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Effect of Acyl Activating Enzyme (AAE) 3 on the growth and development of *Medicago truncatula*

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ABSTRACT

The Acyl-Activating Enzyme (AAE) 3 gene encodes an oxalyl-CoA synthetase that catalyzes the conversion of oxalate to oxalyl-CoA in a CoA and ATP-dependent manner. Although the biochemical activity of AAE3 has been established, its biological role in plant growth and development remains unclear. To advance our understanding of the role of AAE3 in plant growth and development, we report here the characterization of two *Medicago truncatula* AAE3 (*Mtaae3*) mutants. Characterization of a *Mtaae3* RNAi mutant revealed an accumulation of calcium oxalate crystals and increased seed permeability. These phenotypes were also exhibited in the Arabidopsis *aae3* (*Ataae3*) mutants. Unlike the *Ataae3* mutants, the *Mtaae3* RNAi mutant did not show a reduction in vegetative growth, decreased seed germination, or increased seed calcium concentration. In an effort to clarify these phenotypic differences, a *Mtaae3 Tnt1* mutant was identified and characterized. This *Mtaae3 Tnt1* mutant displayed reduced vegetative growth, decreased seed germination, and increased seed calcium concentration as well as an accumulation of calcium oxalate crystals and increased seed permeability as found in *Ataae3*. Overall, the results presented here show the importance of AAE3 in the growth and development of plants. In addition, this study highlights the ability to separate specific growth and development phenotypes based on the level of *AAE3* gene expression.

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1. Introduction

Acyl-activating enzymes (AAEs) play a crucial role in a wide array of metabolic pathways vital to all living organisms by catalyzing the activation of a variety of carboxylic acids. Numerous carboxylate containing metabolites often require activation prior to entry into various biosynthetic or catabolic pathways. Carbohydrates, lipids, amino acids, glucosinolates, cutin, suberin as well as many other secondary metabolites utilize carboxylic acid activation as part of their metabolic processes. To accommodate the large variations in the size and structure of these organic acid substrates, many organisms have evolved large families of AAEs some of which are dependent on coenzyme A and ATP for this activation process. This subset of AAEs are generally categorized as CoA ligases or CoA synthetases.

Based primarily on a highly conserved AMP-binding motif, computational investigations have identified a large number of putative CoA synthetases in the model plant Arabidopsis. The identification of such putative AAEs has allowed for the methodical testing of numerous compounds as potential substrates of the individual CoA synthetases. This approach has led to the determination of substrate specificities for a number of AAEs including AAE3, which has been determined to be an oxalyl-CoA synthetase shown to catalyze the first step in a previously uncharacterized pathway of oxalate catabolism [1]. Since this initial finding, recent studies have shown that yeast [2] and a number of plants including *Medicago truncatula* [3], rice bean [4], rice [5], buckwheat [6], and amaranth [6] express an oxalyl-CoA synthetase suggesting the fundamental importance of this enzyme to plants as well as other organisms.

The importance of AAE3 in enhancing the survival of plants and

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yeast when confronted with certain environmental stresses has been documented in recent reports. In response to biotic stress such as oxalate-secreting micro-organisms, AAE3 was found to reduce the inhibitory growth effects of the secreted oxalate on yeast [2]. In plants, AAE3 was determined to reduce the susceptibility of plants to oxalate-secreting phytopathogens [1,3,7]. In addition, two studies utilizing different plant species reported the importance of AAE3 in regulating aluminum tolerance [4–6]. An interesting aspect of these two latter studies is that in one report, a reduction in AAE3 resulted in a decrease in aluminum sensitivity [5], while in the other report, an increase in AAE3 resulted in a decrease in aluminum sensitivity [4]. This apparent discrepancy will need to be clarified with additional studies before any firm conclusion can be drawn.

Thus far, the importance of AAE3 in plant growth has been confined to a single report in Arabidopsis [1]. Characterization of an *Ataae3* T-DNA mutant showed that lack of *AAE3* expression resulted in reduction in vegetative growth, seed mucilage production, seed germination, and an increase in calcium oxalate accumulation in Arabidopsis. Whether such phenotypes are specific to Arabidopsis or whether a reduction in *AAE3* expression would result in similar phenotypes in other plants remains unknown.

To expand our understanding of the impact of AAE3 in plant growth and development, we report here the characterization of *Mtaae3* RNAi knock-down and *Mtaae3 Tnt1* knock-out mutants. Comparisons of these determined phenotypes to the *Ataae*3 mutants provide additional insights into the responsiveness of each developmental phenotype to changes in the level of *AAE3* gene expression. Like the *Ataae3* T-DNA knock-out mutant, the *Mtaae3* RNAi knock-down mutant showed an increase in calcium oxalate accumulation but lacked other *Ataae3* mutant phenotypes. This apparent discrepancy was clarified through the phenotypic characterization of a *Mtaae3 Tnt1* knock-out mutant, which displayed phenotypes similar to *Ataae3* T-DNA knock-out mutant. These findings show that the level of *AAE3* gene expression is important in determining the severity of the exhibited phenotypes.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Murashige and Skoog (MS) medium was purchased from Caisson Laboratories Inc (North Logan, UT, USA).

2.2. Plant materials and growth conditions

Wild-type (WT, ecotype Columbia, Col-0), two *Ataae3* T-DNA insertional lines, *Ataae3-1* and *Ataae3-2,* were described previously [1]. *M. truncatula* ecotype R108 (wild-type) and *Mtaae*3 RNAi knock-down line (RNAi lines) were reported previously [3]. For *M. truncatula* growth, seeds were removed from their pods, nicked with a razorblade, and sown on MetroMix FS1 synthetic soil mix (Sun-Gro Horticulture, Agawam, MA) under controlled greenhouse conditions. Natural light was supplemented with artificial lighting using a 16 h day/8 h night photoperiod and temperature maintained at 24 °C. In germination assays, WT and mutant seeds were surface-sterilized and incubated in water at 4 °C for 48 h before being plated on one-half strength Murashige and Skoog (MS) medium (plus 0.5% sucrose), which was solidified with 0.8% agar. Germination was scored as the emergence of the radicle from the seed coat. Experiments were performed twice in triplicate (Arabidopsis: 100 seeds and *M. truncatula*: 60 seeds) for each line examined.

2.3. Identification of a Mtaae3 Tnt1 mutant

A *Mtaae3 Tnt1* knock-out allele was identified by searching the *M. truncatula Tnt1* retrotransposon insertional library (https://medicago-mutant.noble.org/mutant/blast/blast.php; [8]) using the *At*AAE3 sequence. Seeds pools containing the *Mtaae3 Tnt1* mutant were ordered and screened for the insertion event by PCR. The primer set used to screen plants from the segregating pool were LTR5: 5′-GCCAAAGCTTCACCCTCTAAAGCCT-3′ and 5-GAATAACCGGAGCCAACGAAG-3'. Homozygous *Mtaae3 Tnt1* plants were identified by PCR using primers 5′-GAA-TAACCGGAGCCAACGAAG-3′ and 5′-CATTCCACTCGGTTCGGTTT-3'which spanned the insertion site. Once the homozygous line was identified, it was backcrossed to R108 a minimum of five times.

2.4. Analysis of aae3 seed phenotypes

To determine seed permeability, both Arabidopsis and *Medicago* WT and *aae3* mutant seeds were incubated in 1% (w/v) 2,3,5-triphenyltetrazolium chloride at 30 °C for 24 h as previously described [1]. After staining, the seeds were rinsed with water before observation under a stereomicroscope.

2.5. Microscopic analysis of calcium oxalate crystal phenotypes

Tissue samples were first imbibed in 5% bleach (sodium hypochlorite) for 2 days, and dehydrated through a grade ethanol series (50%, 70%, and 100%), then ethanol/xylene, and pure xylene to clear the tissues [9]. Images of whole-tissue mounts and seed coats were captured using a CCD72 camera mounted on a Zeiss Axiophot light microscope (Carl Zeiss Microscopy, Jena, Germany).

2.6. Calcium measurements

The calcium concentrations of dry seeds of *M. truncatula* and Arabidopsis were determined by Inductively Coupled Plasma (ICP) atomic emission absorption spectrometry (Soil, Water and Forage Testing Laboratory, Texas A&M University). Statistical analysis was performed using *t*-tests.

2.7. RNA isolation, cDNA synthesis, and qRT-PCR analysis

M. truncatula seeds were germinated and grown on 0.5X MS medium for 10 days at 22 °C under 16 h light and 8 h dark regime. Ten seedlings for each line were pooled for RNA isolation. Total RNA was extracted from WT and *Mtaae3* seedlings using the RNAqueous Kit (Ambion, USA) following the instruction provided by the manufacturer. Total RNA samples were further purified with treatment of DNase I (RNase-free, New England Biolabs). First strand cDNA was synthesized using 1 μg Oligo-dT_{20} and reverse transcriptase (ThermoFisher Scientific, USA). RT-PCR was performed using the primers for MtAAE3: 5′-ATGGAAACCGCTA-CAACCCTCAC-3' (forward) and 5′-TGAAGCTTGAGAGACAAAGTGTTC-3' (reverse); and 5′-ACTCCCACATGCCATCCTTC-3' (forward) and 5′-GACCAGACTCATCATATTCACCCT-3′ (reverse) for the *M. truncatula ACTIN2* gene serving as the internal control.

3. Results and discussion

3.1. Disruption of AAE3 reduces vegetative growth

As a step toward elucidating the physiological roles of AAE3 in plant growth and development, we first compared the growth phenotype of the *Mtaae3* RNAi knock-down mutant [3] to WT

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