at room temperature. The flow-through was discarded and a final wash with 500 μ l of Buffer RPE was performed by adding the solution to the column and spinning for 2 minutes at maximum speed at room temperature. To ensure all ethanol (present in the RPE buffer) was removed from the sample, the column was placed in a sterile 1.5 ml centrifuge tube and spun at maximum speed for 1 minute. It was important to remove all traces of ethanol as it could reduce the total RNA yield during the elution step. The dry column was then placed into a

new, sterile, 2 ml microcentrifuge tube. To elute the RNA sample, two aliquots of 25 μ l of RNase-free water was added to the column, which was then spun for 1 minute at 11,000 r.p.m. at room temperature. From the final RNA sample, 5 μ l was removed to examine using 1% (w/v) agarose gel electrophoresis (to confirm isolation) and the rest was immediately stored at -80°C for later use in cDNA synthesis.

2.6.2 cDNA synthesis

cDNA was synthesized using the protocol and materials from the SuperScript II kit (Invitrogen, Burlington, ON, Canada). All solutions were provided as part of the SuperScript II kit with the exception of 10 mM dNTPs (Invitrogen), sterile distilled water, and RNA (isolated as described above). To a sterile 1.5 ml centrifuge tube the following was added: 1 μ l of dT oligo primer, 2 ng of total RNA (1 to 9 μ l), 1 μ l of 10 mM dNTPs, and sterile distilled water to make the volume up to 12 μ l. This mixture was heated at 65°C for 5 minutes followed by a quick chill on ice (about 5 minutes). The tube was spun on a low speed to collect all contents at the bottom. To the mixture, 4 μ l of 1st Strand Buffer, 2 μ l of 0.1 M DTT (Dithiothreitol), and 1 μ l of RNase Out Inhibitor were added. The mixture was incubated for 2 minutes at 37°C. One microlitre (200 units) of SuperScript Reverse Transcriptase© was added. The solution was mixed by pipetting and incubated for 50 minutes at 37°C. To terminate cDNA synthesis the mixture was incubated for 15 minutes at 70°C. cDNA was stored at -20°C for use in PCR.

2.6.3 Sequence amplification by the polymerase chain reaction

cDNA created from total RNA was used as a template to amplify the ACC deaminase coding sequence (CDS) using gene specific primers. Primers were designed to amplify the ACC deaminase CDS from the 4th nucleotide (to exclude the ATG start codon) to just beyond the stop codon. To clone the CDS into the protein expression vector, pPROTet.E, the restriction sites *Sa*I and *X*baI were added by PCR to the 5' and 3' end of the amplified CDS, respectively. To clone the CDS into the broad host range plasmid, pRK415, restriction sites *X*baI and *K*pnl were attached to the 5' and 3' end of the amplified CDS, respectively. Primers were synthesized by Invitrogen. The sequences were as follows (written as 5' to 3'):

Amplification for cloning into pROTet.E133:

Forward: ACGC *GTCGAC* AGAGGACGAAGCTTGACACTCTCAAGAGTAAA

Reverse: ACGC *TCTAGA* GTGGACACACCATTATTTATCGTAATCGT

Amplification for broad host range plasmid pRK415:

Forward: ACGC *TCTAGA* AGAGGACGAAGCTTGACACTCTCAAGAGTAAA

Reverse: ACGC *GGTACC* GTGGACACACCATTATTTATCGTAATCGT

Underlined bases are extra nucleotides added to ensure complete integration of the restriction sites during amplification. Restriction sites are identified by *italicized* nucleotides.

Primers were rehydrated with 1 x TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA, pH 8) to a concentration of 100 μ M. From this, a small portion was removed and diluted to 10 μ M with sterile distilled water. The 10 μ M primers were used in PCR reactions. A single PCR reaction (20 μ l total volume) contained 2 μ l of 10x PCR Buffer, 0.4 μ l of 10 mM dNTPs, 0.6 μ l of 50 mM

MgCl₂, 1.0 µl of 10 µM forward primer, 1.0 µl of 10 µM reverse primer, 0.2 µl of *Taq* polymerase (5U/µl), 3 µl of cDNA template, and 11.8 µl of sterile distilled water. Reagents, except for water and template, were from Invitrogen. The reaction mixture was mixed by pipetting before being placed in the Eppendorf Mastercycler Gradient PCR machine. All reactions were performed with a negative control (water instead of DNA template). The PCR program was as follows:

1. 94°C, 45 seconds (denaturation)
2. 69°C, 45 seconds (anneal)
3. 72°C, 1 minute 25 seconds (extension)
4. Steps 1 to 3 repeated 29 times.
5. 94°C, 45 seconds
6. 69°C, 45 seconds
7. 72°C, 5 minutes (final extension)

Completed reactions were stored at 4°C for short-term storage and at -20°C for long term storage. PCR products were analyzed by 1% agarose gel electrophoresis.

The correct PCR product was isolated and purified from the agarose gel using a protocol derived from the Gene Clean kit (QBiogene, Carlsbad, CA, USA). The desired band was cut from the gel and placed in a microcentrifuge tube. To this, 1 ml of 6M sodium iodide was added. The tube was placed on a 60°C heat block for about 10 minutes, with periodic mixing, to melt the agarose. To the completely melted solution, 5 µl of Glassmilk© (QBiogene) was added and

mixed. The mixture was placed on ice for 5 minutes to enhance binding of the DNA to the Glassmilk. The mixture was then centrifuged for 30 seconds at maximum speed in a microcentrifuge (about 13,000 r.p.m.) at room temperature to pellet the Glassmilk-bound DNA. The supernatant was removed and the pellet was washed by resuspending in 400 μ l of New Wash (QBiogene), centrifugation for 30 seconds at maximum speed and discarding the supernatant. Washing was repeated twice (for a total of three washes). After removing the last supernatant the pellet was dried by placing the tube on a 50°C heating block for about 5 minutes. The dry pellet was resuspended in 15 to 20 μ l of sterile distilled water and placed back on the 50°C heat block to enhance releasing of the DNA from the GlassMilk. To extract the DNA, the solution was spun at maximum speed for 30 seconds and the supernatant was transferred to a sterile 1.5 ml centrifuge tube. At this step it was critical to avoid carry-over of any GlassMilk as it would interfere with ligations and digestions. To ensure that no GlassMilk remained in the DNA solution, it was spun for 1 minute at maximum speed and the supernatant was transferred to a new, sterile 1.5 ml centrifuge tube. A small portion of the DNA solution (about 2 μ l) was run on 1% agarose gel electrophoresis to confirm isolation of the PCR product. Also, a small portion of the isolated DNA was digested with *Hind*III to confirm that the correct fragment had been isolated (digestion of *Arabidopsis* ACC deaminase with *Hind*III results in a 0.9 kb and a 0.3 kb banding pattern). The final DNA sample was stored at -20°C for cloning (or for use as a probe for northern hybridizations).

2.7 Preparation of competent cells for cloning

Calcium chloride transformation-competent *Escherichia coli* DH5 α cultures were made to clone the *Arabidopsis* CDS. A single colony of *E. coli* DH5 α was inoculated into 50 ml of sterile liquid LB media (Luria Broth, per liter: 10 g Tryptone, 5 g sodium chloride, 5 g yeast extract, add distilled water up to 1 L, autoclave, all ingredients available from Sigma) and grown overnight at 37°C with shaking (approximately 250 r.p.m.). Four millilitres of this culture were used to inoculate 400 ml of sterile liquid LB media in a 2-litre flask. The culture was grown at 37°C with shaking (250 r.p.m.) to an OD₅₉₀ of 0.375. It is important not to grow past this point as overgrowth will decrease the efficiency of transformations. The culture was aliquoted into eight 50 ml pre-chilled, sterile, polypropylene tubes and left on ice for 5 to 10 minutes. The cells were centrifuged for 7 minutes at 1600 x g at 4°C. The centrifuge was allowed to decelerate without the break. The supernatant was poured off and cell pellets were resuspended in 10 ml of ice-cold calcium chloride solution (60 mM calcium chloride, 15% glycerol, 10 mM PIPES [piperazine-N,N'-bis(2-hydroxypropanesulfonic acid)], pH 7, autoclave and store at room temperature, all ingredients available from Sigma). The cells were centrifuged for 5 minutes at 1100 x g at 4°C. The supernatant was discarded and cell pellets were resuspended in 10 ml of ice-cold calcium chloride solution. The resuspended cells were kept on ice for 30 minutes. After incubation on ice, the cells were centrifuged for 5 minutes at 1100 x g at 4°C. The supernatant was discarded and each cell pellet was resuspended in 2 ml of ice-cold calcium chloride

solution. The cells were aliquoted (225 µl) into sterile microcentrifuge tubes and immediately frozen at -80°. Cells were stored at -80°C until use.

2.8 Cloning

The gel-purified PCR product was ligated into the cloning vector pGEM-T Easy (Promega, Madison, WI, USA). The vector is supplied linearized with thymine overhangs. The adenosine overhangs on the PCR products, naturally created by *Taq* polymerase, bind to the thymine overhangs of the vector. Ligation of PCR-purified ACC deaminase CDS into linear pGEM-T Easy vector was performed using T4 DNA ligase provided with the vector. The ligation reaction was set up in a sterile 1.5 ml centrifuge and contained the following: 4 µl of 5x T4 DNA ligase buffer (also supplied with vector kit), 1-4 µl of gel purified PCR product, 1 µl of vector, and sterile distilled water to make the volume up to 10 µl. A ratio of insert to vector of 3:1 was used to ensure maximum ligation. The ligation reaction was spun briefly to collect the contents at the bottom of the tube and then gently mixed by swirling. The reaction was incubated at room temperature for one hour or at 4°C overnight. Transformation-competent *E. coli* DH5α (described in section 2.7) were transformed with the pGEMT-ACCd ligation mixture. Ten microlitres of the ligation mixture and 10 µl of sterile distilled water were added to the bottom of a sterile 15 ml Falcon tube (Fisher Scientific, Nepean, ON, Canada). The tube was incubated on ice for about 5 minutes. An aliquot of frozen competent cells (described in section 2.7) was removed from the freezer and thawed on the bench top. To the ligation mixture, 100 µl of

competent cells were added. The mixture was swirled gently to mix and incubated on ice for 10 minutes. The cell/ligation mixture was then incubated for 2 minutes in a 42°C water bath to transform the cells. Immediately after incubation 1 ml of sterile, liquid LB media was added. The cells were incubated for 1 hour at 37°C with gentle shaking (150 to 200 r.p.m.). A 100 µl aliquot of the cells was then plated on solid (1.5% agar w/v) LB media containing 0.5 mM isopropyl- β -D -thiogalactoside (IPTG), 0.08 mg/ml of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), and 0.1 mg/ml of ampicillin. All supplements were purchased from Sigma. Colour screening was initially used to identify positive clones – a plasmid containing the insert will not be able to break down β -galactosidase in the media and will grow as a white colony. Comparatively, bacteria carrying a vector with no insert will break down the β -galactosidase and grow as blue colonies. Potentially positive clones were picked and grown in liquid LB with 0.1 mg/ml ampicillin.

2.9 Plasmid isolation and digestion

Plasmids were isolated and digested with the appropriate restriction enzymes (depending on what primers the CDS had been amplified with, see section 2.6.3) to release the cloned CDS. Plasmid isolation was performed using the Wizard® Plus Midiprep DNA Purification System (Promega). All solutions mentioned come with the kit but composition will be noted when available. A single colony (believed to be carrying the construct of interest) was used to inoculate 100 ml sterile, liquid LB media. The culture was grown overnight at

37°C with shaking. The cells were pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant was poured off and the tube was blotted (upside down) on paper towel to remove any excess liquid. The pellet was completely resuspended in 3 ml of Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). Three milliliters of Cell Lysis Solution (0.2 M NaOH, 1% SDS) was added and mixed by inverting the tube four times. To the lysed cell mixture, 3 ml of Neutralization Solution (1.32 M potassium acetate, pH 4.8) were added and mixed by inverting the tube four times. The mixture was centrifuged at 14,000 x g for 15 minutes at 4°C. The supernatant (containing plasmid DNA) was carefully decanted into a sterile 50 ml tube. Ten milliliters of Wizard® Midipreps DNA Purification Resin was added to the supernatant. A Midicolumn was attached to a vacuum and then the solution was poured into the Midicolumn. The vacuum was turned on to a maximum of 15 Hg. When all of the sample had passed through the column the vacuum was broken at the source. Fifteen milliliters of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 µM EDTA) was added to the column and the vacuum was applied to pull the solution through the Midicolumn. When all the sample had passed through the vacuum was broken at the source and another 15 ml of Column Wash Solution was added. The vacuum step was repeated and when all the sample had passed through the vacuum was applied for another 30 seconds to dry the column. The lower part of the Midicolumn (below the reservoir) was removed by cutting and placed into a sterile 1.5 ml microcentrifuge tube. The column in the tube was spun for 2 minutes at 10,000 x

g at room temperature to remove any residual Column Wash Solution. The column was transferred to a new microcentrifuge tube and 300 μ l of preheated (70°C) nuclease-free water was added to the column. After a one minute incubation, at room temperature, the column in the tube was spun for 20 seconds at 10,000 x g in a microcentrifuge to elute the DNA. The Midicolumn was discarded. The sample was spun for 5 minutes at 10,000 x g in a microcentrifuge to pellet any residual resin pieces. The supernatant was removed and placed in a new microcentrifuge tube. The plasmid DNA was stored at -20°C for future use. To confirm plasmid isolation, a small aliquot (2 μ l) was analyzed using 1% agarose gel electrophoresis.

To release the cloned CDS from the plasmid a restriction endonuclease digestion was performed. Digestions were performed with XbaI and Sall, or with XbaI and KpnI (depending on the original CDS amplification, see section 2.6.3). Each digestion was with a single restriction endonuclease, therefore after the first digestion samples had to be precipitated to prepare for the second digestion. A 50 μ l digestion reaction was prepared as follows: 10 μ l of plasmid DNA, 34 μ l sterile distilled water, 5 μ l of 5x reaction buffer (supplied with the restriction endonuclease), and 1 μ l of restriction enzyme (Invitrogen). The mixture was gently mixed by pipetting and incubated at 37°C for 1 hour. Digestions were terminated by adding 5 μ l of 5 mM EDTA, pH 8. The sample was then precipitated using ethanol and sodium acetate. To the sample 1.5 volumes of ice-cold 100% ethanol and 1/5 volumes of 3 mM sodium acetate was added. The sample was incubated at -80°C for one hour (or at -20°C overnight). DNA

was collected by centrifugation for 15 minutes at 12,000 x g at 4°C. The pellet was rinsed with 500 ul of cold 70% ethanol and spun again for 2 minutes at 12,000 x g at 4°C. The supernatant was removed and pellets were dried at by placing the tube, open, in a 37°C heating block for about 15 minutes. The pellet was resuspended in approximately 10 ul of sterile distilled water and used for the second digestion (set up as previously described). The final digestions were analyzed using 1% agarose gel electrophoresis. The insert was isolated and purified using the Gene Clean method described above to be ligated into pROTet.E or pRK415.

2.10 Expression of ACC deaminase coding sequence in bacteria

2.10.1 Expression in pPROTet.E expression vector

The ACC deaminase CDS with a *Sall* overhang at the 5' end and an *XbaI* overhang at the 3' end was ligated into the pPROTet.E133 vector that had been linearized with *Sall* and *XbaI* (ligation performed as described in section 2.7, using T4 DNA ligase from Invitrogen). Calcium competent *E. coli* DH5αPRO© (Clontech, Palo Alto, CA, USA) were transformed with the E133-ACCd ligation mixture and plated on solid (1.5% w/v agar) LB media supplemented with 34 µg/ml chloramphenicol and 50 µg/ml spectinomycin. The vector carries chloramphenicol resistance and the DH5αPRO strain carries an autonomously replicating plasmid that provides spectinomycin resistance. Potentially positive clones were selected and grown in liquid LB media with 34 µg/ml chloramphenicol and 50 µg/ml spectinomycin. Plasmids were isolated and

digested with *HindIII* and *EcoRI* (separately) to confirm the presence of the CDS within the plasmid and that it was in the correct orientation. Digestions and the original plasmid isolation were analyzed using 1% agarose gel electrophoresis. Positive clones were stored in a 20% glycerol solution at -80°C for long term storage. Clones were later used to test for the activity of ACC deaminase.

2.10.2 DNA sequence analysis

To confirm that the cloned CDS was within the plasmid and that the sequence had been maintained, plasmids from positive E133-ACCd - containing clones were sequenced. Isolated plasmids were purified by isopropanol precipitation. Samples were incubated in an equal volume of cold 100% isopropanol overnight at -20°C; centrifuged for 15 minutes at 12,000 xg at 4°C to pellet the DNA followed by rinsing the pellet with cold 70% ethanol (v/v in 1x TE Buffer, pH 8) to remove salts; and spun for 2 minutes at 12,000 xg at 4°C. The remaining ethanol was removed and the pellet was dried by heating for about 15 minutes on a 37°C heating block. The final pellet was resuspended in 1x TE Buffer, pH 8. Sequencing of the plasmid was performed by CMRS Inc. (Ottawa, ON, Canada) using the sequencing primers provided with the vector.

2.10.3 Expression of ACC deaminase CDS in pRK415 vector

Amplified ACC deaminase sequences with a 5' *KpnI* overhang and a 3' *XbaI* overhang previously isolated from the pGEMT-easy vector were ligated into the pRK415 vector (kindly provided by Dr. B.R. Glick, University of Waterloo).

The plasmid was linearized with *KpnI* and *XbaI* and the ligation performed as described in section above. Calcium competent *E. coli* DH5 α were transformed with the ligation mixture and grown on solid (1.5% w/v agar) LB media supplemented with 15 μ g/ml tetracycline. The pRK415 plasmid carries the tetracycline resistance gene. Potentially positive clones were picked and grown in liquid LB with 15 μ g/ml tetracycline. Plasmids were isolated and digested with *HindIII* and *EcoRI* (separately) to confirm the presence of the ACC deaminase CDS in the correct orientation. Plasmids and digestions were analyzed using 1% agarose gel electrophoresis.

2.11 Analysis of ACC deaminase activity in bacteria

Bacteria transformed with the pPROTet.E-ACCd and pRK415-ACCd constructs were assessed for their ability to utilize ACC (Penrose and Glick, 2003). These cultures will be referred to as E-ACCd and pRK-ACCd. Control cultures were also tested, consisting of untransformed bacteria or bacteria transformed with the vector only.

2.11.1 Growth on minimal media with ACC as the sole nitrogen source

E. coli DH5 α PRO $\circledR$ carrying E-ACCd and *E. coli* DH5 α carrying pRK-ACCd were assayed for ACC deaminase activity by observing their ability to utilize ACC as a sole nitrogen source. Samples were first grown in rich media and then transferred to DF (Dworkin and Foster) salts minimal media with ACC as the sole nitrogen source. Different antibiotics and inducers were added to all

media depending on the plasmid-ACCd construct. Cultures of *E. coli* carrying pPROTet.E were supplemented with chloramphenicol (34 µg/ml) and spectinomycin (50 µg/ml) and anhydrous tetracycline as the inducer (at concentrations from 0 to 100 ng/ml). Cultures of *E. coli* carrying pRK415 were supplemented with tetracycline (15 µg/ml) and IPTG as the inducer (0.8 mM).

Firstly, 5 mL of rich media was inoculated with a single colony or approximately 5 µl of frozen culture. Cultures were incubated overnight with shaking (about 250 r.p.m.) at 28°C. A 1 ml aliquot was removed and spun at for 2 minutes 8,000 xg at 4°C to pellet the cells. The pellet was washed twice (resuspension of the pellet followed by centrifugation for 2 minutes at 8,000 xg at 4°C) with sterile, liquid DF salts minimal media containing no nitrogen source. DF salts minimal media was prepared to contain, in 1 L, 4.0 g KH₂PO₄, 6.0 g Na₂HPO₄, 0.2 g MgSO₄·7H₂O, 2.0 g glucose, 2.0 ml gluconic acid, 2.0 g citric acid, with trace elements: 1 mg FeSO₄·7H₂O, 1 µg H₃BO₃, 11.19 µg MnSO₄·H₂O, 124.6 µg ZnSO₄·7H₂O, 78.22 µg CuSO₄·5H₂O, 10 µg MoO₃, pH 7.2, and 2.0 g (NH₄)₂SO₄ as a nitrogen source. In the lab, the media was prepared as follows: 1) trace elements (10 mg H₃BO₃, 11.19 mg MnSO₄·H₂O, 124.6 g ZnSO₄·H₂O, 78.22 mg CuSO₄·5H₂O, and 10 mg MoO₃) were dissolved in 100 ml of sterile distilled water and stored at 4°C (for several months), 2) FeSO₄·7H₂O (100 mg) was dissolved in 10 ml of sterile distilled water and stored at 4°C (for several months), 3) The remaining ingredients were added and dissolved one at a time in distilled water, adding 0.1 ml of the trace elements and FeSO₄·7H₂O stock solutions, and autoclaved for no longer than 20 minutes. To select for ACC

deaminase activity, the 2.0 g of $(\text{NH}_4)_2\text{SO}_4$ was replaced with 0.5 M ACC (added to a final concentration of 3 mM), however ACC was added to the media after it had cooled to about 55°C. Alternatively, media with no nitrogen source was prepared without $(\text{NH}_4)_2\text{SO}_4$ or ACC.

The final cell pellet was again resuspended in 1 ml of DF salts minimal media and used to inoculate 50 ml of DF salts minimal media supplemented with 0.2% w/v of ammonium sulfate, and appropriate antibiotics and inducers, in a 250 ml flask. The culture was incubated for 24 hours at 28°C with shaking at 200 r.p.m. A 1 ml aliquot was removed, washed and resuspended as described above and used to inoculate 50 ml of sterile DF salts minimal media supplemented with 3 mM ACC as the sole nitrogen source. The inoculated culture was incubated for 24 to 50 hours with shaking (200 r.p.m) at 28°C. Dilutions of the final culture were plated onto solid (1.5% w/v agar) DF salts minimal media containing 3 mM ACC and appropriate antibiotics. Inoculated plates were incubated for 24 to 96 hours at 28°C to allow colonies to develop. Minimal plates without a nitrogen source were plated with the same cultures as controls.

2.11. 2 ACC deaminase activity assay

ACC deaminase activity was assayed by measuring the amount of α -ketobutyrate produced in cell extracts in the presence of ACC as described by Penrose and Glick (2003). Cultures were grown overnight in 50 ml of LB media with the appropriate antibiotic and inducing supplements. Cells were harvested by centrifugation for 10 minutes at 8,000 x g at 4°C. The cells were resuspended

in 5 ml of 0.1 M Tris-HCl, pH 7.6 (a wash of the cells to remove old media) and spun for 10 minutes at 8,000 x g at 4°C. This wash was repeated twice (for a total of three washes). The final cell pellet was suspended in 2 ml of 0.1 M Tris-HCl, pH 7.6 and transferred to two 1.5 ml-microcentrifuge tubes. Cells were ruptured using freeze-thaw cycles of 5 minutes in liquid nitrogen followed by 5 minutes at 30°C. This was repeated 5 times. Lysates were collected by spinning at 14,000 rpm for 1 hour at 4°C. The supernatant was carefully transferred to a new, chilled, microcentrifuge tube. Approximately 100 µl of lysate was used to measure protein by the Bradford method, the rest was used immediately for the ACC deaminase assay, as previously described in section 2.3. The absorbance of the assay reagents, including the substrate (ACC) and the bacterial extract were taken into account and the final values were used to calculate the amount of α -ketobutyrate produced by the action of ACC deaminase. A α -ketobutyrate standard curve was used to make calculations (see Section 2.3). Final activity was measured as nanomoles of α -ketobutyrate produced per milligram of protein per hour.

3 RESULTS

Functional ACC deaminases have been repeatedly described in a variety of bacteria, especially plant growth promoting bacteria (PGPB) (Glick *et. al.*, 1994) and yeast (Honma and Shimomura, 1978). Most ACC deaminase-containing PGPB utilize the enzyme to degrade ACC from plants during microrhizal associations. To date, however, a functional ACC deaminase has not been identified in plants. The identification, by Dr. S. Regan, of an ACC deaminase-like EST from a cDNA library of poplar stem initiated the search for a functional plant-encoded ACC deaminase.

3.1 A functional ACC deaminase in *Arabidopsis*

Although the EST was identified in poplar, *Arabidopsis thaliana* offers many advantages over poplar as a model system for all types of plant research from molecular genetics to plant physiology. It is a rapidly growing plant with a fully sequenced genome and also forms secondary xylem tissues similar to wood in poplar (Chaffey *et. al.*, 2002). For these reasons, *Arabidopsis* is a good model to study a plant-encoded ACC deaminase and test its potential role during vascular tissue formation in poplar.

3.1.1 An ACC deaminase gene in Arabidopsis

A putative ACC deaminase gene in *Arabidopsis* was identified based on sequence similarity to the poplar ACC deaminase-like EST. In *Arabidopsis* the putative gene is located on chromosome one and has an open reading frame

(ORF) of 1206 nucleotides. The predicted protein of 414 amino acids has a calculated molecular weight of 45 kDa. The predicted protein is similar in size to the ACC deaminase of *Hansenula saturnus* (40 kDa) and *Penicillium citrinum* (41 kDa) (Penrose and Glick, 2001). The ACC deaminase protein from many bacteria form a trimer, but in yeast the final enzyme is a dimer. A trimer would make the final plant-encoded ACC deaminase 135 kDa in size whereas a dimer would result in a 90 kDa protein. The amino acid sequence of the *Arabidopsis* ACC deaminase is between 50% and 60% similar to other known ACC deaminases (Figure 8A) but contains conserved domains with up to 98% similarity to other known ACC deaminases including pyridoxal phosphate-dependent domains and an amino acid transport domain (Figure 8B).

The CDS was amplified from cDNA created from total RNA isolated from whole *Arabidopsis thaliana* plants. The CDS was cloned and ligated into expression vectors to be expressed in *Escherichia coli*. The expression vector pPROTet.E (Clontech) was used in these experiments because it has an anhydroustetracycline-inducible promoter which allows for controlled expression. The CDS was also cloned into a broad host range plasmid, pRK415, and transformed into *E. coli*. pRK415 is a low copy number plasmid that can be weakly induced by IPTG. Constructs will be referred to as pPROTet-ACCd and pRK-ACCd.

Figure 8: Similarity of the *Arabidopsis* putative ACC deaminase amino acid sequence with other proteins. Using facilities on the NCBI website (www.ncbi.nlm.nih.gov/blast), the degree of similarity of the *Arabidopsis* putative ACC deaminase was measured against other protein sequences. **(A)** Amino acid sequence similarity. A BLASTX search (the CDS nucleotide sequence of the *Arabidopsis* putative ACC deaminase was translated into an amino acid sequence – a function of the BLASTX search - and aligned with all other amino acid sequences in the databank) was performed to reveal significant similarity with other known ACC deaminases. Groups of similarity are highlighted by brackets.

* Protein sequence (NP_175275) predicted from bases 1 to 1206 from gene on *A. thaliana* chromosome one, At1g48420. Available at www.ncbi.nlm.nih.gov.

** Vahala (2002)

*** Example: a sequence from the Sargasso Sea (Venter *et. al.*, 2004)

**** Example, threonine dehydratase from the bacteria *Photorhabdus luminescens* (Duchaud *et. al.*, 2003).

(B) Conserved domains within the CDS of the *Arabidopsis* putative ACC deaminase amino acid sequence. A search for conserved domains was performed using the Conserved Domain Database (RPS-BLAST) available on the NCBI website (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The predicted amino acid sequence of the *Arabidopsis* putative ACC deaminase (NP_175275) was probed for conserved domains using the RPS-BLAST database. Note the high degree of conservation with other ACC deaminases and the pyridoxal phosphate dependent domain.

¹ *A. thaliana* amino acid sequence (NP_175275) from gene At1g48420.

² Example: domains from the ACC deaminase of the bacteria *Caulobacter crescentus* (NCBI, 2001). Also available as part of the ACC deaminase family (description [gnl|CDD|12110](#)). Available in the protein database at www.ncbi.nlm.nih.gov.

³ Example: pyridoxal phosphate dependent domain from threonine deaminase from (EC 4.3.1.19) from *Escherichia coli* (Garrison *et. al.*, 1986). Also available as part of the pyridoxal phosphate dependent enzyme family (description [gnl|CDD|25475](#)). Available in the protein database at www.ncbi.nlm.nih.gov.

⁴ Example: Threonine dehydratase (Amino acid transport and metabolism) as part of the amino acid transport and metabolism families, (description [gnl|CDD|10889](#)). Available through the protein database at www.ncbi.nlm.nih.gov.

3.1.2 Expression of *A. thaliana* ACC deaminase in *Escherichia coli*

In bacteria, ACC deaminase cleaves the cyclopropane ring of ACC producing ammonia and α -ketobutyrate. Detection of ACC deaminase activity by a colourimetric assay that measures production of α -ketobutyrate has been previously described and used to confirm the presence of functional ACC deaminases (Penrose and Glick, 2003; Honma and Shimomura, 1978). ACC deaminase activity can be measured and expressed as the amount of α -ketobutyrate produced in the presence of ACC per milligram of protein assayed per hour. The absorbance of assay reagents and test extracts without ACC are taken into account. In addition to α -ketobutyrate production as an indicator of ACC deaminase activity, bacteria with active ACC deaminases are also identified by their ability to utilize ACC as a sole nitrogen source on solid growth media (Honma and Shimomura, 1978; Penrose and Glick, 2003). Bacteria transformed with the *Arabidopsis* ACC deaminase CDS were screened for activity by these methods.

Bacteria transformed with pPROTet-ACCd were grown in rich media supplemented with maximum levels of inducer (100 ng/ml anhydrous tetracycline). Bacterial pellets were washed thoroughly to remove rich media and cell lysate extracts were assayed for ACC deaminase activity. As shown in Figure 9, activity levels in ACC deaminase-transformed bacteria were significantly ($p < 0.01$) higher than background levels recorded in control (ACC deaminase minus) bacteria. Levels of measurable activity were nearly 10x greater than background levels measured in control cultures.

Figure 9: ACC deaminase activity measured in *E. coli* expressing the *Arabidopsis thaliana* ACC deaminase CDS. Cell lysates from cultures carrying the *Arabidopsis* putative ACC deaminase CDS (pPROTet.E-ACCd) and from control cultures (not containing the ACC deaminase CDS) were assayed for ACC deaminase activity. *Activity was measured as nanomoles of α -ketobutyrate produced per milligram of protein per hour in the presence of ACC. The bacterial culture carrying the *Arabidopsis* ACC deaminase CDS was grown under high induction conditions (100 ng/ml of anhydrous tetracycline). Error bars are standard error.

The apparent background activity is believed to be due to other hydrazone molecules present in the sample.

Bacteria containing the pPROTet.E-ACCd construct were also assayed for ACC deaminase activity after being grown under low induction conditions (0 ng/ml anhydrous tetracycline) and compared to activity of cultures grown under strong induction conditions (80 ng/ml anhydrous tetracycline). Cultures induced with anhydrous tetracycline showed a 30% increase in activity levels (data not shown). According to Clontech, 80 ng/ml of anhydrous tetracycline increases vector expression up to 1000x over basal levels. Although expression of the sequence could have reached these levels, activity may have remained low (in comparison) due to the labile nature of the enzyme or unfavourable extract conditions. The induction assays will need to be repeated, however it is clear that ACC deaminase activity is due to the presence of the *Arabidopsis* ACC deaminase CDS in *E. coli* carrying pPROTet.E-ACCd.

In addition to ACC deaminase activity, the ability of bacteria to utilize ACC as a sole nitrogen source is also a positive indication of a functional ACC deaminase. *E. coli* carrying pPROTet.E-ACCd or pRK-ACCd were assessed for the ability to grow on minimal media with ACC. Growth of these cultures was compared to that of untransformed bacteria and bacteria carrying the vectors alone. Table 1 summarizes the results of these growth experiments.

Table 1: Ability of bacterial strains carrying the *Arabidopsis* ACC deaminase CDS to utilize ACC as a sole nitrogen source. *E. coli* carrying no vector, vector alone (either pPROTet.E or pRK415) or the *Arabidopsis* ACC deaminase CDS within an expression vector (pPROTet.E-ACCd or pRK415-ACCd) were tested. In addition to growth on minimal media supplemented with a nitrogen source (ammonium sulfate) or with ACC, cultures were grown on minimal media with no nitrogen source as a control. The degree of growth was based on the quantity of colonies, or size of a bacterial lawn. Bacteria were grown at 28°C for 5 days.

Strain	Growth on minimal media with no nitrogen source	Growth on minimal media with $(\text{NH}_4)_2\text{SO}_4$	Growth on minimal media with ACC
<i>Escherichia coli</i>			
DH5 α	-	++++	-
<i>E. coli</i>			
DH5 α /pPROTet.E	-	++++	+
<i>E. coli</i> DH5 α / pPROTet.E- ACCd	-	++++	+++
<i>E. coli</i>			
DH5 α /pRK415	-	++++	-
<i>E. coli</i> DH5 α /pRK415-ACCd	-	++++	++

Growth of bacterial strains carrying the *Arabidopsis* ACC deaminase was visibly greater than those of control strains. Colonies of ACC deaminase-carrying bacteria on minimal media with ACC were smaller than those that grew on rich media; often less than 0.5 mm in diameter. Colonies grown on minimal media with ammonium sulfate were slightly larger than those on minimal ACC media. An inability for the bacteria to absorb ACC from the media may be the cause of such small colonies (Penrose and Glick, 2001). Regardless of the small size, growth of ACC deaminase-carrying bacterial cultures was greater than that of ACC deaminase minus bacterial cultures when ACC was the sole nitrogen source.

Bacteria carrying pPROTet.E-ACCd showed more growth than those carrying pRK-ACCd, on minimal media with ACC. The pRK415 plasmid is a low copy number plasmid and although it can be induced by IPTG, the levels of expression are not believed to be as high as the induction of sequences in the pPROTet.E vector induced with anhydrous tetracycline.

A few colonies developed on plates inoculated with control strains, however they were few and appeared only after several days of incubation. Other studies have reported growth of bacteria on minimal media with no nitrogen source (Penrose and Glick, 2003) suggesting that the minimal media with or without ACC is not limiting to growth of only ACC deaminase carrying bacteria. Therefore, the degree of growth is a better indicator in this experiment of the presence of a functional ACC deaminase.

E. coli transformed with the *Arabidopsis* ACC deaminase CDS showed ACC deaminase activity in the form of α -ketobutyrate production and the ability to utilize ACC as a sole nitrogen source. These results suggest that the putative ACC deaminase CDS identified in the *Arabidopsis* genome encodes for a functional ACC deaminase.

3.2 ACC deaminase activity in *Arabidopsis* – tissue and developmental specificity?

In *Arabidopsis*, the putative ACC deaminase CDS appears to code for a functional enzyme. The presence and role of ACC deaminase within *Arabidopsis* plants requires evaluation. The first step towards understanding the role of ACC deaminase in plants is to identify in what tissues the gene is expressed. The levels of ACC deaminase in plant tissues were also measured based on α -ketobutyrate production.

3.2.1 ACC deaminase expression in *Arabidopsis*

Expression levels of the *Arabidopsis* ACC deaminase gene (At1g48420) within tissues from whole *Arabidopsis* plants is available through the Massively Parallel Signature Sequencing (MPSS) database¹. This is an online database that summarizes the expression data of multiple microarrays. Sequences are probed for using a 17 base pair oligonucleotide that is unique to that sequence. Up to 95% of genes in *Arabidopsis* can be identified within this

¹ <http://mpss.udel.edu/at/java.html>

database. Abundance of a signature sequence (unique to a single transcript) is quantified as the transcripts per one million (TPM) measured in that sample. Genes that are weakly expressed have TPM values between 1 and 10, whereas highly expressed genes generally have TPMs greater than 1000. The abundance of the *Arabidopsis* ACC deaminase gene transcripts in different tissues, as measured by MPSS, is illustrated in Figure 10. The At1g48420 sequence (the putative ACC deaminase gene) is differentially expressed in the tissues of *Arabidopsis*, suggesting that it has specific developmental or physiological roles in the plant and not just house-keeping functions in the cell. Gene expression is a good indication of a requirement for a gene product. To determine if ACC deaminase was active in *Arabidopsis*, ACC deaminase enzyme activity was measured in protein extracts from various tissues.

3.2.2 ACC deaminase activity in highly vascular tissues of *Arabidopsis*

The first indication that ACC deaminase might be differentially expressed in vascular tissues arose from the expression analysis in the wood forming tissues of poplar, where ACC deaminase expression was high in the phloem and low in developing xylem (recall Figure 6). These vascular tissues cannot be easily separated in *Arabidopsis* as they can in poplar. However, in the analysis of ACC deaminase activity in *Arabidopsis* tissues, the degree of vasculature was kept in mind for the interpretation of the results. Total protein extracts from wild-type *A. thaliana* stem, leaf, and hypocotyl tissues were extracted to measure the level of endogenous ACC deaminase activity.

Figure 10: Expression of the *Arabidopsis* ACC deaminase gene (At1g48420) in tissues of *Arabidopsis thaliana*. Expression data is made available online through the Massively Parallel Signature Sequencing (MPSS) database system. The database provides the results of a 17 base-pair sequence unique to the putative ACC deaminase gene (At1g48420) probed for relative expression abundance, based on transcripts per million expressed (TPM). Most tissues were extracted from plants grown in 16 hours of light for 21 days, with the exception of callus tissue.

Activity was measured by the amount of α -ketobutyrate produced in the presence of substrate, ACC. Of the tissues tested, the hypocotyl tissue likely has the highest amount of vascular tissue because of the activity of a vascular cambium and the production of secondary xylem and phloem. The arrangement and density of xylem and phloem tissues are shown in cross sections of these tissues in Figure 11A. The level of ACC deaminase activity in *Arabidopsis* is shown in Figure 11B. The highest activity was found in the hypocotyls and leaves, but activity was also detected in the stem. It is interesting to note that MPSS data also revealed high expression of the gene in leaf tissue. Activity measured in leaf extracts was significantly ($p < 0.05$) higher than the levels of activity measured in stem extracts.

These results confirm that there is ACC deaminase activity in the tissues of *Arabidopsis*.

3.3 A role for ACC deaminase in ethylene regulation?

The results of expressing the *Arabidopsis* ACC deaminase CDS in bacteria and the measurable activity of ACC deaminase in a variety of *Arabidopsis* tissues confirmed the presence of an ACC deaminase within this plant. It was of great interest to further examine the role of ACC deaminase in specific developmental pathways that are known to be regulated by ethylene. In this section, the effects and activity of ACC deaminase were evaluated during seed germination and seedling development, fruit ripening in tomato, seed development, and vascular tissue differentiation in poplar.

Figure 11: ACC deaminase activity in highly vascular tissues of

Arabidopsis thaliana. Protein extracts from hypocotyl, leaf, and stem tissues were assayed for ACC deaminase activity. (A) Cross sections indicate the arrangement and abundance of xylem and phloem in these tissues. (B) Protein extracts from each tissue were assayed for ACC deaminase activity. *Activity was measured as nanomoles of α -ketobutyrate produced per milligram of protein per hour in the presence of ACC. Error bars are standard error. ** Error not available.

Hypocotyl cross section courtesy of Shuyou Han (Carleton University).

3.3.1 ACC deaminase and ethylene regulation during seed germination

Ethylene is required for many plant developmental pathways such as seed germination, root-hair formation, flower senescence, abscission, and fruit ripening (Abeles *et. al.*, 1992). The effect of ethylene on development can be studied by growing plants in elevated or lowered levels of ethylene. One easily assayed developmental process that requires ethylene is seed germination.

When *Arabidopsis* seedlings are grown in the dark (etiolated) in the presence of elevated ethylene they undergo the triple response: stunted growth of roots and hypocotyls, wide hypocotyls, and an exaggerated apical hook (Wang *et. al.*, 2002). Mutants that are blocked in ethylene perception or some components of the downstream signal transduction pathway do not display the triple response. For example, the *ers-1* mutant of *Arabidopsis* has a mutation in the ERS-1 ethylene receptor that renders it insensitive to changes in ethylene levels (Hua *et. al.*, 1995). When grown on media with ACC, *ers-1* seedlings germinate and grow normally. They do not exhibit the triple response whereas wild-type seeds germinated on the same media do exhibit the triple response (Hua *et. al.*, 1995).

To test whether ACC deaminase regulates the levels of ethylene within the plant, wild-type *A. thaliana* seeds were germinated in the dark, on media containing various levels of L-serine, a known competitive inhibitor of ACC deaminase (Penrose and Glick, 1997; Walsh *et. al.*, 1981). If ACC deaminase is responsible for regulating ethylene levels, we would expect an increased ethylene response (triple response) as the concentration of L-serine reaches the levels required to totally inactivate ACC deaminase.

To ensure that decreases in hypocotyl length are due to changes in endogenous levels of ethylene, and not due to unrelated effects on hypocotyl growth, seeds of the *ers-1* background were germinated and grown on the same plates as the wild-type seedlings. The mutant seeds would be insensitive to alterations in ethylene levels but likely still sensitive to any unrelated effects of L-serine on seedling growth.

When grown on media without L-serine, seedlings of wild-type and *ers1* backgrounds appeared similar in physiology. The length of roots and hypocotyls were similar between plants of both backgrounds and seedlings appeared healthy. The presence of L-serine in the growth media reduced the length of both the hypocotyl and root of wild type seedlings but did not significantly affect these tissues of the *ers-1* mutant. As seen in Figure 12A, as the concentration of L-serine increased, *ers-1* plants showed little change in hypocotyl length. In contrast, wild-type seedlings exhibited reduced hypocotyl and root elongation (Figure 12A and 12B, respectively) as the concentration of L-serine was increased. Wild-type seedlings grown in the presence of L-serine at 1 mM or higher had significantly ($p < 0.01$) reduced growth of hypocotyls and roots compared to wild-type seedlings on control plates, and compared to *ers-1* seedlings grown on control and L-serine plates. Please note that root length of *ers-1* seedlings was not recorded. With 3 mM or more of L-serine, wild-type seedlings exhibited a decrease in hypocotyl and root length of at least 50%. *ers-1* mutants did not exhibit reduced root length when grown in the presence of L-serine.

Figure 12: Effects of competitive inhibition of ACC deaminase on seed germination and seedling growth. *Arabidopsis* seeds of the wild-type and *ers-1* backgrounds were germinated on MS media containing various concentrations of L-serine, a known competitive inhibitor of ACC deaminase. Seedlings were germinated and grown in the dark for 7 days. Changes in hypocotyl length of wild-type and *ers-1* seedlings were measured (A), and changes in root length of wild-type seedlings were measured (B). Error bars are standard error.

A **B**

The maximum decrease in hypocotyl length of *ers-1* seedlings was 2.5 mm below the average length of control seedlings, while the wild-type seedlings commonly exhibited decreases of 8 mm. In addition, wild-type seedlings germinated in the presence of L-serine often exhibited greater curvature of the apical hook.

Although significant reductions in hypocotyls and root length were observed for wild-type seedlings, the plants appeared healthy. Once exposed to light, the growth of wild-type seedlings accelerated, however those on media with L-serine of 3 mM or higher always remained stunted compared to control and *ers-1* seedlings. Although growth of wild-type seedlings was slower and stunted, the proportion of seeds that germinated in the presence of L-serine from 0 to 5 mM was generally constant. *ers-1* seedlings also showed no change in the proportion of seeds germinating in the presence of L-serine.

These results suggest that the inhibition of ACC deaminase activity in germinating seedlings results in an increase in endogenous ethylene levels. This indicates that ACC deaminase may play a role in the regulation of ethylene levels in *Arabidopsis*.

3.3.2 Ethylene regulation - ACC deaminase in ripening tomatoes?

The relationship of ethylene and tomato fruit ripening has been reported and studied for several years. To further investigate the role of ACC deaminase in plants, ACC deaminase activity was measured in tomato fruits during ripening.

During the normal ripening process, tomatoes begin as green fruits, turn pale green and began to enlarge, and then acquire a first spot of yellow/orange colour. After the first spot of colour appears the remaining ripening stages (colour and texture changes) develop rapidly. The tomato turns completely orange, begins to soften and eventually turns bright red. The first appearance of colour is described as the breaker stage and is associated with a burst in ethylene production within the fruit tissue, after which the levels of ethylene decrease (Nakatsuka *et. al.*, 1998; Alexander and Grierson, 2002; Abeles *et. al.*, 1992; Brady *et. al.*, 1987).

Protein extracts from tomatoes picked before, during, and after the breaker stage were tested for ACC deaminase activity based on α -ketobutyrate production in the presence of ACC. Figure 13 shows the changes in

α -ketobutyrate production as ripening progresses, overlaid are predicted ethylene levels during the same ripening stages as suggested by other research (Brady *et. al.*, 1987; Nakatsuka *et. al.*, 1998). Levels of α -ketobutyrate were moderate (in comparison to the peak) in very immature green fruit and then dropped off as the fruit entered the mature green stage of development. Levels continued to drop before the breaker stage. Immediately after the breaker stage a peak of α -ketobutyrate production was measured which slowly tapered off as ripening progressed through dramatic colour and texture changes. These results suggest that an active ACC deaminase may also be present in tomato fruit..

Figure 13: ACC deaminase activity in ripening tomatoes. Total protein extracted from tomatoes at various stages of ripening was assayed for ACC deaminase activity. *Activity was measured as nanomoles of α -ketobutyrate produced per milligram of protein per hour in the presence of ACC. Error bars are standard error and those that cannot be seen are smaller than the data point symbol. The hypothesized levels of ethylene are superimposed and were based on previous reports describing ethylene during tomato fruit ripening (Nakatsuka *et. al.*, 1998; Alexander *et. al.*, 2002; Brady *et. al.*, 1987; Abeles *et. al.*, 1992).

ACC deaminase activity appears to be low during the mature green and pre-breaker stage, likely to allow for the accumulation of ethylene required for the breaker stage. The rise in ACC deaminase activity during the post-breaker stage could presumably decrease ethylene levels in these later stages. Further investigation of an ACC deaminase in tomato is needed, however these results are very intriguing considering the known role for ethylene during tomato fruit ripening and results described for ethylene regulation by ACC deaminase in *Arabidopsis*.

3.3.3 ACC deaminase during seed development

ACC deaminase activity was abundant in highly vascular tissues of *Arabidopsis*. Results also suggest a role for ACC deaminase in ethylene regulation in whole *Arabidopsis* plants and possibly in ripening tomatoes. A relationship between ethylene levels and seed dormancy has been suggested by studies of ethylene mutants (Yee, 2003). To contribute to the theories of a role for ethylene during seed development and the role for ACC deaminase in ethylene regulation, ACC deaminase activity was measured in protein extracts from siliques of *Arabidopsis*.

Siliques, and the seeds within them, were harvested across different developmental stages to determine any possible developmental-specific ethylene regulation by ACC deaminase. As previously shown (Yee, 2003) siliques isolated at six time points contain seeds that fall into four possible developmental stages, premature seeds, precocious germination, dormancy and germination

ready (recall Figure 3). The levels of ACC deaminase activity were measured in siliques of all six time points and there was a peak of ACC deaminase activity at stage 2, when the seed is believed to be entering dormancy (Figure 14). In addition, the levels of activity measured from stage 2 silique extracts was significantly higher than those measured in stem and hypocotyls extracts ($p < 0.05$). MPSS data suggested high expression in siliques (early stage 2) which supports the high activity measurements here. It is interesting to note that very little to no ACC deaminase activity was measured in siliques at the later stages of development, such as stages 5 and 6. It therefore appears that ACC deaminase may be partially responsible for regulating the levels of ethylene during seed development.

3.4 ACC deaminase in the poplar stem

It was previously shown that the ACC deaminase-like EST sequence was expressed at dramatically higher levels in the phloem than in the xylem of the poplar stem (recall Figure 6). An interpretation of these results is that the ACC which is transported in the vascular cambium, and that radiates out to the xylem and phloem tissues, is utilized by ACC deaminase to keep ethylene levels low in the phloem. In contrast to the apparent high levels of ACC deaminase expression in the phloem, the expression of ACC oxidase is highest in the xylem, suggesting that ethylene levels would be high in this tissue.

Figure 14: ACC deaminase activity during seed development.

Total protein extracts from *A. thaliana* siliques, and the seeds within them, collected across the six time points of growth (corresponding to four developmental stages, recall Figure 3) were assayed for ACC deaminase activity. *Activity was measured as nanomoles of α -ketobutyrate produced per milligram of protein per hour, in the presence of ACC. Error bars are standard error. Error bars of stage 5 and 6 are too small for the scale.

To test whether this apparent increase in ACC deaminase expression in the phloem coincides with an increase in ACC deaminase activity, the levels of activity were measured in the poplar stem.

3.4.1 ACC deaminase activity in the poplar stem

The stem of poplar was divided into three easily separated fractions containing developing xylem, cambium/phloem, and cortex tissues. Protein was isolated from each fraction and the extracts were assayed for ACC deaminase activity based on α -ketobutyrate production. Figure 15 clearly shows a higher level of activity in the phloem/cambium tissues compared to nearly no activity detected in the xylem. Levels of activity in the phloem/cambium were over 50x greater than that of xylem tissue, and just over 1.5x the level of activity in cortex tissue.

Changes in activity across vascular tissues are similar to the ACC deaminase gene expression measured in the stem, with the exception of cortex measurements. Expression of the ACC deaminase-like EST was not detected in cortex tissue, but moderate levels of activity were measured in cortex protein extracts. At this time it is uncertain of the reason why ACC deaminase expression in the cortex does not correlate well with activity levels, but additional expression studies that are currently underway in the laboratory of our collaborator (Bjorn Sundberg, Sweden) may clarify these results.

Figure 15: ACC deaminase activity in vascular tissues of poplar stem.

Total protein extracts from xylem, cambium/phloem, and cortex were assayed for ACC deaminase activity. *Activity was measured as nanomoles of α -ketobutyrate produced per milligram of protein per hour in the presence of ACC. Error bars are standard error.

Spatially, developing xylem and phloem are very close together – only a few cambial cell layers separate the two tissue layers. Low levels of ACC deaminase activity in developing xylem but high levels in neighbouring phloem further suggest that ethylene levels are being tightly regulated in a tissue specific manner. High ACC oxidase activity (Andersson-Gunneras *et. al.*, 2003) in developing xylem and low levels of ACC deaminase activity suggest that ethylene is required at a specific developmental stage of xylem formation. In this regard, ACC deaminase in the stem of poplar may be regulating ethylene levels in a tissue and developmentally-specific manner.

3.4.2 *Is it an ACC deaminase?*

The full gene was not available from poplar for cloning and expression in bacteria, so a few additional experiments were performed to prove that α -ketobutyrate produced in the poplar assays was in fact due to ACC deaminase activity. Because the cambium/phloem fraction of the poplar stem showed the highest enzyme activity, it was used for these tests.

Firstly, ACC deaminase activity was measured over the course of time to confirm that activity was stimulated by the presence of ACC. Levels of activity dramatically increased between 1 and 5 minutes after incubation with ACC, reached a peak around 15 minutes, and decreased again (Figure 16A). A maximum level of measurable product after 15 minutes was expected as current protocols recommend an incubation time of 15 minutes with the substrate.

Figure 16: Control assays to confirm ACC deaminase activity in the poplar stem. Control assays were performed to ensure that the α -ketobutyrate production measured in poplar extracts was indeed due to ACC deaminase activity. **(A)** The change in activity* over time, in the presence of ACC (3 mM), in phloem protein extract. Error bars are standard error. **(B)** ACC deaminase activity* in the presence of a competitive inhibitor. Protein extracts from poplar phloem were incubated with ACC and various concentrations of a competitive inhibitor, L-serine. Error bars are standard error.

*Activity was measured as nanomoles of α -ketobutyrate produced per milligram of protein per hour.

A **B**

The decrease in activity after 20 minutes could be the result of an unstable environment in the reaction mixture, an indication that the poplar ACC deaminase protein is unstable, or because the enzyme has utilized all the available substrate. Assays using a range of substrate concentrations are required.

Secondly, ACC deaminase activity was measured after boiling the protein extract. High temperatures generally denature protein structure and render enzymes inactive. In most extracts tested, boiling for greater than 30 seconds eliminated enzyme activity (data not shown).

These control assays verified that levels of α -ketobutyrate produced in the presence of ACC were indeed due to an active enzyme, believed to be an ACC deaminase.

To confirm that the production of α -ketobutyrate was due to ACC deaminase activity, known competitive inhibitors of ACC deaminases were added to assay mixtures. DNTB (5,5'-dithiobis(2-nitrobenzoic acid)) (Penrose and Glick, 1997) and L-serine are known inhibitors of ACC deaminase (Penrose and Glick, 1997; Honma, 1985; Walsh *et al.*, 1981). In bacteria, the presence of L-serine has been reported to reduce and even eliminate ACC deaminase activity (Walsh *et al.*, 1981). Based on enzyme kinetics in the presence of L-serine, it is listed as a competitive inhibitor. The affinity of ACC deaminase for L-serine is also comparable to the affinity for ACC (Walsh *et al.*, 1981).

Various concentrations of L-serine were added to phloem extracts before the addition of ACC.

The change in enzyme activity in the presence of L-serine is illustrated in Figure 16B. As the concentration of L-serine in the assay mixture increased the amount of α -ketobutyrate produced decreased. Addition of L-serine at a final concentration of 10 mM resulted in at least a 50% decrease in activity compared to activity in control samples. Addition of DNTB, an inhibitor of pyridoxal phosphate-dependent enzymes (Penrose and Glick, 1997) also resulted in reduced, and often no, α -ketobutyrate production in phloem protein extracts (data not shown). Elimination of activity by an ACC deaminase competitive inhibitor strongly suggests that activity levels previously measured are the product of a functional ACC deaminase.

A functional ACC deaminase encoded by the putative *Arabidopsis* ACC deaminase gene confirms that ACC deaminase is present in the plant. The results suggest that ACC deaminase activity is needed to regulate ethylene levels by controlling the amount of ACC available for ethylene biosynthesis. Although the CDS was only cloned from *Arabidopsis*, activity assays in other plants, specifically poplar and tomato reveal an ACC deaminase activity in these plants as well. More importantly, the ACC deaminase activity generally coincides with developmental processes in these plants that require ethylene but is specifically active at times or within specific tissues that do not require ethylene.

4 DISCUSSION

The research presented here has provided strong evidence for the existence of an ACC deaminase in plants. A standardized ACC deaminase activity assay has identified activity in *Arabidopsis*, poplar, and tomato. A putative ACC deaminase gene was isolated from *Arabidopsis* (At1g48420) and shown to encode a bona fide ACC deaminase. In *Arabidopsis*, the ACC deaminase showed involvement in ethylene regulation and activity correlated with specific stages of seed development, seed germination and the development of highly vascular tissues such as leaves and hypocotyls. In the poplar stem, ACC deaminase activity and expression was correlated with phloem development. In tomatoes, ACC deaminase activity also correlated with a specific stage of fruit ripening. The significance of these findings and the potential role of ACC deaminase in regulating ethylene levels in plants is discussed.

4.1 A functional plant-encoded ACC deaminase

Since it had never been characterized in plants it was of great interest to determine if a functional ACC deaminase existed. For ease of use, *Arabidopsis* was chosen as a model plant to initiate the identification of a functional plant-encoded ACC deaminase. A complete CDS sequence for a putative ACC deaminase was easily isolated from *Arabidopsis*.

4.1.1 The *Arabidopsis* ACC deaminase gene

Within the *Arabidopsis* genome there appears to be only one ACC deaminase-like gene, on chromosome one (revealed by searches using the NCBI BLAST database, recall section 3.1.1), so it is not believed to be part of a multigene family. The predicted amino acid sequence shares up to 60% similarity with other known ACC deaminases. Differences in amino acid sequence are thought to be in non-conserved regions as there was very strong (up to 98%) similarity of conserved domains between the *Arabidopsis* amino acid sequence and other known ACC deaminases. Conserved domains found in other ACC deaminases, a pyridoxal-phosphate dependent domain, and an amino acid transport domain are present in the *Arabidopsis* ACC deaminase amino acid sequence. The degree of conservation strongly suggested that the putative gene encoded for an ACC deaminase. Sequence alignments suggest that a pyridoxal phosphate binding domain is encoded for across the entire amino acid sequence and that an amino acid transport-like domain is encoded for near the centre of the amino acid sequence. This domain may be where ACC cleaving and deamination occurs. This could also be where L-serine binds to competitively inhibit ACC.

4.1.2 A functional ACC deaminase – expression in bacteria.

Expression of ACC deaminases from plant growth promoting bacteria in *E. coli* has proven to be a useful tool to confirm that a gene codes for a functional enzyme (Grichko and Glick, 2000; Campbell and Thompson, 1996; Penrose and

Glick, 2001; Shah *et. al.*, 1998). For this reason, the *Arabidopsis* putative ACC deaminase CDS was cloned into *E. coli* under the regulation of an inducible expression vector. Significantly higher levels of activity in cultures carrying the *Arabidopsis* ACC deaminase suggested that the CDS coded for a functional enzyme (recall Figure 9). Background activity measured in control cultures is probably due to the indicator, 2,4-dinitrophenylhydrazine, reacting with endogenous amino ketones (Friedman and Haugen, 1943). The amount of measurable activity increased when expression induction by anhydrous tetracycline was increased, suggesting that measurable activity in test cultures was due to the presence of the *Arabidopsis* ACC deaminase CDS. A need for high induction to obtain measurable activity may be indicative of the nature of the *Arabidopsis* ACC deaminase. Purification of the enzyme from transformed bacterial cultures, or from plant tissues, will be useful to study the integrity and kinetics of this plant-encoded ACC deaminase.

Although high activity was recorded from *E. coli* transformed with the *Arabidopsis* ACC deaminase, their ability to utilize ACC as a sole nitrogen source on solid media was weak (recall Table 1). Colonies of ACC deaminase-carrying bacteria took about 4 days to develop and never showed growth as strong as those on rich media or minimal media supplemented with ammonium sulfate. Slow growth has been observed in other experiments (Campbell and Thomson, 1996; Penrose and Glick, 2001) and could be due to the minimal amount of growth supplements available in the media. It has also been suggested that some strains of *E. coli* may not be able to absorb ACC from the media (Penrose

and Glick, 2001). Even though growth on minimal media was not strong, the ability to measure ACC deaminase activity in transformed bacteria indicates that the *Arabidopsis* ACC deaminase gene encodes for a functional enzyme.

4.2 Regulation of ethylene levels by ACC deaminase

To understand the role of ethylene during plant development it is important to determine how, when, and where its production is regulated. Based on the ACC degrading activity of ACC deaminases it was thought that the plant-encoded ACC deaminase may be involved in ethylene regulation. Often, physiological changes in response to a change in enzyme activity can be used to infer a relationship of that enzyme with a particular function. For ACC deaminase, it was thought that a role in ethylene regulation might be identified when its activity was inhibited *in vivo*.

ACC deaminases from yeast and *Pseudomonas* strains show some reactivity with D-isomers of several amino acids, such as serine and alanine (Minami *et. al.*, 1998). However, reactivity with L-isomers is generally less and L-serine in particular is a strong competitive inhibitor of ACC deaminases (Minami *et. al.*, 1998). The affinity of *Hansenula satumus* ACC deaminase for L-serine is only slightly less than that for ACC (Minami *et. al.*, 1998). Because a decrease in ACC deaminase activity in the presence of L-serine has been observed in microorganisms, the role of ACC deaminase in *Arabidopsis* ethylene regulation was studied by inhibition with L-serine.

4.2.1 ACC deaminase down regulates ethylene biosynthesis

Inhibition of ACC deaminase *in vivo* in *Arabidopsis* by the addition of L-serine to growth media proved to be a useful experiment. The observation of triple response-like characteristics in wild-type seedlings germinated on media containing L-serine (recall Figure 16) suggests that ACC deaminase inhibition caused an increase in endogenous ethylene levels. This strongly indicates that the ACC deaminase functions in regulating ethylene biosynthesis by controlling the amount of ACC available.

The stunted physiology of wild-type seedlings was similar to descriptions of the appearance of *ctr1* (constitutive triple response 1) mutants of *Arabidopsis*. The CTR1 receptor is thought to be a negative regulator of ethylene perception (Johnson and Ecker, 1998). *ctr1* mutants exhibit triple response characteristics under normal levels of ethylene and even in the presence of ethylene-binding inhibitors (Kieber and Ecker, 1993). Mature *ctr1* plants remain stunted with small rosettes and leaves due to the inhibitory effects of ethylene on cell elongation (Kieber *et. al.*, 1993). Similarly, even after exposure to light the wild-type seedlings grown with L-serine would remain stunted and their rosettes never grew as large as control plants. *ctr1* plants show such phenotypes as a result of an inability to down regulate the ethylene response (Kieber *et. al.*, 1993). Ethylene is known to have inhibitory effects on cell elongation including during leaf development (Abeles *et. al.*, 1992). The effects on growth observed in wild-type seedlings in these experiments are believed to be the result of an inability to down regulate ethylene biosynthesis because of ACC deaminase inhibition.

The ethylene insensitive mutant, *ers1*, was a useful control for these experiments. A mutation in the N-terminal transmembrane domain of the *ERS1* ethylene receptor results in the inability to perceive ethylene causing the ethylene insensitive phenotype (Wang *et. al.*, 2002). Even under long-term exposure to ethylene, *ers1* plants grow normally and do not exhibit stunted growth. *ers1* plants also tend to have larger rosette leaves because they are unable to transmit the inhibiting affects of ethylene on cell elongation (Hua *et. al.*, 1995). Elevated ethylene levels caused by ACC deaminase inhibition did not significantly affect the growth of roots, hypocotyls, or cotyledons of the *ers1* seedlings. Slight changes in hypocotyl length were observed at various concentrations of L-serine but the maximum decrease did not appear to be significant when compared to the changes observed in wild-type seedlings. In addition, compared to the correlated increase of stunting observed in wild-type seedlings as L-serine concentration increased, decreases in hypocotyl length of *ers1* seedlings was not correlated to increasing concentrations of L-serine. Stable germination potential of both *ers1* and wild-type seedlings, ranging between 34% to 80% germination for both across all concentrations of L-serine, suggests that ACC deaminase inhibition by L-serine is not detrimental to germination. For these reasons the changes observed in wild-type seedlings are attributed to an increase in ethylene levels by specific L-serine inhibition of ACC deaminase and not other affects caused by L-serine.

These experiments confirmed that ACC deaminase is involved in down regulating ethylene levels. This role for the *Arabidopsis* ACC deaminase may be used to infer a role for ACC deaminase detected in other tissues and plants.

4.3 Tissue-specific ethylene regulation by ACC deaminase – ethylene in plant development

The roles of ethylene in plant growth and development have been confirmed in some situations but remain ambiguous in others. Fruit ripening, for example, has been strongly associated with ethylene. The role of ethylene in seed development and dormancy, however, has some support but conclusive evidence is pending. Identifying a role for ACC deaminase in ethylene regulation (discussed above) provides insight as to why ACC deaminase may be active in different tissues at various developmental stages.

4.3.1 Ethylene regulation in highly vascular tissues of Arabidopsis

MPSS expression data suggested the presence of ACC deaminase in a variety of tissues. Activity was detected in the highly vascular tissues tested (recall Figure 11). Among these, activity was measured to be highest in the leaf, suggesting a need to down-regulate ethylene here. Ethylene down regulation might be necessary to allow for the development of specific types of cells within the leaf or for leaf development as a whole. Rosette leaf (leaves formed at the base of the plant) development is known to be affected by ethylene. The emergence of young leaves is associated with enhanced ethylene levels and as leaves mature ethylene levels decrease (Smalle *et. al.*, 1999; Abeles *et. al.*,

1992). Rosette leaves used in this assay are considered to be in transition from immature to adult and adult based on their age. ACC deaminase activity in these leaves may function to regulate ethylene levels during the developmental change from young to adult rosette leaves. It will be interesting to measure ACC deaminase activity as leaves emerge and age. It is hypothesized that ACC deaminase activity would be low during early leaf development, when ethylene levels are proposed to be up-regulated (Smalle *et. al.*, 1999) and would increase as leaves mature. In land plants ethylene has been mostly found to inhibit cell expansion, which is associated with leaf expansion (Abeles *et. al.*, 1992). The affects of ethylene on cell wall structure are thought to be involved in its control of cell expansion, although the exact mechanisms are still unknown. Regulation of ethylene levels by ACC deaminase in growing leaves may be linked to regulating cell expansion during growth. The same functions for ACC deaminase in the leaf may also be true for those in the stem; down-regulation of ethylene in a cell specific manner or to promote shoot elongation. However, a definitive role for ACC deaminase in cell specific developments or tissue developments cannot be identified at this time.

ACC deaminase activity was also measured in hypocotyl extracts and was only slightly lower than those measured in leaf extracts. The vascular cambium in hypocotyls is quite active and a large amount of xylem and phloem are produced. Ethylene regulation by ACC deaminase during hypocotyl growth could be for a variety of reasons. Some down regulation of ethylene biosynthesis may be occurring to regulate cell elongation and expansion. However, ACC deaminase

may also be present to down regulate ethylene in specific vascular tissues or cell types. It has been suggested that ethylene is needed in xylem-specific developments such as secondary cell wall formation and programmed cell death (Dolan, 1997; De Jong *et. al.*, 2002; He *et. al.*, 1996; Sitbon *et. al.*, 1999; Zhong *et. al.*, 2002) therefore it is hypothesized that ethylene is produced in xylem tissues but perhaps not in phloem and cortex. There is a great deal of phloem and cortex within the *Arabidopsis* hypocotyl (recall Figure 11). If ethylene is down regulated in developing and maturing phloem then it would be expected that a significant amount of ACC deaminase activity would be present in these tissues and thus measured in assays of hypocotyl extracts. Unlike in poplar, vascular tissues of *Arabidopsis* cannot be easily separated. *In situ* hybridization of ACC deaminase within the *Arabidopsis* hypocotyl may reveal cell or region specific expression. In addition, anti-sense technologies could be used to down regulate endogenous levels of ACC deaminase to assess the effects of ACC deaminase on the development of specific vascular tissues within the hypocotyl.

The presence of ACC deaminase activity in highly vascular tissues of *Arabidopsis* may be interpreted as a correlation between ethylene regulation and vascular tissue formation. Regulation of ethylene by ACC deaminase in specific tissues and even specific cell types may be occurring. However, because activity within specific vascular tissues could not be measured and it is known that ethylene is involved in many processes of plant development, the ACC deaminase activity in highly vascular tissues of *Arabidopsis* may not be limited to just vascular tissue formation.

4.3.2 ACC deaminase during seed development – ethylene and dormancy

During seed development it is thought that ethylene levels are low to allow entrance into dormancy and then elevated to release seeds from dormancy (Yee, 2003; Kepczynski and Kepczynski, 1997). ACC deaminase activity measured in extracts from siliques and their seeds harvested across the six time points of development may provide some insight about the pattern of ethylene biosynthesis during seed development. ACC deaminase activity was highest in stage 2 and early stage 3 siliques, when seeds are believed to be entering dormancy, and very low at the later stages of development, when seeds have maximum germination potential (recall Figure 14). This suggests that ethylene levels are kept low when seeds are entering dormancy and allowed to accumulate later in development so seeds can exit dormancy. The hypothesized regulation of ethylene levels by ACC deaminase during seed development is illustrated in Figure 17.

4.3.3 An ACC deaminase in tomato? Ethylene regulation during tomato fruit ripening

The role of ethylene during ripening of tomato fruit has been well characterized. Ethylene levels are low in immature fruits, peak at the onset of ripening (breaker stage), and then decrease during the later stages of ripening (Alexander and Grierson, 2002; Brady *et. al.*, 1987; Nakatsuka *et. al.*, 1998). The burst of ethylene production at the breaker stage is thought to initiate downstream changes of ripening (Wenbo *et. al.*, 2002). This is evidenced in mutants that have reduced ethylene levels.

Figure 17: Ethylene regulation by ACC deaminase during seed development. ACC deaminase activity measured in siliques and seeds harvested across 6 time points of development suggests a developmental specific pattern of ethylene regulation. Results suggest that ACC deaminase is active when seeds are entering dormancy to maintain low levels of ethylene. However, as seeds prepare to exit dormancy ACC deaminase activity is low to allow ethylene to accumulate.

Hypothesized ethylene levels are adapted from Yee (2003).

For example, the *Never-ripe* (*Nr*) mutant of tomato fails to ripen completely due to a reduced ability to perceive ethylene (Nakatsuka *et. al.*, 1998). In addition, tomato plants transformed with ACC deaminase from a *Pseudomonas* strain have severely delayed ripening due to reduced ethylene biosynthesis (Klee *et. al.*, 1991). In both cases it is apparent that a rise in ethylene levels is required to stimulate down stream ripening processes. Conversely, elevated levels of ethylene past the breaker stage cause fruits to over-ripen and rot - just leave a tomato in a bag with a banana, or a ripe tomato!

A unique pattern of ethylene biosynthesis during ripening suggests that there would be mechanisms to regulate where, when, and how much ethylene is made. The pattern of ACC deaminase activity, based on α -ketobutyrate production, in ripening tomatoes was quite interesting (recall Figure 13). Low levels of activity in late-immature fruit may be to allow for ethylene biosynthesis to increase for the upcoming breaker-stage. A post-breaker peak of ACC deaminase activity may serve to reduce ethylene biosynthesis after ripening has been initiated.

An ACC deaminase-like sequence has not been identified from available sequences in tomato, however some short ESTs had very low sequence similarity to the *Arabidopsis* ACC deaminase (NCBI). The ESTs were from various tissues including callus, shoot, radicle, and fruit, however none had identified functions. ACC deaminase activity proved to be a valid indication of a functional enzyme in *Arabidopsis* and it is likely that a functional ACC deaminase also exists in tomato as a mechanism to regulate ethylene levels during, at least,

fruit ripening. Measurement of ACC deaminase activity in ripening tomatoes, presented here, also substantiated a role of ACC deaminase in ethylene regulation

The nature of ethylene, when and where it is present within a tissue, the multitude of down-stream effects of ethylene, and the cross talk between ethylene and a variety of other hormones all contribute to the complexity of its role in plant development. Understanding the mechanisms by which ethylene is regulated may offer some insight as to when and where the hormone is needed. The results discussed in this section revealed that ethylene regulation by ACC deaminase is occurring in a variety of tissues at many different developmental stages. Some hypothesized relationships of ACC deaminase and ethylene regulation during plant developments is illustrated in Figure 18.

4.4 ACC deaminase in poplar

It was recently shown (Andersson-Gunneras *et. al.*, 2003) that ACC oxidase activity is restricted to developing xylem during tension wood formation in poplar and that expression and activity was not abundant in neighbouring developing phloem, suggesting that ethylene is required for proper xylem formation but not phloem formation. It was also shown that ACC is made available at stable levels at the vascular cambium. Therefore it is reasonable to believe that a regulatory mechanism exists to prevent ethylene biosynthesis where it is not required.

The identification of an ACC deaminase-like sequence expressed in the phloem tissue, but not in the xylem, opens up the possibility that ACC deamination is a method to decrease ethylene levels.

4.4.1 Regulation of ethylene biosynthesis in vascular tissues of poplar stem by ACC deaminase – tissue specificity

ACC deaminase activity was measured in various vascular tissues by the amount of α -ketobutyrate produced in the presence of substrate (ACC). A loss of activity after boiling protein extracts and inhibition of activity by L-serine confirmed that α -ketobutyrate production was indeed the result of ACC deaminase activity. High ACC deaminase activity in developing phloem and very low activity in developing xylem (recall Figure 15) corroborated the previously recorded gene expression levels. Active ACC oxidase (Andersson-Gunneras *et al.*, 2003) and the lack of ACC deaminase activity in developing xylem indicates that ethylene is produced during xylem formation. High ACC deaminase activity in developing phloem indicates that ethylene biosynthesis is probably down regulated in these tissues. Tissue-specific regulation of ethylene biosynthesis is a good indication that ethylene has tissue-specific functions during vascular tissue formation. It is thought that ethylene is needed during xylem development to cue later stages of xylem-specific maturation, and therefore ethylene biosynthesis would be down regulated by ACC deaminase where xylem is not forming.

Figure 18: The role of ACC deaminase during plant developments. Based on differential ACC deaminase activity measured in a variety of plant tissues, some roles for ACC deaminase regulation of ethylene are hypothesized. **(A)** High levels of activity in the hypocotyl may be limited to non-xylem tissue perhaps because ethylene is needed to cue xylem-specific developments. **(B)** ACC deaminase activity in leaves may down regulate ethylene to allow for leaves to make the developmental transition from immature to adult, perhaps to promote cell expansion, or perhaps its activity is limited to non-xylem cells. **(C)** During seed development, ACC deaminase maintains low ethylene levels when seeds are entering dormancy. **(D)** In ripening tomato it is hypothesized that ACC deaminase is most active post-breaker to regulate ethylene after the climacteric burst.

ACCd – ACC deaminase

PCD – programmed cell death

4.4.2 Why ethylene might be needed in developing xylem

Structurally, xylem and phloem are quite different. Xylem vessels are dead at maturity and contain thick, lignified secondary cell walls. Mature phloem contains bundles of phloem fibers that do contain thick secondary cell walls but they are not highly lignified. Phloem cells are also alive at maturity. Xylogenesis is the maturation from immature xylem into mature wood tissue by the processes of cell division from the vascular cambium, cell elongation and radial expansion, development of secondary cell walls including lignification, and programmed cell death (Mellerowicz *et. al.*, 2001). ACC oxidase but no ACC deaminase in the xylem strongly suggests that ethylene is produced in developing xylem. Tension wood formation in angiosperm trees and compression wood in conifers has been used to study the mechanisms and molecular signals involved in wood formation. When tilted there is an increase in the number of fibers and xylem vessels formed in tension wood and compression wood that has been associated with increased ACC and ethylene biosynthesis (Andersson-Gunneras *et. al.*, 2003; Yamamoto and Kozlowski, 1987a). The increase in tracheid production during tension and compression wood formation is also associated with an increase in cambial activity and increased auxin (Andersson-Gunneras *et. al.*, 2003; Little and Eklund, 1999). The ability to stimulate tracheid formation by the application of etherel (an ethylene-like substance) to tree stems also suggests a role for ethylene in wood formation (Yamamoto and Kozlowski, 1987a). Enhancing levels of endogenous ethylene by including ethylene precursors such as L-

methionine or ACC in growth media has been shown to enhance xylogenesis in lettuce pith parenchyma explants (Miller and Roberts, 1984). These studies, just to name a few, have clearly shown an association between ethylene and xylem formation, and therefore wood formation.

Perhaps ethylene cues the production of xylem from the vascular cambium and signals downstream developmental stages for maturation into mature wood tissue. Lignified secondary cell wall formation and programmed cell death are key developmental stages that occur during xylem maturation, but not during phloem maturation. These developmental processes have also been associated with ethylene. During secondary cell wall formation the orientation of cellulose microfibrils in the inner layer of secondary cell walls of elongating xylem can become disorganized by changes in ethylene levels (Dolan, 1997). Also, results of Zhong *et. al.* (2002) suggest that an increase in lignin deposition in secondary cell walls of xylem cells is associated with enhanced ethylene production in an *Arabidopsis* mutant. Other experimental evidence also indicates that ethylene stimulates peroxidase activity which is involved in the oxidative polymerization of lignin precursors to form lignin (Sitbon *et. al.*, 1999). In addition, some genes involved in lignin biosynthesis have been found to be ethylene-inducible (Sitbon *et. al.*, 1999).

During the last phase of maturation, xylem cells undergo programmed cell death (PCD). Experimental evidence suggests that an ethylene-induced signal during PCD affects the influx of calcium ions into cells which is a cue for PCD. This has been observed during PCD in aerenchyma formation in roots of maize

(He *et. al.*, 1996). An increase in hydrogen peroxide in cells is also a sign of PCD. De Jong *et. al.* (2002) found that hydrogen peroxide production was blocked when ethylene synthesis inhibitors were applied to tomato cultures during chemically-induced PCD. Although application of ethylene alone was not sufficient to induce hydrogen peroxide production, ethylene was found to be a potentiator of chemically-induced PCD. Ethylene has also been linked with stimulating the release of reactive oxygen species from mitochondria of cells during the initiation PCD (De Jong *et. al.*, 2002).

Other processes that involve PCD, such as senescence, have been shown to rely on ethylene either as an inducer or potentiator. For example, petal and ovary senescence in peas is delayed when ethylene is inhibited whereas ethylene treatment induces DNA breakdown (a signal of PCD) and senescence (De Jong *et. al.*, 2002). Genes involved in the initiation of PCD during senescence have also been suggested to be induced by ethylene (Greenberg, 1996).

A variety of experimental results have suggested that ethylene is needed during many stages of xylem development from initiation of tracheid formation, secondary cell wall formation, to programmed cell death. The pattern of ACC deaminase activity measured in the stem of poplar suggests that ethylene biosynthesis is down regulated in non-xylem tissues such as the phloem

4.5 Future directions - understanding plant-encoded ACC deaminases

Results discussed here have illustrated that a functional ACC deaminase exists in some plants. The general trend of ACC deaminase activity within some tissues and during some developments was examined, but a great deal of study is still required to really understand this plant-encoded ACC deaminase.

4.5.1 ACC as a mechanism of ethylene regulation

The conjugation of ACC to malonyl ACC (MACC) and 1-(γ -L-glutamyl-amino) cyclopropane-1-carboxylic acid (GACC) has been reported in tomato fruit, apple fruit, and conifer tissues (Tan and Bangerth, 2000; Martin and Saftner, 1995; Martin *et. al.*, 1995; Wilksch *et. al.*, 1998). It has been suggested as a possible mechanism to divert ACC from ethylene biosynthesis during fruit development. However, experimental data does not strongly support this theory. MACC activity was shown to be relatively constant across the stages of tomato ripening, from green to ripe, with a slight increase in the amount of ACC malonyl transferase, MACC, and GACC levels at the same time in which ACC oxidase activity and ethylene biosynthesis were increasing (Martin and Saftner, 1995). This does not suggest that conjugation is a strong diversion of ACC from ethylene biosynthesis. Conjugation of ACC may be a very weak regulatory mechanism, but there is no evidence that these conjugates control how much ethylene is produced during any types of plant developments. In addition, because conjugates can be converted back into ACC for ethylene biosynthesis, it is not a permanent reduction of ACC. For these reasons it is not believed that conjugates of ACC are a strong mechanism to regulate ethylene biosynthesis. In

comparison, ACC deaminase activity in plants discussed here suggests that it plays a significant role in tissue and developmental specific regulation of ethylene levels. The conversion of ACC to α -ketobutyrate and ammonia is irreversible. ACC deaminase activity was measured at times when ethylene levels are believed to be low for developmental and tissue specific purposes. It will be interesting to identify ACC deaminase in other plants to determine if it is a conserved mechanism of ethylene regulation.

4.5.2 Analyzing properties and enzyme kinetics

Activity in plant tissues was quite low compared to activity measured in ACC deaminase containing bacteria. Activity in microorganisms has been reported across a wide range, between $0.2 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{hour}^{-1}$ and $15 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{hour}^{-1}$ (Grichko and Glick, 2000). Levels measured in plant protein extracts and bacterial extracts were anywhere from 2 times and up to 1000 times less than the activity measured in microorganisms. Low α -ketobutyrate production could be the result of several factors. The crude protein extracts most likely contained proteases and oxidizing agents which would damage enzymes. In addition, the amount of ACC deaminase solubilized within the total protein extract may be very low: this would depend on the efficiency of extraction and the percent of total protein comprised by ACC deaminase. It is also important to consider that the native levels of activity *in vivo* might be naturally low and this would result in even lower levels of measurable activity *in vitro*. ACC deaminases from microorganisms have been found to have relatively low

affinities for ACC (Penrose and Glick, 2001), especially compared to other enzymes such as ACC oxidase (Penrose and Glick, 1997). This could also be true for plant ACC deaminases. Improvement of the protein extraction method to rid the extract of phenols, proteases, and oxidizing agents will be important for future testing. It will be critical to have purer protein extracts to further characterize the functionality of ACC deaminase in plants. The goal of these experiments, however, was to determine if a functional ACC deaminase exists.

Further characterization of the *Arabidopsis* ACC deaminase might involve determining what regulates gene expression. ACC deaminase from *Enterobacter cloacae* contains 5' upstream regions that are responsive to both ACC and leucine (Li and Glick, 2001). Although expression was induced in the presence of ACC and leucine, leucine alone was sufficient to induce expression (Li and Glick, 2001). Cloning of the *Arabidopsis* gene including parts of the upstream region, followed by analysis of expression in the presence of different regulatory factors will aid in understanding the regulatory pattern of the plant-encoded ACC deaminase.

The research of this thesis successfully identified a plant-encoded ACC deaminase that appears to have a role in ethylene regulation. Results suggest that ACC deaminase is involved in seed development and germination, development of highly vascular tissues in *Arabidopsis*, ripening in tomato, and during phloem formation in poplar. As a regulatory mechanism, it is expected that ACC deaminase is active within plants at most stages of growth but that the

amount of activity varies depending on the requirement for ethylene biosynthesis during a particular development. It was of great interest to identify a functional plant-encoded ACC deaminase that may be a mechanism for ethylene regulation during vascular tissue formation in poplar. This evidence will add to the debate about ethylene biosynthesis and the involvement of ethylene in wood formation.

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