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Genetic, biochemical and physiological studies of acetyl-CoA metabolism via acyl-condensation

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Genetic, biochemical and physiological studies of acetyl-CoA metabolism via acyl-condensation

By

Huanan Jin

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Plant Biology

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2010

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CHAPTER I. GENERAL INTRODUCTION

PHA AND ITS METABOLISM

Polyhydroxyalkanoates (PHAs) are polyoxoester polymers and are deposited within the cell mainly in the form of proteins and lipids bound granular inclusions that a lot of microbes accumulate as carbon and energy source when carbon source is provided in excess but limited in additional nutrient(s) (such as phosphorus, nitrogen, sulfur etc. is limiting) (Madison and Huisman, 1999; Kim and Lenz, 2001). In addition, PHAs are involved in the transport of calcium, phosphate and DNA by forming a channel with calcium polyphosphate spanning the membrane, therefore enhancing transformation efficiency and maybe also involving in regulation of intracellular calcium concentration and calcium signaling (Reusch et al., 1986; Reusch et al., 1987; Reusch and Sadoff, 1988). Furthermore, PHAs take part in resistance to stresses (radiation, desiccation and osmotic pressure) (Tal and Okon, 1985).

The PHAs’ monomer units are 3-hydroxyacids of which carbon chain lengths range from 4 to 14 carbons. Since the discovery of polyhydroxybutyrate (PHB, homopolymer) in *Bacillus megaterium* (Lemoigne, 1926), there is great interest in PHAs as biodegradable plastics that have a lot of applications (van der Walle1, 2001). In response to these applications, intensive studies have been carried out to understand the regulation of PHAs biosynthesis, diversity produced by different microbes (Kessler and Witholt, 2001; Kim and Lenz, 2001; Steinbüchel, 2001) and to maximize and diversify PHAs produced by using recombinant organisms of over-expressing biosynthetic PHA genes or by metabolic engineering (Kraak et al., 1997; Fiedler et al., 1998; Jung, 2000; Klinke, 2000; Klinke et al., 2000b; Han et al., 2004). For example, the British chemical company Imperial Chemical
Industries (ICI) commercialize PHA in the name of "Biopol" produced by fermentation, which is copolyester composed of 3-hydroxybutyrate and 3-hydroxyvalerate, since the 1980s.

PHB is the most common type of PHAs naturally produced in most microbes. However, since the homopolymer is brittle and hard to process, these poor physical and chemical properties limit homopolymer’s commercial use. Compared to PHB, heteropolymers that are composed of mixtures of 3-hydroxyacid-monomers of different carbon chain lengths are less stiff and easier to process (van der Walle1, 2001). This has resulted in an increasing interest in producing heteropolymers with improved qualities by genetic or media manipulations.

There are three types of proteins involved in the synthesis, maintenance and degradation of PHA granules (Klinke et al., 2000a): PHA biosynthetic enzymes, PHA granules associated proteins and PHA depolymerases.

Many genes are involved in PHAs biosynthesis. PHA synthase is the enzyme that synthesizes PHAs. PHA synthases can be divided into four classes (Rehm, 2003). Class I and II are homodimer PhaC (product of \textit{phaC} gene). Class III is multi-heterodimer PhaCE (product of \textit{phaC} gene and \textit{phaE} gene). Class IV also is heterodimer PhaCR (product of \textit{phaC} gene and \textit{phaR} gene). However, Class IV subunit PhaR is required for PhaCR activity, unlike class III, subunit PhaE is not required. PHA synthases have broad substrate specificity and can utilize different carbon-chain length monomers. Nevertheless, class I, III and IV prefer to incorporate short chain monomer (four to five carbon length monomers) and class II is preferentially active towards medium chain monomer (six to fourteen carbon length monomers). Furthermore, all PHA synthases use R-configuration substrates only (Steinbüchel. and Hein., 2001). In spite of PHA synthases’ preferences for different carbon length monomers, studies have shown that the diversity of metabolic pathways of the
organism and carbon sources provided in media considerably contribute to the variety of PHAs produced by an organism (Brandl et al., 1989; Ballistreri, 1995; Kraak et al., 1997; Fiedler et al., 1998; Jung, 2000; Klinke, 2000).

Other PHA biosynthetic enzymes generate 3-hydroxyacyl-CoA substrate for PHA synthases (Fig. 2.). There are several pathways that have been identified to be involved in the substrate generation (Steinbüchel. and Hein., 2001). One pathway is the operon phaABC pathway which involves condensation of two acetyl-CoA molecules to form an acetoacetyl-CoA, which is subsequently reduced to 3-hydroxybutyryl-CoA. In this pathway, 3-ketothiolase (the product of the phaA gene) and acetoacetyl-CoA reductase (the product of the phaB gene) catalyze the first and second step, respectively. The second pathway produces precursors by three enzymes, NADH-dependent acetoacetyl-CoA reductase, crotonase and phaJ, sequentially converting acetoacetyl-CoA to 3-hydroxybutyryl-CoA (Reiser et al., 2000). Another common pathway to generate PHA precursor is from the intermediates of fatty acid β-oxidation: (I) (S)-3-hydroxyacyl-CoA can be converted to (R)-3-hydroxyacyl-CoA by an epimerase; (II) 3-ketoacyl-CoA can be reduced to (R)-3-hydroxyacyl-CoA by a reductase; and (III) trans-2,3-enoylacyl-CoA can be hydrated to (R)-3-hydroxyacyl-CoA by phaJ. Also, amino acid catabolism can generate the PHA precursors. A recently discovered noteworthy pathway is the metabolic link between fatty acid de novo biosynthesis with biosynthesis of PHA, catalyzed by PhaG (a 3-hydroxy-decanoyl-[acyl-carrier-protein]:CoA transacylase) (Rehm et al., 1998).

In vivo, PHA granules are covered by a 4-nm-thick layer, which sequesters hydrophobic PHA from cytoplasm and is composed of proteins and phospholipids (Hanley et al., 1999). There are two systems of PHA bound proteins: PhaP-PhaR and PhaF-PhaI. PHA
accumulating organisms have one of these two systems. In the PhaP-PhaR system, PhaP (phasin) is a predominant protein present in the granules (accounting for 90% of PHA bound proteins, and about 5% of total cellular protein) (Wieczorek et al., 1995; York et al., 2001b). This small amphiphilic protein PhaP is an analogy to olesins that form a layer around triacylglycerol inclusions present in seeds of plants. PhaR, which forms a tetramer, binds to the phaP promoter, hereby repressing phaP expression in the absence of PHA. When organisms begin to synthesize PHA, PhaR binds to ongoing PHA oligomer and this leads to releasing the repression of phaP. However, the affinity of PhaP for PHA granules is higher than that of PhaR. Thus in the later stages of PHA synthesis, excess PhaR can bind to the PhaP promoter again, preventing more PhaP synthesis than required. Also, PhaR can serve as auto-repressor by binding to PhaR promoter, thus preventing the synthesis excess PhaR (Pieper-Furst et al., 1994, 1995; Wieczorek et al., 1996; Maehara et al., 1999; Maehara et al., 2001; York et al., 2001b; York et al., 2001a; Potter et al., 2002). In the PhaF-PhaI system, phaI and phaF are in one operon and in a sequence of I-F. However, two promoters are present. One is located upstream of phaI and this promoter transcribe phaI and phaF as an operon. The other is located upstream of phaF and transcribe phaF. In the absence of phaI protein, PhaF, which is a histone-like bifunctional protein, binds to phaC promoter and the operon promoter, turning off phaC and the operon expression. Thus, only the phaF promoter is active. When cultivation conditions permit organisms to synthesize PHA (such as nitrogen is limiting and carbon source is in excess.), PhaF DNA binding capacity is decreased (Maybe other transcription factors compete for the same DNA region.) and the operon promoter begins to transcribe the phaIF. After the onset of phaI synthesis, PhaI binds to PhaF, resulting in conformational change of PhaF. This conformational change sequesters PhaF
DNA binding domain and consequently depresses the repression of phaC and the phaIF operon, whereas, the complex phaF-phal binds to PHA as major PHA binding proteins (Prieto et al., 1999).

There are two types of PHA depolymerases (encoded by phaZ genes): intracellular depolymerase and extracellular depolymerase. Correspondingly, there are two forms of granules: native PHA (with intact layer) or intracellular PHA which can be degraded by intracellular depolymerase and denatured PHA (without layer or damaged layer) or extracellular PHA, which can be degraded by extracellular depolymerase, released from PHA accumulating organisms after death (Handrick et al., 2004a). Organisms have one type or both.

Since a lot of proteins participate in PHA metabolism, it is expected that regulation of PHA metabolism is a complex network. Regulation of PHA metabolism takes place at three levels: transcriptional level, enzymatic and metabolic level and PHA maintenance and biodegradation level.

At transcriptional level, a lot of transcription factors are involved in expression of PHA biosynthetic genes (Miyake et al., 1997). For example, when microorganisms are grown on nutrient limiting media, two-component systems (NtrB-NtrC and PhoR-PhoB, etc.) activate the PHA biosynthetic genes expression. The NtrC dimer as an activator together with sigma factor $\sigma^{54}$ (NtrA, RpoN) separately binds to upstream element and the promoter of PHA biosynthetic genes to activate gene expression in response to limiting nitrogen (Sun et al., 2000; Hoffmann and Rehm, 2005). In addition, $\sigma^{54}$ (NtrA, RpoN) is a negative regulator of PhaF in Pseudomonas putida and Pseudomonas aeuginosa. Under phosphorus-limiting condition, PhoB as an activator binds to pho box of the promoter of PHA biosynthetic genes.
to trigger gene expression (Schembri et al., 1995). In *Pseudomonas oleovorans*, PhaF binds to promoter of *phaC1* in the absence of PHA, hereby inhibiting transcription of *phaC1*. In addition, carbon sources also affect transcription of PHA biosynthetic genes. When *Pseudomonas aeruginosa* was grown on gluconate under nitrogen limiting conditions, $\sigma^{54}$ was required for PHA accumulation, but it was not indispensable when the bacteria used octanoate as carbon source though PHA production was higher in wild type than that in $\sigma^{54}$ mutant. In another bacteria *Pseudomonas oleovorans*, the extent of *phaC1* promoter activity depends on carbon source in media (Prieto et al., 1999).

At enzymatic and metabolic level, several enzymes are subject to allosteric regulation or post-transcriptional modification (Stinbuchel, 1991; Miyake et al., 1997). In the cyanobacterium *Synechococcus sp.* phaC is active only under nitrogen limiting condition in the light. It is proposed that phaC is activated by acetyl phosphate (Miyake et al., 1997). In addition, posttranslational modification is required for phaC activity in *Alcaigenes eutrophus* (Gerngross et al., 1994). Studies have shown that high ratio of NAD(P)H/NAD(P) stimulates PHA synthesis and free Coenzyme A inhibits $\beta$-ketothiolase (product of *phaA*), furthermore, NAD(P)H inhibits citrate synthase (Lee, 1995; Haywood, 1998). Another work showed that *R. eutropha* isocitrate dehydrogenase leaky mutant with low TCA cycle activity produced PHA faster than wild type (Park, 1996). In *Pseudomonas putida*, isocitrate lyase mutant produced more medim-chain length PHAs than wild type (Klinke, 2000). Another work showed that over-expression of alginate biosynthetic gene decreased PHA accumulation (Hoffmann and Rehm, 2004). Inhibition of fatty acids $\beta$-oxidation by acrylic acid enhanced PHA production and mutants deficient in $\beta$-oxidation produced more PHA (Han et al., 2004). In addition, a study showed that there was a significant competition for reducing powers
between hydrogen evolution and PHB accumulation in *R. rubrum* Ha (Hustede et al., 1992). These studies have indicated that PHA accumulation is increased by enhancing reducing equivalents and carbon flow to the PHA pathway.

By introducing PhaG (a transacylase) an enzyme that links fatty acid *de novo* biosynthesis to the PHA biosynthesis from *Pseudomonas putida*, a new pathway of medium chain length PHA biosynthesis was established in recombinant *Pseudomonas fragi* (Fiedler et al., 2000). In *Pseudomonas oleovorans*, medium chain length PHA accumulation was decreased in *phaD* mutant, an unknown functional gene (Klinke et al., 2000a). In *Pseudomonas sp.*, there are two PHA biosynthetic genes loci *phb* and *pha*. By using complementation studies and heterologous expression, they found that *phb* locus encodes *phb*BAC operon that is specific for synthesizing homopolymer PHB and *pha* locus encodes two PhaCs PhaC1 and PhaC2, that are responsible for synthesizing heteropolymer PHA (Matsusaki et al., 1998). In *Pseudomonas oleovorans*, over-expression of *phaC1* gene significantly increased PHA production under non-nitrogen limiting conditions. However, under nitrogen limitation, PhaC1 activity was not the rate-limiting step of PHA synthesis (Kraak et al., 1997). Another two studies indicated that over-expression of *phaC* alone or with *phaJ* not only increased PHA production but also changed the ratio of monomers of PHA in *Aeromonas hydrophila* and *Aeromonas caviae*, whereas, *phaJ* alone had little effect (Fukui et al., 2001; Han et al., 2004). Over-expression experiments showed that in *Alcaligenes eutrophus*, the biosynthesis rates of PHAs were controlled by β-ketothiolase (product of *phaA* gene) and acetoacetyl-CoA reductase (product of *phaB* gene). However, the amount and molar fraction of monomers was determined by PhaC activity (Jung, 2000). In contrary to above results, by using $^{13}$C-NMR, a study showed that acetoacetyl-CoA reductase
was the key enzyme controlling the PHA amount and the molar fraction of monomers instead of phaC in *Alcaigenes eutrophus* (Doi et al., 1992). Alternatively, other studies showed that β-ketothiolase was the key enzyme of PHA biosynthesis in *Alcaigenes eutrophus* and *Azotobacter beijerinckii* by inhibiting effects of intermediates of the PHA pathway (Senior and Dawes, 1971; Oeding and Schlegel, 1973).

At PHA maintenance and biodegradation level, several studies have shown that PhaP promotes further PHA synthesis by increasing the surface/volume ratio of PHA granules. In *Ralstonia eutrohpha*, phaP mutant contains only one single, large granule and the molecular weight of PHA increases, whereas, over-expressing PhaP strain leads to formation of more, smaller PHA granules with decreased molecular weight than the wild type (York et al., 2001a). The same phenotypes have been observed in heterologous hosts (Matsusaki et al., 1998). PhaP might activate PHA synthase by protein-protein interaction when they are associated with PHA inclusions (Fukui et al., 2001). In addition, PhaP also is required to mobilize PHA in *R. rubrum* (Handrick et al., 2004b). These studies have indicated that PhaP plays an important role in the dynamic accumulation and utilization of PHA.

Studies showed that PHA depolymerase (product of *phaZ* gene) was repressed in the presence of a soluble carbon source. After the soluble carbon source was exhausted, the repression of PHA depolymerase was derepressed (Schembri et al., 1995). However, another study showed that PHA depolymerase was always active and that PHA was synthesized and mobilized simultaneously (Doi et al., 1992). In *Ralstonia eutrohpha*, PHA depolymerase null mutant experiments indicated that the accumulation patterns of depolymerases mutants were very similar to that of wild type in a nutrient-limited mineral salt medium. However, in a rich medium, except ∆phaZ1 that accumulated a little higher PHA, the other mutants (∆phaZ2,
$\Delta$phaZ3, $\Delta$phaZ1$\Delta$phaZ2, $\Delta$phaZ1$\Delta$phaZ3, $\Delta$phaZ1$\Delta$phaZ2$\Delta$phaZ3) accumulated lower PHA than wild type in the log phase. However, mutants ($\Delta$phaZ1, $\Delta$phaZ1$\Delta$phaZ2, $\Delta$phaZ1$\Delta$phaZ3, $\Delta$phaZ1$\Delta$phaZ2$\Delta$phaZ3) remained much higher PHA content than wild type in the stationary phase and mutants of $\Delta$phaZ2 and $\Delta$phaZ3 remained PHA content as low as wild type (York et al., 2003). These results imply that there may be a feedback between PHA synthesis and degradation. In addition, the roles of PHA depolymerases in PHA degradation during active synthesis of PHA remain to be answered.

**RHODOSPIRILLUM RUBRUM**

*R. rubrum* is gram negative, motile and purple bacterium. This non-sulfur microbe is spiral shaped with a length of 3µm to 10µm and a width of 0.5µm to 1.5µm (Truper and Pfenning, 1978). It is capable of growth on a variety of organic compounds under aerobic or anaerobic conditions in the presence or absence of light (Truper and Pfenning, 1978). Moreover, it is metabolically versatile and accumulates several chemicals within the cell including PHA, polysaccharides and polyphosphates (Stanier et al., 1959). A variety of genetic tools have been well developed for use in this microorganism. Indeed, *R. rubrum* has attracted considerable interest for industrial and academic communities: 1) PHA production, 2) hydrogen evolution, 3) model system for investigating photosynthetic system and 4) mechanisms of the nitrogen fixation (Brock, 2000). *R. rubrum* can accumulate up to about 50% dry weight of PHA when carbon is in excess and other nutrient(s) is limiting (Brandl et al., 1989). In addition, *R. rubrum* has the capacity to utilize CO as a sole carbon, energy source and H$_2$ as electron source chemolithoautotrophically under anaerobic conditions.
(Truper and Pfenning, 1978). This mechanism of CO oxidation has been well genetically and biochemically characterized in *R. rubrum*. A CO-sensing heme transcriptional activator, CooA, which is a member of cyclic AMP receptor protein family, binds to the upstream of promoter regions of *CooFSCTJ* and *CooMKLXUH* in the presence of CO (Shelver et al., 1995). Consequently, these two operons are highly expressed. Two Ni-Fe-S-enzymes, an O₂-labile carbon monoxide dehydrogenase encoded by the operon *CooFSCTJ* and an O₂-labile CO-tolerant hydorgenase encoded by the operon *CooMKLXUH* anaerobically oxidize CO to CO₂ by the following reaction: CO+H₂O→CO₂+H₂ (Fox et al., 1996b; Fox et al., 1996a). In the presence of O₂, photosynthesis system is genetically suppressed and therefore it is colorless. However, the repression mechanism for the photosynthesis system is still unclear. When grown under the anaerobic conditions, *R. rubrum* immediately begins to synthesize the photosynthesis apparatus including membrane proteins, bacteriochlorophylls, etc. (Kiley and Kaplan, 1988). The photosynthetic systems are integrated into intracytoplasmic membrane systems arising from invagination of the plasma membrane (Brock, 2000). Consequently, the bacterium becomes photosynthetically active and purple. CO₂ is fixed mainly via Calvin cycle in *R. rubrum* (Louise, 1967, 1969). The photosynthetic pigment of *R. rubrum* is different from that of plants. It possesses bacteriochlorophyll a that maximally absorbs light at wavelength of 364 nm, not chlorophyll a with a maximum absorption at wavelength of 430 nm (Brock, 2000). In addition, it does not evolve O₂ as the by-product of photosynthesis (Louise, 1967; Balows, 1992; Brock, 2000). *R. rubrum* as well is a nitrogen fixer. Since the nitrogen fixation process is very energy demanding, the nitrogenase enzyme complex, which catalyzes the conversion of atmospheric nitrogen to ammonium, is highly regulated at the transcriptional and
posttranslational level. In *R. rubrum*, the post-translational regulation of nitrogenase was particularly well characterized. Nitrogenase is inactivated by covalently attaching ADP-ribose to the arginine residue 101 on nitrogenase reductase upon exposure of cells to darkness or ammonia (Kanemoto and Ludden, 1984). Upon exhaustion of the fixed-nitrogen source or in presence of light, the activity of nitrogenase can be restored by a glycohydrolase removing ADP-ribose (Lowery and Ludden, 1988).

**ACETOACETYL-COA THIOLASES**

Thiolases can be divided into two classes based on their specificities for the chain length of substrates. Thiolase I (3-ketoacyl-CoA thiolases, KAT, EC 2.3.1.16) exhibit broad activity towards various 3-ketoacyl-CoA substrates, whereas thiolase II (acetoacetyl-CoA thiolases, AACTs, EC 2.3.1.9) specifically catalyze condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. 3-ketoacyl-CoA thiolases are localized to mitochondria and peroxisomes and catalyze the cleavage of acyl-CoA esters resulting in the formation of acetyl-CoA and two carbon shorter acyl-CoA ester in fatty acid beta-oxidation. In contrast, acetoacetyl-CoA thiolases are generally found in the cytosol and are shown to be indispensable. Acetoacetyl-CoA thiolases, which are involved in different pathways (Fig.1), have been isolated from many organisms. In plants, AACTs are found in cytosol, where they catalyze the first step of forming mevalonate-derived isoprenoids which play an essential role in membrane component (phytosterols), growth and development (brassinosteroids), disease resistance (mono-, sesqui- and di-terpenoid phytoalexins) and isoprenic units for farnesylation of a series of proteins (Chappell, 1995). In bacteria, AACTs catalyze the first
step biosynthesis of polyhydroxyalkanoates (PHA), which are carbon and energy storage macromolecules. In yeast, AACT encoded by ERG10, which is essential for yeast survival (Hiser et al., 1994), generates precursors for ergosterols and was predicted to be homotetramer based on molecular weight revealed by gel infiltration (Kornblatt and Rudney, 1971). However, AACTs are not exclusively found in cytosol and do not function only in mevalonate biosynthesis. In mammals, both cytosolic and mitochondrial AACTs exist. Cytosolic AACTs are involved in the essential mevalonate pathway that generates cholesterol while the mitochondrial AACTs are involved in synthesis and degradation of ketone bodies. In addition, the mitochondrial AACTs were shown to be involved in isoleucine catabolism in mammals. High resolution crystal structure of human cytosolic AACT has been resolved. It is shown to be a homotetramer of exact 222 symmetry (Kursula et al., 2005).

Fig. 1. AACTs take part in different biosynthetic pathways in different organisms.
DISSERTATION ORGANIZATION

This dissertation consists of five chapters. The first chapter is a general introduction of PHA metabolism, *Rhodospirillum rubrum* and thiolases. Chapter 2 is a manuscript to be submitted to FEMS Microbiology Letters. This chapter is on metabolic engineering of *Rhodospirillum rubrum* for enhancing PHA production and investigation of the regulatory mechanism of PHA biosynthesis when grown on RRNCO medium by over-expressing 6 PHA biosynthetic genes individually or in combination. All experiments and data described in this chapter were carried out by myself under the supervision of Dr. Basil J. Nikolau. Chapter 3 is a manuscript in preparation for the submission to Journal of Bacteriology. The manuscript describes the characterization of three PHA polymerases and one (R)-specific 2-enoyl-CoA hydratase of *Rhodospirillum rubrum* by generating deletion mutants. All experiments and data presented in this chapter were conducted by myself under the supervision of Dr. Basil J. Nikolau. Chapter 4 is a manuscript to be submitted to the Plant Journal. This chapter is on functional characterization of the two AACT-coding genes in *Arabidopsis thaliana*. Except the phytosterol analysis that was conducted by Dr. Song Zhihong, all other experiments and data described in this chapter were performed by myself under the supervision of Dr. Basil J. Nikolau. Chapter 5 is the general summary of the results presented in chapters 2, 3 and 4.
REFERENCES


CHAPTER II. METABOLIC ENGINEERING OF RHODOSPRILLUM RUBRUM FOR
ENHANCING PHA PRODUCTION AND INVESTIGATION OF THE
REGULATORY MECHANISM OF PHA BIOSYNTHESIS WHEN GROWN ON
CARBON MONOXIDE MINERAL SALT MEDIUM

A manuscript to be submitted to FEMS Microbiology Letters

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ABSTRACT

To study the regulatory mechanism of PHA biosynthesis and possibly enhance PHA production when grown on carbon monoxide and carbon dioxide mineral salt medium (RRNCO), which is simulating synthesis gas medium, \textit{R. rubrum} strains were cultured in nitrogen-limiting RRNCO medium and were examined for PHA production. Six PHA biosynthetic genes (\textit{phaC1AB}, \textit{phaC2}, \textit{phaC3}, \textit{phaJ}) were over-expressed individually or in combination in \textit{R. rubrum} and nine over-expressing strains and one control strain harboring empty vector were generated. PHA content analyses revealed that over-expressing \textit{phaC1} and \textit{phaC2} alone significantly enhanced PHA production, whereas over-expressing \textit{phaC3} and \textit{phaJ} alone did not have significant effect on PHA production. PHA content analyses and western blot experiments showed that over-expressing each gene of operon \textit{phaC1AB} alone significantly increased PHA production and over-expressing \textit{phaB} resulted in the increasing of the PHA content the most. Furthermore, the data showed that the expression level of \textit{phaB}
controlled PHA production and that PhaB was the key enzyme of the operon \textit{phaC1AB} PHA biosynthetic pathway. Western blot experiments suggested that \textit{phaB} was subject to post transcriptional regulation when transcribed in the whole operon form. Under these conditions, strain AB-9 over-expressing \textit{phaA} and \textit{phaB} accumulated 30% of cellular dry weight, 2.5 times of control strain’s PHA content. However, the monomer composition of PHA of all strains did not significantly change (0.3%-1% 3-hydroxyvalerate). In addition, growth experiments showed that PHA enhanced \textit{R. rubrum} growth under these conditions.

Keywords: \textit{Rhodosprillum rubrum}; Polyhydroxyalkanoates; carbon monoxide

**INTRODUCTION**

PHAs are biodegradable polymers and deposited within cells as inclusion bodies that a lot of microbes accumulate as carbon and energy source when grown on unbalanced nutrient growth conditions (Madison and Huisman, 1999; Kim and Lenz, 2001; Steinbüchel. and Hein., 2001). There has been great interest in PHAs as biodegradable plastics that have a variety of applications (Steinbüchel, 2001). A lot of bacteria can produce PHAs, including \textit{Rhodosprillum rubrum} (Steinbüchel. and Hein., 2001). This gram negative, photosynthetic purple non-sulfur bacterium can accumulate PHA composed of 3-hydroxybutyrate and 3-hydroxyvalerate (Brandl et al., 1989). However, the limitation of microbe fermentation to produce commercial PHA is the expensive carbon source in media. Synthesis gas (syngas) fermentation not only overcomes the expensive carbon source of media but also bypasses the high cost and poor yields of enzymatic hydrolysis of waste biomass into simple sugars that are suitable for fermentation. The syngas approach uses carbon monoxide and hydrogen
production from gasifying waste biomass instead of sugars as the building blocks for biosynthesis of biochemicals by fermentation. Gasification is the high temperature (750-800°C) conversion of waste carbon into flammable gas mixtures, known as synthesis gas (syngas), composed of carbon monoxide, hydrogen, methane, nitrogen, carbon dioxide, and smaller quantities of higher hydrocarbons (Guehenneux G., 2005). One way to convert syngas to biochemicals is through microbe fermentation. *R. rubrum* can utilize CO as a sole carbon and energy source under anaerobic conditions (Kerby et al., 1995). When exposed to CO, two Ni-Fe-S-enzymes, an O2-labile carbon monoxide dehydrogenase (CODH, the holoenzyme is encoded by the operon *CooFSCTJ*) and an O2-labile CO-tolerant hydorgenase (the holoenzyme is encoded by the operon *CooMKLXUH*) is highly induced (Fox et al., 1996b; Fox et al., 1996a). CO is metabolized by *R. rubrum* according to the following reaction (Bonam et al., 1989): CO+H2O→CO2+H2. CO2 is fixed mainly via Calvin cycle in *R. rubrum* (Anderson and Fuller, 1967). H2 can be metabolized by uptake hydrogenase to provide electron source (reducing power) to fix CO2 (Stanier et al., 1959; Anderson and Fuller, 1967). Thus, *R. rubrum* is of great potential for PHA production from syngas. Several studies have been conducted on growth of *R. rubrum* and on the PHA production utilizing syngas (Najafpour G., 2003; Do et al., 2007). However, no study was carried out to investigate the regulatory mechanism of PHA biosynthesis and enhance PHA production through metabolic engineering of *R. rubrum*. This research addressed these questions when grown on the nitrogen-limiting formulated RRNCO (Kerby et al., 1995), a carbon monoxide and carbon dioxide mineral salt medium, which is a good mimic of syngas.
MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and culture conditions

The strains of *Rhodosprillum rubrum* and *Escherichia coli* and the plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table 2. *E. coli* was grown at 37°C in LB medium. *R. rubrum* was grown photoheterotrophically under 2000 Lux light intensity at 25°C in SMN medium (supplemented malate-ammonium medium; rich medium for *R. rubrum*) (Kerby et al., 1992). To assess PHA production, a 0.5 ml aliquot of a normalized SMN culture (5 O.D.) was collected by centrifugation at 13,000×g for 2 min; then the cells were washed once with RRNCO medium (ammonium chloride was omitted) (Kerby et al., 1995) and resuspended with 0.5 ml RRNCO medium and transferred to 50 ml RRNCO medium. The culture was shaken at 150 rpm at 25°C under 5000 Lux light intensity with a carbon monoxide head space in 121 ml stoppered serum bottles. 4ml cells were harvested at 54, 66, 84, 98, 120, 146-hour cultivation for PHA content analysis. Cell density was determined using a spectronic 20D+ spectrophotometer at 680 nm (Thermo Fisher Scientific Inc., Waltham, MA). Antibiotics were used for selection of plasmids as follows: ampicillin 100 µg/ml (*E. coli*); kanamycin, 25 µg/ml (*R. rubrum*) or 50 µg/ml (*E. coli*). IPTG and X-gal were used at concentrations of 20 and 40 mg/ml, respectively.

2.2. Gas chromatographic analysis of PHA

Liquid cultures were collected by centrifugation at 6000×g for 10 min and cells were washed once with 10 mM Tris-HCl buffer (pH 7.5) and lyophilized overnight. PHA content and compositions were determined as described by Brandel et al. (Brandl et al., 1988). Hexanedioic acid was added as an internal control. The methyl esters were assayed by
GC-MS with an Agilent 6890 GC equipped with a DB-WAX column (30m×0.25 mm ID, 0.5 µm) interfaced to a 5973 mass spectrometer and an electron impact ionization detector (Agilent Technologies, Santa Clara, CA). The GC/MS data files were deconvoluted by NIST AMDIS software. The PHA content was calculated as the percent of cell dry weight. 3-hydroxybutyric acid was used to construct standard curve. Data were analyzed using SAS software and regression equations were used to determine the PHA content curves that were analyzed using two-way ANOVA or T-test. P-value less than 0.05 was used to evaluate the statistical significance difference.

2.3. DNA isolation and manipulation

Genomic DNA was isolated from *R. rubrum* as described by Kerby et al. (Kerby et al., 1992). Plasmids were isolated from *E. coli* cells grown in LB medium by using QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Agarose gel electrophoresis and transformation of *E. coli* were carried out as described by Sambrook and Russell (Sambrook and Russell, 2001). PCR products were cloned into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA).

2.4. Construction of plasmids

The plasmids for expressing protein in *E. coli* BL21-AI were constructed as recommended by Invitrogen Gateway technology. The plasmids pUX19-PT-pha genes and pUX19-PT-GUS were constructed as follows. Using PCR, *XbaI* and *NdeI* sites were first introduced into the upstream and downstream regions of the promoter of operon *CooFSCTJ*. The PCR product was purified and ligated into the TA cloning vector, pPCR2.1. The resulting vector was digested with *NdeI* and *SacI* and the promoter fragment was purified and cloned into pUX19 (Zhang et al., 2001) digested with *XbaI* and *NdeI*, forming vector
pUX19-P1. The terminator of CooFSCTJ operon, the PHA biosynthetic genes and the GUS gene were prepared and ligated sequentially into the vector pUX19-P1 with the same method, forming vectors pUX19-PT-(pha genes). The primers for amplifying fragments from *R. rubrum* genomic DNA are listed in Table 2.

2.5. Conjugation

The pUX19-PT-(pha genes) plasmids were mobilized from *E. coli* 17-1 into *R. rubrum* by conjugation conducted as described by Jihong Liang (Liang et al., 1991). *R. rubrum* conjugants were selected on kanamycin containing MN medium. Single colony of *R. rubrum* conjugant was isolated by repeated transfer on kanamycin containing MN medium. Vector integration sites were identified by PCR.

2.6. GUS activity assay

GUS strain cells were collected and resuspended in GUS assay buffer (1 mM EDTA, 50 mM NaHPO₄-Na₂PO₄, 0.1% Triton X-100, Ph 7.0) and were disrupted ultrasonically. GUS activity was determined as described by Sanjukta Aich etc. (Aich et al., 2001)

2.7. Protein purification and Preparation of antibodies

Recombinant His-tag or GST-tag fusion proteins were affinity-purified via their tags from *E. coli* BL21-AI strains harboring pDEST15-(pha-gene) or pDEST17-(pha-gene), grown in the presence of 0.2% L-arabinose. Recovered proteins further purified via SDS-PAGE. The recombinant protein bands was excised from gels, crushed in PBS solution and used to immunize mice to generate antibodies against each protein.

2.8. SDS-PAGE and Western immunoblot analysis

Proteins were separated by SDS-PAGE in 12.5% polyacrylamide gels. Proteins were electrophoretically transferred to a nitrocellulose membrane (Sambrook et al.)
and Russell, 2001). Gels were loaded with aliquots containing of equal amounts of protein; protein concentrations were determined using the Bio-Rad Dc Protein Assay Kit, using BSA to generate the standard curve. PHA biosynthetic proteins were immunologically detected with a combination of primary mice antibodies directed against the PHA proteins, and secondary HRP-conjugated anti-mouse IgG antibody (Bio-Rad). The blot was developed with Amersham (Little Chalfont, Buckinghamshire, UK or Piscataway, NJ) ECL western blot detection reagents. For quantitative analysis, an Epson Perfection 2400 scanner (Shiojiri-shi, Nagano-ken, Japan) was used to scan the x-ray film. The signal intensities were quantified by ImageJ software.

RESULTS

3.1. GUS activity measurement to test the efficacy of the CO inducible system

Previous studies have shown the presence of three PHA polymerases PhaC1 (Jin), PhaC2 (Hustede et al., 1992) and PhaC3 (Reiser et al., 2000) and one (R)-specific 2-enoyl-CoA hydratase (PhaJ) (Clemente et al., 2000) in *R. rubrum* (Table 3). PhaJ converts trans-2, 3-enoylacyl-CoA to the substrate (R)-3-hydroxyacyl-CoA of PhaC (Madison and Huisman, 1999). The sequence identities of PhaC1 to PhaC2, PhaC1 to PhaC3 and PhaC2 to PhaC3 are 14.3, 18.4 and 50.2%, respectively (Jin). In addition, phaC1 is in an operon form (*phaC1-phaA-phaB*) (Jin). The products of this operon *phaC1-phaA-phaB* sequentially catalyze condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA and polymerization of 3-hydroxybutyryl-CoA (Madison and Huisman, 1999). In order to
investigate the effect of over-expressing these PHA biosynthetic genes on the PHA production, we have over-expressed, individually and in combination, these genes by fusing them individually to the *R. rubrum* native promoter of the *cooFSCTJ* operon, which is a strong, carbon monoxide inducible promoter (He et al., 1999). We also generated the strain with empty vector (V-15) as the control strain. In addition, we constructed the P<sub>cooF</sub>:GUS vector and generated the strain to test the efficacy of the carbon monoxide inducible system. The result of strain GUS-13 showed that GUS activity was increased by four hundred times after 24 hours carbon monoxide induction. Based on the GUS experiment, it indicated that the over-expression system worked well.

3.2. Metabolic engineering of *R. rubrum* through over-expressing PHA biosynthetic genes

The recombinant *phaA, phaB, phaC1, phaC2, phaC3* and *phaJ* were constantly highly expressed under the control of *CooFSCTJ* promoter in the presence of carbon monoxide. When the pUX19-CooF-pha-gene vector was introduced into *R. rubrum*, two types of recombination occurred: recombination at *CooF* promoter site and at PHA structural gene site. Take the vector pUX19-CooF-phaC1 for example, when recombination occurred in *CooF* promoter, only *phaC1* was under control of *CooF* promoter and only *phaC* can be highly induced by carbon monoxide and when recombination occurred in *phaC*, the *phaC* and the following PHA structural genes *phaA* and *phaB* were under control of *CooF* promoter and thus can be induced by carbon monoxide since they are in one operon and in an order of *phaC1-phaA-phaB*. Based on this strategy, ten different strains have been constructed (including empty vector control and GUS positive control) (Table 4.) The strains were cultivated in nitrogen-limiting RRNCO medium in presence of carbon monoxide. Growth curve of each strain was measured in the time course experiments (Fig. 1) and the
ability to accumulate PHA of each strain was determined at the last six time points (Fig. 2).

The results showed the highest PHA content of each strain was obtained after 66-hour-growth. The monomer composition of PHA did not significantly change (0.3%-1% 3-hydroxyvalerate) when over-expressing PHA biosynthetic genes as compared to the control strain. At the same time, western blot experiments were conducted to study the expression levels of all PHA biosynthetic genes (Fig. 2).

3.3 The effect of over-expressing phaCs and phaJ on PHA production

Three strains C1-13, C2-2 and C3-4 that over-expressed phaC1, phaC2 and phaC3, respectively, were investigated regarding PHA content. Strains C1-13 and C2-2 accumulated significantly more PHA than the control strain (Fig. 1A and 1B). However, over-expressing phaC3 had little effect on PHA production. Western blot experiments showed that the expression levels of phaC1 of strain C1-13 and phaC3 of strain C3-4 were at least 50 times higher than that of the control strain. The protein level of phaC2 of strain C2-2 was 25 times higher than that of the control strain. However, over-expressing phaC2 resulted in more significantly increasing in PHA production than over-expressing the other two phaCs. In addition, over-expressing phaJ did not significantly affect PHA production (Fig. 1C and 1D).

3.4 The effect of over-expressing the operon gene phaC1-phaA-phaB individually or in combination on PHA production

The effect of over-expressing of various combinations of phaC1AB genes on PHA content was investigated using strains A-11 (over-expressing phaA), B-1 (over-expressing phaB), AB-9 (over-expressing phaA and phaB) and C1AB12 (over-expressing phaA, phaB and phaC1). Strain A-11 and B-1 accumulated significantly more PHA than that of the control strain, whereas strain B-1 produced significantly more PHA than did strain A-11.
Western blot results showed that the expression level of \textit{phaA} increased by 5 times in strain A-11 and that of \textit{phaB} increased by 50 times in strain B-1 as compared to that of control strain by quantifying protein expression levels. However, strain AB-9 over-expressing \textit{phaA} and \textit{phaB} together accumulated similar PHA content to strain B-1 although the expression level of \textit{phaA} of AB-9 is five times higher than that of strain B-1. Furthermore, though the whole operon \textit{phaC1AB} of strain C1AB-12 was induced, the PHA content of strain C1AB-12 was significantly lower than that of strain AB-9. The expression level of \textit{phaC1} of strain C1AB-12 is at least 50 times higher than that of strain AB-9. However, the expression level of \textit{phaB} of strain C1AB-12 was 7 times lower than that of strain AB-9. These results indicated that the expression level of \textit{phaB} controlled PHA production and that PhaB was the key enzyme of the operon PHA biosynthetic pathway under these growth conditions. \textit{phaB} expression level of strain AB-9 was 7 times higher than that of strain C1AB-12 in spite of them being under control of the same promoter and the expression levels of \textit{phaA} were similar. In addition, over-expressing \textit{phaC1} alone did not affect \textit{phaB} expression, whereas, over-expressing \textit{phaC1} in the whole operon form decreased the expression level of \textit{phaB}. These results suggested that \textit{phaB} was subject to post transcriptional regulation when transcribed in the whole operon form.

3.5 The correlation of cell density and PHA production of each over-expressing strain

Interestingly, the results showed that the cell densities of the strains were positively correlated with PHA content (Fig. 3.). Strain AB-9 accumulated highest PHA content and reached 28.98% of cellular dry weight (CDW) at 66-hour-time point. Correspondingly, strain AB-9 reached the highest cell density among all strains. Strain B-1 and C2-2 accumulated lower PHA content than strain AB-9 and grew slower and reached lower O.D. than that of
AB-9, and so on. The data implied that PHA accumulation contributed to *R. rubrum* growth under nitrogen-limiting RRNCO medium.

**DISCUSSION**

Elucidating the mechanisms affecting PHA content when grown on syngas as carbon source would facilitate the industrial use of *R. rubrum*. In this study for the first time, we evaluated the effect of over-expressing PHA biosynthetic genes of *R. rubrum* on increasing PHA production and investigated the regulatory mechanism of PHA biosynthesis when grown on nitrogen-limiting RRNCO medium, which is a simulating synthesis gas medium. Our results showed that over-expressing *R. rubrum* strains can increase PHA accumulation from about 15% cellular dry weight (CDW), which was composed of 99.4% 3-hydroxybutyrate and 0.6% 3-hydroxyvalerate. This study contributed to evaluation of the potential of *R. rubrum* for PHA production from syngas.

Over-expressing *phaJ* did not significantly affect PHA production. This result is consistent with previous studies in *Aeromonas hydrophila* and *Aeromonas caviae* (Fukui et al., 2001; Han et al., 2004). The step catalyzed by PhaJ is not considered a rate-limiting step in PHA synthesis. A previous study has showed that over-expression of *phaC1* did not significantly increase PHA production under nitrogen limitation conditions in *Pseudomonas oleovorans* (Kraak et al., 1997), whereas another two studies indicated that over-expression of *phaC* increased PHA production in *Aeromonas hydrophila* and *Aeromonas caviae* (Fukui et al., 2001; Han et al., 2004). Our data clearly showed that over-expressing *phaC2* resulted in increasing PHA production the most, though over-expressing *phaC1* also increased PHA
production. In addition, over-expressing \textit{phaC3} had little effect on PHA production. It is likely that PhaC2 had the highest activity and different PhaCs are probably responsible for utilizing different carbon sources. Among the six genes, \textit{phaB} had the most significant effect on PHA content and our result is consistent with a study that showed PhaB was the key enzyme controlling the PHA amount in \textit{Alcaigenes eutrophus} (Doi et al., 1992). Alternatively, other studies showed that β-ketothiolase was the key enzyme of PHA biosynthesis in \textit{Alcaigenes eutrophus} and \textit{Azotobacter beijerinckii} by inhibiting effects of intermediates of the PHA pathway (Senior and Dawes, 1971; Oeding and Schlegel, 1973). In addition, our data implied that the expression level of \textit{phaB} controlled PHA content and \textit{phaB} was subject to post transcriptional regulation when transcribed in the whole operon form. These mechanisms may provide a fine regulation of PhaB level to control PHA production and help \textit{R. rubrum} to be more flexible adaptation to environmental change. However, the mechanism of regulation still needs further investigation.

Our results clearly showed that the capacity of PHA enhancing \textit{R. rubrum} growth was proportional to the PHA content when these strains which grown in nitrogen-limiting RRNCO medium (Fig. 3). This result is consistent with previous study that has shown that PHAs enhance resistance to stresses (radiation, desiccation and osmotic pressure) (Tal and Okon, 1985). A study has indicated that PHA enhances resistance to stresses by PHA degradation that results in an increasing of the major regulator, guanosine-tetraphosphate (ppGpp) of stress in \textit{Pseudomonas oleovorans} (Lo´pez, 1995).
ACKNOWLEDGMENTS

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REFERENCES


Jin, H.a.N., B. J. Characterization of Three PHA Polymerases and One (R)-specific 2-enoyl-CoA Hydratase of Rhodospirillum rubrum ATCC11170. manuscript.


### TABLES AND FIGURES

#### Table 1. Bacterial strains, plasmids.

<table>
<thead>
<tr>
<th>Strains, plasmid or oligonucleotides</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td><strong>strains</strong></td>
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<td><em>R. rubrum</em> ATCC 11170</td>
<td>Source of PHA</td>
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<tr>
<td><em>E. coli</em> DH5α</td>
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#### Table 2. Oligonucleotides used in this study

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<td>PUS-Xbal</td>
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<td>PDS-NdeI</td>
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<td>CooF_SCTJ terminator</td>
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<td>TDS-XhoI</td>
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<td>phaJUS-NdeI</td>
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<td>phaJDSS-sacI</td>
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<td>phaC3DS-XhoI</td>
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*the italized sequences indicate the restriction sites.*
Table 3. PHA biosynthetic genes

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<th>Gene symbol</th>
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<tr>
<td>3-ketothiolase</td>
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<td>phaC polymerase</td>
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<td>phaC2</td>
<td>Rru_A2413</td>
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<td>phaC-like polymerase 2</td>
<td>PhaC3</td>
<td>Rru_A1816</td>
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<td>(R)-specific trans-2,3-enoylacyl-CoA hydratase</td>
<td>phaJ</td>
<td>Rru_A2964</td>
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Table 4. Different recombinant R. rubrum strains

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<th>R. rubrum strains</th>
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<tr>
<td></td>
<td></td>
<td>cooFSCTJ</td>
</tr>
<tr>
<td>phaC</td>
<td>CAB-12 (the whole operon is induced)</td>
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</tr>
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<td></td>
<td>C-13 (only the phaC is induced)</td>
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<tr>
<td>phaA</td>
<td>A-9 (phaA and phaB are induced)</td>
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<td></td>
<td>AB-11 (only the phaA is induced)</td>
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<td>phaB</td>
<td>B-1 (only the phaB is induced)</td>
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<td>Vector control</td>
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Fig. 1. Growth curves of each strain and PHA production of each strain at last six time points. Data represent averages from triplicate biological samples. AB-9 (+), C3-4 (*), C1AB-12 (■), C2-2 (◇), A-11 (△), J-10 (◆), Control (×), B-1 (▲), C1-13 (□). The range of error was within ±5% of each value.
Fig. 1. (continued)

**E**

- **Y-axis**: O.D. (680 nm)
- **X-axis**: Time (h)

**F**

- **Y-axis**: PHA (% CDW)
- **X-axis**: Time (h)
Fig 2. Protein expression levels of each strain at last six time points.

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<th>Strains</th>
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</table>

$R^2 = 0.942$

Fig 3. The correlation of PHA content and cell density at the 66-hour-time point. AB-9 (+), C3-4 (*), C1AB-12 (■), C2-2 (◇), A-11 (△), J-10 (◆), Control (×), B-1 (▲), C1-13 (□).
CHAPTER III. CHARACTERIZATION OF THREE PHA POLYMERASES AND ONE (R)-SPECIFIC 2-ENOYL-COA HYDRATASE OF RHODOSPIRILLUM RUBRUM ATCC11170

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ABSTRACT

PHA polymerases (PhaCs) synthesize polyhydroxyalkanoates polymer from PHA precursor (R)-3-hydroxyacyl-CoA and (R)-specific 2-enoyl-CoA hydratase (PhaJ) converts the intermediate of β–oxidation, trans-2,3-enoylacyl-CoA, into PHA precursor (R)-3-hydroxyacyl-CoA. Three PHA polymerases and one (R)-specific 2-enoyl-CoA hydratase were identified in R. rubrum. Here we report their characterization of each gene in vivo. The function of each phaC and phaJ was examined by generating R. rubrum deletion mutants (ΔphaC1, ΔphaC2, ΔphaC3, ΔphaJ, ΔphaC1ΔphaC2, ΔphaC1ΔphaC3, ΔphaC2ΔphaC3, ΔphaC1ΔphaC2ΔphaC3). PHA production and composition of these mutants were analyzed when grown on acetate or hexanoate as a sole carbon sources, respectively. The growth experiments of mutants show that PHA is required for R. rubrum optimal growth when grown on acetate as carbon source and that PHA enhances bacteria growth in the stationary phase when grown on hexanoate as carbon source. The genetic and chemical analyses reveal that PhaC2 is the major enzymes for synthesize PHA in vivo when
grown in either carbon source and responsible for integrating 3-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxyhexanoate monomers into polymer and that PhaC1 encoded by phaCAB operon locus and PhaC3 are specific for integrating 3HB into polymer. In addition, non-additive contribution of PhaC1 and PhaC3 to PHA content indicates that they interact with each other. Absence of 3HHx monomer in PHA of ∆phaJ mutant demonstrates that PhaJ is responsible for converting trans-2, 3-enoylacyl-CoA into (R)-3-hydroxyacyl-CoA in vivo. Furthermore, the data indicate that presence of 3HHx monomer in PHA results in continuous accumulation of PHA in R. rubrum. In addition, deletion of phaJ caused a severe effect on R. rubrum growth when grown on hexanoate as carbon source.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyester polymers, which are deposited within cells mainly in the form of protein- and lipid-bound granular inclusions that many microbes use as a means of storing carbon and energy (Steinbüchel et al., 1995; Madison and Huisman, 1999; Kessler and Witholt, 2001; Steinbüchel. and Hein., 2001). Additional functionalities that have been ascribed to PHAs include the transport of calcium phosphate and DNA across membranes, calcium signaling (Reusch et al., 1986; Reusch et al., 1987; Reusch and Sadoff, 1988); and resistance to stresses, such as radiation, desiccation and osmotic pressure (Tal and Okon, 1985).

PHAs have technological applications as biodegradable plastics (Anderson and Dawes, 1990). Moreover, because these applications are dependent on the chemo-physical properties
of the PHA polymer, which are themselves dependent on the monomeric constituents and their relative order in the polymer, intensive studies have been carried out to understand the mechanisms of PHA biosynthesis (Madison and Huisman, 1999; Kessler and Witholt, 2001; Steinbüchel and Hein., 2001). These studies have established that PHA biosynthesis and accumulation is enhanced when microbes are in excess-carbon source but limited for other nutrient(s), such as phosphorus, nitrogen, or sulfur (Steinbüchel et al., 1995; Madison and Huisman, 1999; Kessler and Witholt, 2001; Steinbüchel and Hein., 2001). In addition, the type of PHA polymer that is produced depends on the diversity of substrates that are availability to the PHA polymerase (product of the \( \text{phaC} \) gene) that assembles the polymer (Fig. 1) (Matsusaki et al., 1998; Antonio et al., 2000).

The substrate for all known PhaC enzymes is \((R)\)-configured 3-hydroxyacyl-CoA of different acyl-chain lengths (from 4 to 14 carbons; Fig. 2). There are several metabolic pathways have been identified for generating a diverse pool of such substrates. One pathway is encoded by the \( \text{phaABC} \) operon that involves the condensation of two acetyl-CoA molecules to form an acetoacetyl-CoA, which is subsequently reduced to 3-hydroxybutyryl-CoA. In this pathway, 3-ketothiolase (the product of the \( \text{phaA} \) gene) and acetoacetyl-CoA reductase (the product of the \( \text{phaB} \) gene) catalyze the first and second reaction, respectively. Another pathway that produces these precursors consists of three enzymes: NADH-dependent acetoacetyl-CoA reductase, crotonase and \((R)\)-specific 2-enoyl-CoA hydratase (PhaJ), which sequentially catalyzing acetoacetyl-CoA (Reiser et al., 2000). Another set of reactions that can generate precursors of PHA biosynthesis uses intermediates of fatty acid \( \beta \)-oxidation as substrates: (I) \((S)\)-3-hydroxyacyl-CoA can be converted to \((R)\)-3-hydroxyacyl-CoA by an epimerase; (II) 3-ketoacyl-CoA can be
reduced to (R)-3-hydroxyacyl-CoA by a reductase; and (III) trans-2,3-enoylacyl-CoA can be hydrated to (R)-3-hydroxyacyl-CoA by phaJ (Madison and Huisman, 1999). Finally, a recently discovered pathway is the metabolic link between fatty acid de novo biosynthesis with biosynthesis of PHA, catalyzed by phaG (a 3-hydroxy-decanoyl-[acyl-carrier-protein]: CoA transacylase) (Rehm and Steinbuchel, 1998).

Most bacteria produce PHAs that are composed of monomers that are either of short chain length (C3-C5) or medium chain length (C6-C14) (Anderson and Dawes, 1990; Baek Y.K., 2001; Steinbüchel. and Hein., 2001), although several have been found to produce PHAs with a broader range of monomers (Brandl et al., 1989; Haywood et al., 1991; Lu et al., 2004). The purple and phototrophic bacterium *Rhodospirillum rubrum* is known to produce PHA containing both short and medium chain length monomers (Brandl et al., 1989; Liebergesell et al., 1991; Herbert W. Ulmer, 1994). This metabolically versatile bacterium can grow under aerobic or anaerobic conditions, in the presence, or absence of light but with an organic source of carbon-substrate (Truper and Pfenning, 1978; Tabita, 1995). In addition, *R. rubrum* can accumulate up to about 50% dry weight of PHA (Brandl et al., 1989). Previous studies and genome sequencing have revealed that the presence of three PHA polymerases PhaC1, PhaC2 (Hustede et al., 1992) and PhaC3 (Reiser et al., 2000) and one PhaJ (Clemente et al., 2000) in *R. rubrum* (table 3). Because of the metabolic flexibility of *R. rubrum*, it offers potential to be used in the conversion of many different carbon-sources to PHA and therefore there are considerable industrial interests in exploring this potential (Do et al., 2007; Smith et al., 2008). In spite of the fact that several studies of PHA metabolism and PHA diversity in *R. rubrum* were conducted when grown different carbon sources (Brandl et al., 1989; Handrick et al., 2004a; Handrick et al., 2004b), no investigation was
performed to study the PHA production and diversity contributed by each PHA polymerase PhaC and PhaJ in *R. rubrum*. In this work, we characterized the function of each PhaC and PhaJ *in vivo* and studied the effect of each PhaC and PhaJ on PHA production and diversity under nitrogen-limiting mineral salt medium anaerobically. In addition, we also examined PHA functions in *R. rubrum*.

**MATERIALS AND METHODS**

**Chemical and enzymes.** All chemicals were obtained from Sigma-Aldrich Corporation (St. Louis, MO) and Fisher Scientific Inc. (Pittsburgh, PA). All DNA-manipulating enzymes were obtained from Invitrogen Corporation (Carlsbad, CA).

**Bacterial strains, plasmids.** The strains of *Rhodosprillum rubrum* and *Escherichia coli*, and the plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table 2.

**Bacteria growth conditions.** *E. coli* was grown at 37°C in LB medium. *R. rubrum* was grown under 2000 Lux light intensity at 25°C in SMN medium (supplemented malate-ammonium medium; rich medium for *R. rubrum*) (Kerby et al., 1992). To assess PHA production, a 0.2 ml aliquot of a normalized SMN culture (5 O.D.) was collected by centrifugation at 13,000×g for 2 min; the cells were washed once with RRNCO medium (ammonium chloride, hydrogen sulfide and carbon dioxide were omitted) (Kerby et al., 1995) and resuspended with 0.2 ml RRNCO medium and transferred to 20 ml RRNCO medium (using either 10 mM acetate or 5 mM hexanoate as the carbon source). The culture was shaken at 150 rpm at 25°C under 5000 Lux light intensity. The cultures were grown
anaerobically with an argon head space in 18×150 mm anaerobic tubes (Bellco Biotechnology, Vineland, NJ). Aliquots of 4-ml were withdrawn from the cultures at 0, 72, 96, 120, 192-hours post-inoculation for biochemical analyses. Cell density was determined by monitoring $A_{680}$ using a Spectronic 20D+ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). When needed, gentamicin (25 µg/ml) or kanamycin (25 µg/ml) was added to media to maintain selection for plasmids. IPTG and X-gal were used at concentrations of 20 and 40 mg/ml, respectively.

**GC analysis of PHA.** Cells were collected from liquid cultures by centrifugation at 6000×g for 10 min, and following washing with 10 mM Tris-HCl buffer (pH 7.5) the cell-pellets were lyophilized, and stored at -70°C until analysis. PHA content and composition was determined as described by Brandel et al. (Brandl et al., 1988). Hexanedioic acid was added as an internal standard. Derivatized samples were concentrated under a stream of nitrogen gas when necessary. The methyl esters were assayed by GC-MS with an Agilent 6890 GC equipped with DB-WAX column (30m×0.25 mm ID, 0.5 µm) interfaced to a 5973 mass spectrometer and an electron impact ionization detector (Agilent Technologies, Santa Clara, CA). In the GC, helium (1.2 ml/min) was used as the carrier gas, and the temperature gradient was programmed from 80 to 200°C at 10 °C/min. In the MS, the operating parameters were set to 70 eV for the ionization voltage and the interface temperature was at 280°C of. The temperatures of the injector and detector were 250°C and 240°C, respectively. The GC/MS data files were de-convoluted with the NIST AMDIS software, and searched against the NIST compounds library. The PHA content was calculated as the percent of cell dry weight. 3-hydroxybutyric acid was used to construct standard curve.
**Statistical analysis.** Data were analyzed using SAS software and regression equations were used to determine the PHA content curves and growth curves that were analyzed using two-way ANOVA or T-test. P-value less than 0.05 was used to evaluate the statistical significance difference.

**DNA isolation and manipulation.** Genomic DNA was isolated from *R. rubrum* as described by Kerby et al. (Kerby et al., 1992). Plasmids were isolated from *E. coli* cells grown in LB medium by using QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Agarose gel electrophoresis and transformation of *E. coli* were carried out as described by Sambrook and Russell (Sambrook and Russell, 2001). PCR products were cloned into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA).

**Construction of gene-deletion-plasmids.** The 1018-bp DNA fragment upstream of the *phaC1* ORF was amplified by PCR with the primers mC1USf-SacI and mC1USR-XbaI, and this amplification introduced SacI and XbaI sites at the 5’ and 3’ ends of the product. The PCR product was purified and ligated into the TA-cloning vector, pPCR2.1-TOPO. The resulting vector was digested with SacI and XbaI and the fragment was purified and cloned into pJQ200SK, digested with SacI and XbaI, forming the vector pJQ200SKmC1U. The 1023-bp DNA fragment downstream of the *phaC1* ORF was cloned into the vector pJQ200SKmC1U via the same strategy, forming the ΔphaC1 gene-deletion-vector, pJQ200SKmC1UD. The same procedure was used to construct vectors pJQ200SKmC2UD, pJQ200SKmC3UD and pJQ200SKmJUD, which were used to create gene-deletion alleles for *phaC2*, *phaC3*, and *phaJ* genes. The primers used for amplifying *R. rubrum* genomic DNA fragments for these latter vectors are listed in Table 2.
Conjugation. The three phaC and phaJ gene-deletion-plasmids were mobilized from E. coli strain 17-1 into R. rubrum by conjugation (Liang et al., 1991). R. rubrum conjugants were selected on gentamicin-containing MN medium (MN medium is SMN medium in which yeast extract and casein enzyme hydrolysate are omitted). Single-colony R. rubrum conjugants were isolated by repeated transfer on gentamicin-containing MN medium.

Construction of phaCs and phaJ deletion mutants of R. rubrum

The deletion strains of R. rubrum (ΔphaC1, ΔphaC2, ΔphaC3, ΔphaJ, ΔphaC1ΔphaC2, ΔphaC1ΔphaC3, ΔphaC2ΔphaC3, ΔphaC1ΔphaC2ΔphaC3) were generated by homologous recombination via a two-step procedure using the suicide vector pJQ200SK(Quandt and Hynes, 1993). The molecular confirmation of each deletion allele was achieved by PCR amplifying each allele with flanking primers (Table 2) and determining its nucleotide sequence. Primers imC1f and imC1r were used to confirm the ΔphaC1 allele; primers imC2f and imC2r were used to confirm the ΔphaC2 allele; primers imC3f and imC3r were used to confirm the ΔphaC3 allele; and primers imJf and imJr were used to confirm the ΔphaJ allele.

RESULTS

Previous studies have shown the presence of two PHA polymerases PhaC2 (Hustede et al., 1992) and PhaC3 (Reiser et al., 2000) and one PhaJ (Clemente et al., 2000) in R. rubrum. We blasted R. rubrum genome with phaC2 and phaC3 sequences and found that there are three phaCs in R. rubrum genome except phaC2 and phaC3 (table 3). The other one (phaC1) is in an operon form (phaC1-phaA-phaB). Figure 3 shows the alignment of the deduced amino acids sequences of PhaC1, PhaC2 and PhaC3 using Clustal W. The sequence
identities of PhaC1 to PhaC2, PhaC1 to PhaC3 and PhaC2 to PhaC3 are 14.3, 18.4 and 50.2%, respectively. To investigate the functions of these phaCs and phaJ, single deletion mutants ($\Delta$phaC1, $\Delta$phaC2, $\Delta$phaC3, $\Delta$phaJ), double deletion mutants ($\Delta$phaC1$\Delta$phaC2, $\Delta$phaC1$\Delta$phaC3, $\Delta$phaC2$\Delta$phaC3) and triple deletion mutant ($\Delta$phaC1$\Delta$phaC2$\Delta$phaC3) were generated as described in Materials and Methods. PhaJ of R. rubrum shows activities towards less than C$_8$ trans-2, 3-enoylacyl-CoA in vitro (Reiser et al., 2000). Our results show that WT R. rubrum accumulated less than 1% PHA and 14.6% of cell dry weight (CDW) on heptanoate and hexanoate as carbon source after 96-hour cultivation, respectively. More than 5mM hexanoate in medium resulted in inhibition of growth (data not shown). In addition, R. rubrum is more preferential to accumulate PHA using short- and medium-chain fatty acids as carbon sources than using sugars and succinate, etc., as carbon sources (Stanier et al., 1959; Brandl et al., 1989). Furthermore, Brandl et al. have shown that R. rubrum produces 3HB and 3HV copolymer and 3HB, 3HV and 3HHx copolymer using acetate and hexanoate as carbon sources, respectively (Brandl et al., 1989), and so using these two carbon sources enable us to elucidate the functions of each phaCs and phaJ. The effects of phaCs and phaJ on PHA production and bacteria growth were examined in nitrogen-limiting mineral salt medium containing acetate or hexanoate as a sole carbon sources for over 8 days anaerobically, respectively. The results from the experiments are shown in Fig. 4A-H, 5A-H and Table 4.

**Effect of single phaC deletion on PHA production and composition.** WT, $\Delta$phaC1 and $\Delta$phaC3 mutants reached the maximum PHA production after 72-hour growth using acetate as carbon source (Fig. 4A). The PHA content of $\Delta$phaC1 and $\Delta$phaC3 mutants were similar to that of WT. Interestingly, the strains WT and $\Delta$phaC1 or $\Delta$phaC3 mutants continuously accumulated PHA in the stationary phase when grown on hexanoate.
Furthermore, the $\Delta$phaC1 and $\Delta$phaC3 mutants significantly accumulated more PHA than wild type, PHA content increasing by 36% and 25% significantly after 192-hour growth, respectively. The PHA content of the $\Delta$phaC2 mutant reached the maximum production after 96-hour cultivation when grown on either carbon source. $\Delta$phaC2 mutant accumulated only 2.99% PHA of CDW, 22.1% of wild type PHA content (13.52% of CDW) and accumulated 2.53% PHA of CDW, 19% of wild type PHA content (13.2% of CDW) after 96-hour growth when grown on acetate and hexanoate as carbon source, respectively. The data show that PhaC2 is the major enzymes for synthesizing PHA in vivo when grown in either carbon source.

Simultaneously, the effect on PHA composition of these mutants was determined under the same growth conditions. The results from the experiments are shown in Table 4. There were no data showing significant differences of monomer composition of the PHA during different time points for each strain (data not shown). The composition ratio of 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) monomers of the $\Delta$phaC2 mutant was significantly decreased (less than 0.1% when grown in acetate or hexanoate as a sole carbon source), in contrast to WT (0.38%, 4.87% grown in acetate or hexanoate, respectively). Furthermore, The 3HV and 3HHx monomer ratio of the $\Delta$phaC1 and $\Delta$phaC3 mutants significantly increased when grown in hexanoate as carbon source (the ratio of 3HV and 3HHx monomers 6.68% and 6.8%, respectively), compared to WT (4.87%). In order to show if the ratio decrease of 3HV and 3HHx monomers in $\Delta$phaC2 mutants was caused by detection limit because $\Delta$phaC2 mutant contained less PHA amount, 20%-25% of WT PHA content after 96-hour growth, we concentrated $\Delta$phaC2 mutant samples 5 times and both medium chain monomers were still not detectable (both monomers ratio less than 0.1%).
another experiment, we analyzed the cells (9.6 mg and 10.0 mg) from 20 ml 96-hour cultures for each WT and \( \Delta \text{phaC2} \) mutant, respectively. WT contained 4.3% 3HV and 3HHx monomers and no 3HV and 3HHx monomers were detected in \( \Delta \text{phaC2} \) mutant. So ratio change of 3HV and 3HHx monomers in \( \Delta \text{phaC2} \) mutants was not caused by detection limit. The data indicate that PhaC2 is responsible for integrating 3HB, 3HV and 3HHx monomers into polymer and PhaC1 and PhaC3 are specific for integrating 3HB monomer into polymer.

**Effect of double and triple phaC deletion on PHA production.** No PHA was detected in the \( \text{phaC1}\Delta \text{phaC2} \) double mutant and \( \Delta \text{phaC1}\Delta \text{phaC2}\Delta \text{phaC3} \) triple mutant when grown on either carbon source (Fig. 4B-C). The \( \Delta \text{phaC2}\Delta \text{phaC3} \) mutants reached the maximum production of PHA content after 120-hour cultivation and accumulated only 1.00% and 0.68% PHA of CDW when grown on acetate and hexanoate as carbon source, respectively. The data also show that the contribution of PhaC1 and PhaC3 to PHA content was not additive (The PHA content of the mutant \( \Delta \text{phaC2} \), \( \Delta \text{phaC1}\Delta \text{phaC2} \) and \( \Delta \text{phaC2}\Delta \text{phaC3} \) were 2.99%, 0%, 1.00%, respectively, when using acetate as carbon source and respectively and 1.75%, 0%, 0.68%, respectively, when using hexanoate as carbon source after 120-hour growth.). These results reveal that PhaC1 and PhaC3 interact with each other. WT and \( \Delta \text{phaC1}\Delta \text{phaC3} \) gained the maximum PHA production after 72-hour growth using acetate as carbon source. However, PHA content of the\( \Delta \text{phaC1}\Delta \text{phaC3} \) double mutant was 25% significantly higher than that of wild type after 72-hour growth. In addition, the consumption of PHA was much faster in WT than that of \( \Delta \text{phaC1}\Delta \text{phaC3} \) mutants during the stationary phase when grown on acetate. The strains WT and \( \Delta \text{phaC1}\Delta \text{phaC3} \) continuously accumulated PHA in the stationary phase when grown on hexanoate. Furthermore, \( \Delta \text{phaC1}\Delta \text{phaC3} \) mutant accumulated more PHA than wild type, PHA content increasing by
34% significantly after 192-hour growth, though the double mutant \( \Delta \text{phaC1} \Delta \text{phaC3} \) contained less PHA than WT after 72-hour growth using hexanoate as carbon source. The data show that PhaC1 and PhaC3 negatively affect PhaC2 when grown in either carbon source.

**Effect of PHA content on growth.** Interestingly, the growth curves of these mutants were closely related to PHA content they contained (Fig. 6A and 6B). The PHA content of \( \Delta \text{phaC1}, \Delta \text{phaC3} \) and \( \Delta \text{phaC1} \Delta \text{phaC3} \) mutants were similar to or higher than that of WT and the growth curves of all these mutants were similar to that of WT when grown on either carbon source (Fig. 4A-C, 4E-G and 5A-C, 5E-G). The final O.D.s and growth speeds of the \( \Delta \text{phaC2} \) background mutants were proportional to PHA content that they contained since they accumulated much less PHA than WT when grown on acetate. The \( \Delta \text{phaC2} \) mutant that accumulated lower PHA content than WT grew slower and reached lower final O.D. than that of WT. The \( \Delta \text{phaC1} \Delta \text{phaC2} \) mutant that accumulated further lower PHA content grew further slower and had further lower final O.D.. The \( \Delta \text{phaC1} \Delta \text{phaC2} \) mutant and \( \Delta \text{phaC1} \Delta \text{phaC2} \Delta \text{phaC3} \) triple mutant of which no PHA was detected grew slowest and had lowest final O.D.. These \( \Delta \text{phaC2} \) background mutants had lower cell densities than those of WT and the other mutants in the stationary phase and the final O.D. of these strains were proportional to PHA content they contained using hexanoate as carbon source. The final cell density of \( \Delta \text{phaC2} \) was lower than that of WT and the cell densities of \( \Delta \text{phaC1} \Delta \text{phaC2}, \Delta \text{phaC2} \Delta \text{phaC3}, \Delta \text{phaC1} \Delta \text{phaC2} \Delta \text{phaC3} \) were further lower than that of WT after 240-hour growth. These results reveal that PHA is required for *R. rubrum* optimal growth when grown on acetate as carbon source and that PHA enhances bacteria growth in the stationary phase when grown on hexanoate as carbon source.
Effect of *phaJ* deletion mutants on growth and PHA production. No 3HHx monomer was detected in the $\Delta$phaJ mutant when grown in hexanoate as a sole carbon source (Table 4). The data prove that PhaJ is responsible for converting the intermediate of fatty acid beta-oxidation, trans-2, 3-enoylacyl-CoA, into (R)-3-hydroxyacyl-CoA. Surprisingly, the deletion of *phaJ* led to a severe effect on *R. rubrum* growth when grown on hexanoate and $\Delta$phaJ mutant had the lowest cell densities for the whole 240-hour time course experiment, though PHA content of this mutant was similar to that of WT and the growth curve was also similar to that of WT when grown on acetic acid (Fig. 4D, 4H, 5D, 5H).

Effect of PHA composition on PHA accumulation. A quite interesting observation was that *R. rubrum* strains accumulated PHA in the log phase and then degraded it soon in the stationary phase when PHA composed of only short chain monomers (3HB and 3HV) and *R. rubrum* strains continuously accumulated PHA in the stationary phase when the monomers of PHA containing HHx (PHA produced by all strains when grown on acetate and by $\Delta$phaC2 background mutants in which no HHx monomer of PHA was detected when grown on hexanoate) (Fig. 4A-H and Table 4). In order to further confirm that the presence of HHx resulted in continuous accumulation of PHA, we also measured the PHA content of $\Delta$phaJ mutant at different time points since no HHx was detected in the $\Delta$phaJ mutant. Just as expected, the PHA content of $\Delta$phaJ mutant decreased soon in the stationary phase, though it reached the maximum production after 72-hour growth and accumulated as high as 8.7% PHA of CDW, 68% of WT PHA content (12.7% of CDW) when grown on hexanoate.
DISCUSSION

*R. rubrum* expresses three different PhaCs, designated PhaC1, PhaC2 and PhaC3. The genetic and chemical evidences presented in this study clearly show different contribution of each PhaCs of the same class to PHA composition for the first time. Previous study shows that two loci that encode two classes of PHA synthases with different substrate specificity were responsible for producing homopolymer PHB and heteropolymer PHA in *Pseudomonas sp.*, respectively (Matsusaki et al., 1998). However, it is less likely in *R. rubrum* that absence of 3HV and 3HHx monomers in PHA of ∆phaC2 mutant was due to enzyme specificity since all three PhaCs are classified as class I based on *R. rubrum* genome annotation (Sequencing Center Univ of Wisconsin and Institute, 2005) and class I have the broad substrate specificity and are preferentially active toward short-chain and less active towards medium-chain length monomers (Rehm, 2003). One possible explanation is that PhaC2 forms a complex with enzymes of fatty acid beta-oxidation and the other two enzymes are excluded to access the substrate. In addition, the PHA content of ∆phaC2 mutant was significantly decreased, compared to WT. We used antibodies that we generated to analyze protein expression level and found that PhaC2 was highly expressed and PhaC1 and PhaC3 were unable to be detected in WT (data not shown). The major contribution of PhaC2 to PHA content may be attributed to highly expression level. On the other hand, ∆phaC1 or ∆phaC3 background mutants (∆phaC1, ∆phaC3 and ∆phaC1∆phaC3) contained significant higher percent PHA than WT. We analyzed PhaC2 protein expression level with western blot and found no significant change at the protein level in the ∆phaC1 or ∆phaC3 background mutants (data not shown). The activity of PhaC2 may be higher than those of PhaC1 and PhaC3 that
compete for substrate with PhaC2, resulting in higher PHA content than that of WT. Furthermore, our data indicate that the contribution of PhaC1 and PhaC3 to PHA content was not additive in $\Delta$phaC2 background mutants. It is possible that these two enzymes form heterodimer that has higher activities than homodimers of PhaC1 and PhaC3 individually. The heterodimer of PhaC1 and PhaC3 maybe represents a new class of PHA polymerases since all class I found so far are homodimers. However, further experiments need to be carried out to prove this hypothesis. The net rate of PHA accumulation results from the equilibrium of the synthesis and degradation (both occur simultaneously) (Doi et al., 1990; Doi et al., 1992). The consumption rate of PHA was much faster in WT than those of $\Delta$phaC1, $\Delta$phaC1$\Delta$phaC3 and $\Delta$phaJ mutants during the stationary phase when grown on acetate as carbon source. These $\Delta$phaC1 and $\Delta$phaC1$\Delta$phaC3 mutants may have higher rate of PHA synthesis than that of WT and $\Delta$phaJ mutant may have lower rate of PHA degradation than that of WT since PhaJ catalyzes reversible reaction of trans-2,3-enoylacyl-CoA to (R)-3-hydroxyacyl-CoA (Moskowitz and Merrick, 1969) and also is involved in PHA degradation. In addition, because no PHA was detected in the triple mutant, we analyzed the liquid media from which cells were removed by centrifuge and found that the media of triple mutant contained higher 3-hydroxybutyric acid (20 µg/ml) than that of WT (6.7 µg/ml) in liquid medium.

Previous studies have shown that PHAs enhance resistance to stresses (radiation, desiccation and osmotic pressure) (Tal and Okon, 1985). Our results clearly show that PHA is required for optimal growth when grown on acetate as carbon source and that PHA enhances bacteria growth in the stationary phase when grown on hexanoate as carbon source anaerobically. PHA serves as an electron sink and carbon reservoir and provides a
mechanism for cellular homeostasis of electron and carbon during nutrients unbalanced growth. In such scenario, PHA formation scavenges excess carbon source and is beneficial to bacteria growth. A study has pointed that mutation of PhaC results in little or no growth of Ralstonia eutropha under PHA-producing conditions (York et al., 2001b). Another study shows that an increase in level of the major regulator guanosine-tetraphosphate (ppGpp) of the stress was associated with PHA degradation and that this phenomenon was not observed in the mutant of PHA degradation deficiency of Pseudomonas oleovorans (Lo´pez, 1995). Our data show that the capacity of PHA enhancing bacteria growth in the stationary phase was proportional to the PHA content these strains contained when grown on hexanoate as carbon source for the first time. However, the mechanism still remains unclear and needs further investigation. In addition, our data indicate that presence of HHx monomer resulted in continuous PHA accumulation in the stationary phase. It is possible that R. rubrum is more adaptive to degrade PHA composed of short chain monomers during the stationary phase. PHA Depolymerase (PhaZ1) of R. rubrum has been showed that it is specific for degrading PHA composed of short-chain-length monomers (3HB and 3HV), but not medium-chain-length PHA in vitro (Handrick et al., 2004a).

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REFERENCES


### TABLES AND FIGURES

**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<td>Strain for conjugative transfer of plasmids into <em>R. rubrum</em>; <em>recA pro hsdR RP4-2-Tc::Mu-Km::Tn7</em></td>
<td>ATCC</td>
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<tr>
<td><strong>R. rubrum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 11170</td>
<td>Wild type</td>
<td>ATCC</td>
</tr>
<tr>
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</tr>
<tr>
<td>koC2</td>
<td>ΔphaC2</td>
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</tr>
<tr>
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</tr>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pPCR2.1-TOPO</td>
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<td>Invitrogen</td>
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<tr>
<td>pJQ200SK</td>
<td>Gene deletion vector for <em>R. rubrum</em>; encodes sacB; Gm&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pJQ200SKC1UD</td>
<td><em>phaC1</em> gene-deletion-plasmids; Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJQ200SKC3UD</td>
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<td>This study</td>
</tr>
<tr>
<td>pJQ200SKJUD</td>
<td><em>phaJ</em> gene-deletion-plasmids; Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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*Km<sup>r</sup>, kanamycin resistant; Ap<sup>r</sup>, ampicillin resistant; Gm<sup>r</sup>, gentamicin resistant.*
TABLE 2. Primers used in this study

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<td>Jr</td>
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* Restriction sites designed into sequence are indicated in bold and italicized.
### TABLE 3. \textit{phaC}s and \textit{phaJ} genes

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<thead>
<tr>
<th>Enzyme</th>
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<tr>
<td>\textit{phaC} polymerase</td>
<td>\textit{phaC1}</td>
<td>Rru_A0275</td>
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<td>\textit{phaC}-like polymerase 1</td>
<td>\textit{phaC2}</td>
<td>Rru_A2413</td>
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<tr>
<td>\textit{phaC}-like polymerase 2</td>
<td>\textit{PhaC3}</td>
<td>Rru_A1816</td>
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<td>(R)-specific trans-2,3-enoylacyl-CoA hydratase</td>
<td>\textit{phaJ}</td>
<td>Rru_A2964</td>
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### TABLE 4. PHA composition of \textit{R. rubrum} strains

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Strains</th>
<th>PHA composition (wt%)</th>
<th>3HB (C\textsubscript{4})</th>
<th>3HV (C\textsubscript{5})</th>
<th>3HH\textsubscript{x}(C\textsubscript{6})</th>
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<tr>
<td>Acetate</td>
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<tr>
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<td></td>
<td>koC2 (\textit{phaC2})</td>
<td>100% &lt; 0.1% &lt; 0.1%</td>
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</tr>
<tr>
<td></td>
<td>koC3 (\textit{phaC3})</td>
<td>99.3% 0.7% &lt; 0.1%</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>koJ (\textit{phaJ})</td>
<td>99.8% 0.2% &lt; 0.1%</td>
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<tr>
<td></td>
<td>koC1C2 (\textit{phaC1}\textnormal{\textit{phaC2}})</td>
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<tr>
<td></td>
<td>koC1C3 (\textit{phaC1}\textnormal{\textit{phaC3}})</td>
<td>99.3% 0.7% &lt; 0.1%</td>
<td></td>
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<tr>
<td></td>
<td>koC2C3 (\textit{phaC2}\textnormal{\textit{phaC3}})</td>
<td>100% ND ND</td>
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<tr>
<td></td>
<td>koC1C2C3 (\textit{phaC1}\textnormal{\textit{phaC2}}\textnormal{\textit{phaC3}})</td>
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<tr>
<td>Hexanoate</td>
<td>WT</td>
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<td></td>
<td>koC1 (\textit{phaC1})</td>
<td>93.3% 0.9% 5.8%</td>
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<tr>
<td></td>
<td>koC2 (\textit{phaC2})</td>
<td>100% &lt; 0.1% &lt; 0.1%</td>
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<tr>
<td></td>
<td>koC3 (\textit{phaC3})</td>
<td>93.2% 1.1% 5.8%</td>
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<tr>
<td></td>
<td>koJ (\textit{phaJ})</td>
<td>99.7% 0.3% &lt; 0.1%</td>
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<tr>
<td></td>
<td>koC1C2 (\textit{phaC1}\textnormal{\textit{phaC2}})</td>
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<td>93.6% 0.8% 5.6%</td>
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<tr>
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<td>100% ND ND</td>
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<tr>
<td></td>
<td>koC1C2C3 (\textit{phaC1}\textnormal{\textit{phaC2}}\textnormal{\textit{phaC3}})</td>
<td>NA NA NA</td>
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</tbody>
</table>

\textsuperscript{a} 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HH\textsubscript{x}, 3-hydroxyhexanoate; NA, non applicable; ND, non detected.

\textsuperscript{b} Samples were collected at 96-hour-time point. Data represent averages from triplicate biological samples.
FIG 1. Proposed pathways for biosynthesis of PHA in bacteria. (1) 3-ketothiolase (PhaA); (2) acetoacetyl-CoA reductase (PhaB); (3) PHA synthase (PhaC); (4) NADH-acetoacetyl-CoA reductase; (5) crotonase; (6) (R)-specific 2-enoyl-CoA hydratase (PhaJ); (7) epimerase; (8) 3-ketoacyl-CoA reductase; (9) acyl-ACP:CoA transacylase (PhaG).

FIG 2. The structure of PHAs. R can be branched-chain and aromatic groups and the length of R group ranges from 1- to 14-carbon. $R_1$ and $R_2$ can be the same or different.
FIG. 3. Alignment of PhaC3, PhaC2 and PhaC1 from *R. rubrum*. Black shading indicates identical residues and gray shading indicates similar residues.
FIG. 4. PHA contents of *R. rubrum* Strains when grown in acetate as carbon source (A-D) and hexanoate as carbon source (E-H). WT (+), ΔphaC1 (*), ΔphaC2 (■), ΔphaC3 (◇), ΔphaJ (△), ΔphaC1ΔphaC2 (◆), ΔphaC1ΔphaC3 (×), ΔphaC2ΔphaC3 (▲), ΔphaC1ΔphaC2ΔphaC3 (○). Data represent averages from triplicate biological samples.
FIG. 4. (continued)
FIG. 5. Growth curves of *R. rubrum* strains when grown in acetate as carbon source (A-D) and hexanoate as carbon source (E-H). WT (+), ΔphaC1 (*), ΔphaC2 (■), ΔphaC3 (◇), ΔphaC1ΔphaC2 (◇), ΔphaC1ΔphaC3 (×), ΔphaC2ΔphaC3 (▲), ΔphaC1ΔphaC2ΔphaC3 (□). Data represent averages from triplicate biological samples. The range of error was within ±5% of each value.
FIG. 6. The relationship of cell densities and PHA content of *R. rubrum* strains when grown in acetate as carbon source (A) after 72-hour cultivation and the correlation of final cell densities and PHA content of *R. rubrum* strains when grown in hexanoate as carbon source (B) after 240-hour cultivation. WT (+), ΔphaC1 (*), ΔphaC2 (■), ΔphaC3 (◇), ΔphaJ (△), ΔphaC1ΔphaC2 (●), ΔphaC1ΔphaC3 (●), ΔphaC2ΔphaC3 (▲), ΔphaC1ΔphaC2ΔphaC3 (□). Data represent averages from triplicate biological samples. The range of error was within ±5% of each value.
CHAPTER IV. REVERSE GENETIC CHARACTERIZATION OF ACETOACETYL-COA THIOLASES IN ARABIDOPSIS REVEALS THEIR IMPORTANCE FOR PLANT GROWTH AND DEVELOPMENT

A manuscript to be submitted to The Plant Journal

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Department of Biochemistry, Biophysics and Molecular Biology\textsuperscript{1}, Interdepartmental Plant Biology\textsuperscript{2}, Iowa State University and The Ames Laboratory of US Department of Energy\textsuperscript{3}, Ames, Iowa 50011

ABSTRACT

Acetoacetyl-CoA thiolase (AACT, EC 2.3.1.9) catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA that is the precursor of mevalonate-derived isoprenoids. BLASTP analyses have identified two AACT genes in the Arabidopsis genome, At5g47720 (AACT1) and At5g48230 (AACT2). Two T-DNA insertion alleles at AACT1 gene and one T-DNA insertion allele at AACT2 gene have been characterized. These characterizations indicate that although both genes are expressed (as evidenced by western analysis); mutation in AACT2 is embryo lethal whereas null alleles of AACT1 are viable and show no apparent growth phenotypes. Furthermore, segregation analysis and genetic complementation demonstrates that mutations in AACT2 affect male transmission, and in vivo pollen germination and elongation. Complementation of yeast AACT knock-out mutant \textit{Δ}erg10 showed that both Arabidopsis AACTs are functional. Promoter::GUS fusion experiments
indicate that *AACT1* is primarily expressed in the vascular system and *AACT2* is highly expressed in root tips, young leaves, top stems and anthers. *AACT2*-RNAi lines show pleiotropic phenotypes, including reduced apical dominance, elongated life span and flowering duration, sterility, dwarfing, reduced seed yield and shorter root length. Microscopic analysis reveals that dwarfing is caused by smaller cell size and less cell number and loss of pollen coat resulted in sterility. In addition, Microscopic examination shows faster degeneration of tapetum cells. These phenotypes were rescued when they were grown in the presence of mevalonate. Phytosterol analysis of *AACT2*-RNAi plants showed reduced sterol content and altered composition in the seedling roots. The accumulation of these sterols was restored to wild type levels when the plants were fed with mevalonate. In contrast, no significant phytosterol changes were detected in the *aat1* mutant. These results indicate that *AACT2* is an essential in plant growth and development and cannot be replaced by *AACT1*.

**INTRODUCTION**

The biosynthesis of isoprenoids is a common metabolic pathway that occurs in all plants, fungi, aminals and bacteria. In higher plants, isoprenoids, which are composed of structurally diverse large family of compounds, are synthesized from IPP precursor generated from two independent biosynthetic pathways: cytosolic MVA pathway and plastidic MEP pathway. The cytosolic MVA pathway is primarily responsible for the production of sesquiterpenes, triterpenes, phytosterols, and brassinosteroids. The plastidic MEP pathway produces
gibberellins, abscisic acid, carotenoids, chlorophyll side chain and quinines (Newman and Chappell, 1999). In cytosol, acetoacetyl-CoA thiolases (AACTs, EC 2.3.1.9) catalyze Claisen-type condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, which is the precursor of mevalonate-derived isoprenoids. In this MVA pathway, hydroxymethylglutaryl-CoA reductases (HMGRs) which are considered to be the key step in controlling the biosynthesis of mevalonate-derived isoprenoids (Bach, 1995), have been intensively investigated as to the regulation of their roles in plant growth and development (Enjuto et al., 1994; Enjuto et al., 1995; Lumbreras et al., 1995; Suzuki et al., 2004). In addition, many genes involved in this pathway have also been studied and characterized (Cunillera et al., 1996; Cunillera et al., 1997, 2000; Lluch et al., 2000). In sharp contrast, less attention has paid to AACT than the other enzymes of this pathway. Genes encoding acetoacetyl-CoA thiolases have been isolated from many organisms, ranging from bacteria to higher eukaryotes. In bacteria, AACTs catalyze the first step biosynthesis of polyhydroxyalkanoates (Anderson and Dawes, 1990). In yeast, AACT encoded by ERG10, which is essential for yeast survival (Hiser et al., 1994), generates precursor for ergosterols and was predicted to be homotetramer based on molecular weight revealed by gel infiltration(Kornblatt and Rudney, 1971). In mammals, cytosolic AACTs are involved in the essential mevalonate pathway that generates cholesterol. High resolution crystal structure of human cytosolic AACT has been resolved (Kursula et al., 2005). It is shown to be a homotetramer. There are many positively charged residues at the interdimer surface leading towards the CoA-binding pocket, which is possibly important for the efficiently capturing substrates. Comparison of the human and bacterial Zoogloea ramigera thiolases show that the geometrical structure of the catalytic triads (Cys 92, His 353, Cys 383) and the two
oxyanion holes are highly conserved. In plants, AACT catalyzes the first step of forming mevalonate-derived isoprenoids which play an essential role in membrane component, growth and development, disease resistance and isoprenic units for farnesylation of a series of proteins (Newman and Chappell, 1999). Comparison of the deduced amino acid sequences of the AACTs from several plants reveals that AACTs are highly conserved and share about 50% amino acid sequence identity (Dyer et al., 2009), which is consistent with the concept that AACT is essential gene in eukaryotes. In spite of these findings, very little is known about their roles of AACTs in the biosynthesis of isoprenoids in plants and significance in the plant growth and development. In addition, there is general agreement that paralogous genes may control the synthesis of the necessary isoprenoid compounds required for different purposes at different growth conditions or in different tissues of the plant at different stages of growth and development (Bach et al., 1999; Newman and Chappell, 1999). Genomic analysis revealed two AACT paralogous genes in Arabidopsis. In order to investigate of the role of AACTs in the plant metabolism, growth and development and the significance of each paralogous gene in plants, we characterized each Arabidopsis AACT1 and AACT2 genes. In this paper, we report the isolation and characterization of AACT1 and AACT2 T-DNA insertion lines and generated antibodies and promoter::GUS lines to characterize the expression patterns of each gene. In addition, we demonstrate that both genes encode functional AACT by complementation of the yeast ortholog AACT Δerg10 mutant. Moreover, we also show that AACT2 is essential in Arabidopsis and knocking-down of AACT2 expression results in pleiotropic phenotypes. Exogenous mevalonate, an acetyaceto-CoA-derived compound, complements the AACT2-suppressed phenotype. Our findings indicate that significant amount of AACT-generated isoprenoid pool is required for
plant normal growth and development and that no isoprenoids generated from other biosynthetic pathways can compensate for reduction of this pool.

RESULTS

Both AACTs complemented Yeast Δerg10 Mutant

Search of the Arabidopsis genome revealed two genes encoding acetoacetyl-CoA thiolases. The loci of these genes are At5g47720 and At5g48230, respectively, locating within 0.22 Mb of each other on chromosome 5. ClustalW showed that these two genes shares 78.4% sequence identity at amino acid level (Figure 1). We designated these two genes AACT1 (At5g47720) and AACT2 (At5g48230). Yeast complementation studies were performed to establish the function of each AACT. In yeast, AACT is essential to survival; AACT deletion (Δerg10) mutant proves to be lethal. Therefore, the Δerg10 heterozygous diploid S. cerevisiae strain was used to conduct complementation experiments. A pDEST52-based vector with each AACT open reading frame fused to the galactose-inducible-GAL1 promoter was introduced into this heterozygous diploid strain, respectively. Then the transformed diploid was forced to sporulate to obtain transformed haploid cells. The haploid Δerg10 mutant with each open reading frame of AACT1 and AACT2 grew on induction YPG medium, but not on non-induction YPD medium (Figure 2). This result clearly establishes that both AtAACT1 and AtAACT2 proteins have AACT enzymatic activity.

AACT1 and AACT2 exhibit different expression patterns and levels

To examine precisely the spatial and temporal expression pattern of the two AACT genes in tissues and organs, we fused fragments of 0.8-kb AACT1 gene promoter and 1.5-kb AACT2
gene promoter to the GUS reporter, respectively. \textit{AACT1} has a relatively short intergenic region preceding the start codon, and in this case about 0.8 kb was included in the promoter::GUS fusion construct. \textit{AACT2} has long intergenic region and 1.5 kb upstream of the translational start site was included. These two constructs were transformed into wild-type \textit{Arabidopsis} ecotype Columbia plants. At least 10 independent transgenic lines were generated and analyzed (T2 generation) for each construct. 8-day-old seedling and 6-week-old plant were assayed using 3 plants per independent transgenic line. Plants were grown under 24-hour light cycle in order to avoid possible variations in the GUS expression associated with the light cycle. A representative sample is shown in Figure 3. The \textit{AACT1} promoter::GUS construct transformants showed strong GUS staining in vascular system of various organs. The histochemical analysis of seedlings showed intense staining in the vascular system of roots and cotyledons, but not in young initiating true leaves (Figure 3a and c). In adult transgenic plants, GUS activity was clearly detected in the vascular system of young leaves, fully expanded leaves, stems, flowers and funiculi of 8-day-after-flowering siliques (Figure 3e, g, I, k, m, o, p, s and t). In contrast, \textit{AACT2} showed different expression patterns from \textit{AACT1}. \textit{AACT2} is strongly expressed in root tips of seedlings (Figure 3d). In the aerial parts of seedlings, significant GUS activity was observed in new emerging leaves of seedlings, but not in cotyledons (Figure 3b). In buds, GUS was exclusively expressed in anther (Figure 3f and h). In addition, young siliques, leaves and stems of adult plants were highly stained (Figure 3m, q and u). However, mature organs, fully expanded leaves, mature siliques, flowers and stems of the first internode, exhibited less GUS activities (Figure 3j l, n, r, and v). The expression levels of two \textit{AACT} genes were also assessed by western blot using total protein of different organs (leaves, roots, stems, inflorescences and siliques) from
6-week-old plants and of 16-dya-old seedling (Figure 4). The expression levels of AACT2 were much higher than those of AACT1 in leaves, stems, inflorescences and siliques, whereas in roots, both genes showed similar expression levels. In addition, the protein level of AACT2 was slightly lower in 2-day-dark treatment seedlings than in light-grown seedling.

**Generation of AACT1 and AACT2 Antibodies**

To study each function of AACT1 and AACT2, polyclonal antibodies for each AACT were generated by immunizing mice with AACT1 and AACT2 recombinant proteins. Western blot analyses showed that each antibody recognized both purified recombinant proteins. In addition, analysis of total protein extracted from *Arabidopsis* leaves revealed two bands at about 43.3k (AACT1) and 40.9kD (AACT2) with either antibody. Since each antibody recognized both proteins, the higher titer AACT1 antibody was used for western blot analysis in this study.

**T-DNA insertion AACT1 mutant does not show visible phenotype under normal growth conditions and stress growth conditions**

As the first step to investigate the function of the AACT genes in plant growth and development, we characterized homozygous T-DNA insertion mutants acquired from ABRC (Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH). There are two AACT1 T-DNA insertion alleles SAIL_448_H10 (*aact1-1*) and SALK_008505 (*aact1-2*). Revealed by PCR-amplifying both ends of the T-DNA insertion using T-DNA primers in combination with gene-specific primers, two copies of tail-to-tail T-DNA insertion location in the SAIL_448_H10 line was between the 11th exon and the 12th exon of AACT1 and a 142-bp genomic DNA including intron between these two exons was deleted by T-DNA insertion (Figure 5a). The T-DNA insertion site of SALK_008505 was between the
11th exon and intron (Figure 5a). The insertion resulted in a 29-bp deletion of this exon and intron junction of AACT1. The absence of the full-length transcripts was evidenced by RT-PCR in aact1-1 and aact1-2 mutant alleles (Figure 5b). Furthermore, western blot with AACT1 antibody showed that the corresponding band of AACT1 was undetectable in each aact1 mutant (Figure 5c). Thus, we concluded that both aact1-1 and aact1-2 are null alleles.

As the initial step to characterize a loss-of-function mutation, segregation analysis was performed by PCR-genotyping the progeny from one heterozygous plant. Out of 96 progeny from one heterozygous aact1-1 plant, 25 were wild type, 47 were heterozygous and 25 were homozygous. For the aact1-2 allele, PCR-genotyping of 97 progeny from one heterozygous aact1-2 plant showed that 26 were wild type, 43 were heterozygous and 27 were homozygous. Both alleles showed a normal Mendelian ratio inheritance (1:2:1). These data showed that transmission of each mutant aact1 allele through female and male gametophyte was not affected. In addition, both aact1-1 and aact1-2 mutants did not show visible phenotype under the growth conditions used in this study. Moreover, analysis of phytosterol showed no significant phytosterol changes were detected in the both aact1-1 and aact1-2 mutants (data not shown). Since we found that AACT1 was uniquely expressed in vascular system, we assumed this gene may be involved in stress resistance. A series of stress treatments, including salt (150mM NaCl), osmotic (300mM mannitol), UV-B, heat, cold and drought stresses, were carried out to test this hypothesis. However, no visible phenotype was detected after these stress treatments (data not shown).

**AACT2** mutation results in embryo lethality and reduces pollen germination and pollen tube elongation *in vivo.*
For AACT2, we analyzed four T-DNA insertion alleles EMB1276, SALK_036024, SALK_010613 and WiscDsLox1E12 acquired from ABRC. For SALK_036024 allele, we found the T-DNA insertion located to be in 3’ UTR of AACT2 gene. However, the AACT2 mRNA of SALK_036024 allele did not have significant difference from that of wild-type allele (data not shown). Furthermore, western blot analysis of AACT2 protein level showed that homozygous mutant plants accumulate similar level of AACT2 as wild-type plants (data not shown). In the PCR analysis of the other three lines, only the T-DNA insertion of EMB1276 allele was detected and we failed to detect T-DNA insertion in the other two alleles. The T-DNA insertion in the line EMB1276 was between the third exon and intron of AACT2 and resulted in a 25-bp deletion of this exon and intron junction of AACT2, established by PCR-amplifying both ends of the T-DNA insertion (Figure 6a). This T-DNA insertion allele was designated aact2-1 and was subjected to further characterization. The glufosinate ammonium resistance in aact2-1 allele was found to be active and we used this antibiotic selection for identifying the presence of this allele in the complementation experiments. 257 progeny from three heterozygous aact2-1 plants were genotyped by PCR. Out of these plants, no homozygous plant was recovered; 118 were wild type and 139 were heterozygous. Chi-square analysis showed that the ratio of wild type and heterozygous plants (1.17:1) was significantly deviated from 2:1, a normal Mendelian segregation ratio (p-value<0.01). These data indicated that transmission efficiency of aact2-1 allele through female or male gametophyte was reduced. In addition, analysis of 30 siliques from 10 heterozygous aact2-1 plants revealed 18.3% aborted seeds, whereas, sibling wild type plants only harbored 1.9% aborted seeds (Figure 6b-f). To clearly determine the timing of seed abortion in aact2-1 mutant, Meinke group has analyzed the developing seeds from aact2-1
heterozygous plants using differential interference contrast microscope after clearing of dissected embryo with Hoyer’s reagent (Tzafrir et al., 2004). They found that aact2-1 homozygous embryo was arrested before globular stage. These data showed that disruption of AACT2 resulted in embryo lethality. However, the heterozygous plants did not show any other visible phenotype under the normal growth conditions, compared to wild type. To study transmission efficiency of aact2-1 allele through female or male gametophyte, reciprocal crosses were performed. Genotyping 77 progeny from WT ♀ (+/+ × aact2-1 ♂ (+/-) crossing revealed that 61 were wild type and 16 were heterozygous. 60 were wild type and 79 were heterozygous out of 139 progeny from aact2-1 ♀ (+/-) × WT ♂ (+/+ ) crossing. These data showed that transmission of aact2-1 allele through male gametophyte was significantly reduced (p-value<0.01), but not through female gametophyte (p-value>0.1). In order to investigate the developmental stage(s) of male gametophyte impaired by aact2-1 allele, different stages of male gametophyte were examined. Microscopic analysis of tetrads, the products of meiosis, from young buds and DAPI stained pollens from mature flowers of the heterozygous aact2-1 plants revealed that tetrads were normal and that pollens had two generative nuclei and one vegetative nucleus, similar to those of sibling wild type (data not shown). We also examined the pollen morphology under scanning electron microscope. The morphology of pollens from aact2-1 heterozygote was normal. In addition, Alexander staining test of about 1000 pollen from each genotype showed no significant difference in pollen viability between pollens from wild type and aact2-1 heterozygous sibling plants (data not shown). Because of big variation in vitro pollen germination, we were unable to detect significant difference between pollens from aact2-1 heterozygous plants and wild type plants. In order to avoid potential effect caused by extent of pistil maturation and stress
applied to flowers when removing stamen, pistils of male-sterile plants (ms1-1/ms1-1) (Wilson et al., 2001) were used for conducting *in vivo* pollen germination. 18 hours later after pollination, Alexander’s staining (Alexander, 1969) was applied to the pistils. Non-germinated pollens were stained light blue and germinated ones were stained purple (Figure 6g-h). 87.2% pollens from wild type plants germinated, which was significantly higher than 70.5% pollen germination rate from aact2-1 sibling heterozygous plants (p-value<0.05) (Figure 6i). The data indicated that mutation of AACT2 affected pollen germination *in vivo*. In addition, to show if mutation of AACT2 affected pollen tube elongation *in vivo*, we also analyzed the distribution of aborted seeds along the silique. The distribution of aborted seeds along siliques of heterozygous plants has been used to identify mutation that affects pollen tube growth (Meinke, 1982). If the gene is essential for pollen tube elongation, less aborted seeds are found at the bottom half of siliques because mutant pollen tubes are less competitive than wild type pollen tubes. 34 siliques from 11 heterozygous plants (2-4 siliques per plant) were analyzed and 1311 seeds in total were mapped in these siliques. Percentage in top half of aborted seeds was 60.0% that was significantly higher than 40.0% aborted seeds in bottom half (p-value<0.05). To further confirm the loss of AACT2 gene function was responsible for embryo lethality, seeds abortion, defective transmission efficiency of aact2-1 allele including pollen germination and pollen tube elongation *in vivo*, complementation experiments were performed to show if these phenotypes could be rescued by transformation with the wild type AACT2 allele. For the complementation study, one pCambia1300-based plasmid containing approximately 5 kb genomic DNA containing AACT2 with its native promoter was transformed into aact2-1 heterozygous plants. The construct contains hygromycin resistant gene as the selection
marker. For screening homozygous \textit{aact2-1} allele in T1 plants transformed with 5-kb-\textit{AACT2}-genomic-DNA, one of gene-specific primer P25 flanking the T-DNA insertion of \textit{aact2-1} allele was designed to be in the genome region beyond the 5 kb fragment, so the wild type genomic \textit{AACT2} DNA can be distinguished from the transformed 5 kb fragment. 3 \textit{aact2-1} homozygous plants were recovered from the 53 independent T1 plants transformed with 5-kb-\textit{AACT2}-genomic-DNA. The data demonstrated that the loss of \textit{AACT2} gene function result in the embryo lethality phenotype of \textit{aact2-1}. In order to prove that the disruption of \textit{AACT2} was responsible for defective transmission efficiency of \textit{aact2-1} allele, we generated T2 plants with the desired genotype (\textit{Hyg'}/\textit{Hyg'}; \textit{aact2-1}/+) as follows. First, we screened the progeny from each T1 plants with hygromycin and obtained 8 \textit{aact2-1} heterozygous T1 lines harboring single insertion locus of 5-kb-\textit{AACT2}-genomic-DNA (The ratio of resistant progeny: sensitive progeny is 3:1). Then, 96 T2 glufosinate ammonium and hygromycin resistant plants from these 8 T1 lines (12 T2 plants from each T1 line) were screened in order to obtain homozygous 5-kb-\textit{AACT2}-genomic-DNA insertion and \textit{aact2-1} heterozygous T2 plants by test of the antibiotics resistance of the progeny of T2 plants (T3 plants). The progeny of desired T2 plants were all hygromycin resistant and 3/4 progeny were glufosinate ammonium resistant. Finally, 2 T2 plants with the desired genotype from 2 independent T1 lines were obtained and used to test the transmission efficiency of \textit{aact2-1} allele. Of 96 T3 plants from one T2 plant, 24 were wild type, 49 were \textit{aact2-1} heterozygous and 23 were \textit{aact2-1} homozygous revealed by PCR-genotyping. Out of 105 progeny from the other T2 plant, 28 were wild type, 50 \textit{aact2-1} heterozygotes and 27 \textit{aact2-1} homozygotes. Presence of wild type \textit{AACT2} copy restored the distorted segregation ratio of progeny from \textit{aact2-1} heterozygote to normal Mendelian segregation ratio for both independent lines. This
result clearly proved that the loss of AACT2 gene function resulted in the defective transmission efficiency of aact2-1 allele.

**AACT2 RNAi plants show pleiotropic phenotype**

Because the disruption of AACT2 gene resulted in the embryo lethality, transgenic RNAi lines were generated to further study the role of AACT2 in plant growth and development by knocking down AACT2. For this study, a pAGRIKOLA-based plasmid obtained from NASC (The European Arabidopsis Stock Center) containing an inverted 180-bp sequence of AACT2 open reading frame under the control of 35S promoter and octopine synthase terminator was used for generating AACT2-RNAi lines (Hilson et al., 2004). This 180-bp sequence of AACT2 contained in this construct was designed specifically for AACT2 and comparison of this 180-bp sequence to whole Arabidopsis genome sequence by Blastn algorithm did not show any significant identity with any other genome sequence including AACT1 sequence. Therefore, the AACT2 RNAi was designed to specifically target the AACT2 gene. 82 independent transgenic glufosinate ammonium-resistant AACT2-RNAi lines were generated by floral dipping. Considering the variable effect of RNAi, we classified these lines into 4 groups based on their phenotype severity, designated wild-type or mild, moderate, severe and very severe. Among these 82 plants, 45 showed wild-type phenotype, 25 were mild or moderate, 7 were severe and 5 were very severe (Figure 7a). To determine whether the defective phenotype in these RNAi AACT2 plants was correlated with a reduction of AACT protein level, 2 independent lines from each group were selected for western blot analysis except very severe lines since no enough tissue for western blot analysis. Western blot analysis showed that the protein level of AACT2 extracted from inflorescence of 6-week-old plants with obvious altered phenotype was decreased dramatically (Figure 7b); however the
protein level of AACT1 in these RNAi lines remained the similar level to that of wild type plants (Figure 7b). Hence, the results indicated that altered phenotypes were associated with reduced AACT2 protein levels. One severe line (designated AACT2i-1) was used for further characterizing and quantifying phenotype. AACT2i-1 plants displayed obviously pleiotropic phenotype. Firstly, AACT2i-1 plants showed reduced apical dominance with increased branching (Figure 8a). This is quite obvious in inflorescence stems. AACT2i-1 plants had more secondary stems that resulted in bushy-like inflorescence. In addition, root lengths of AACT2i-1 plants were markedly reduced when compared to that of wild type plants (Figure 13a). Secondly, AACT2i-1 plants were also found to have crinkly, smaller and thinner leaves and shorter petioles (Figure 8b and d). AACT2i-1 plants showed shorter and thinner stems and the height of AACT2i-1 plants was only about 50% those of wild type plants (Figure 8c and e), resulting in dwarfing phenotype. Thirdly, AACT2i-1 plants showed elongated life span and flowering duration and continued to flower up to approximately 20-week-old, almost twice as long as that of wild type plants (Figure 8g). Finally, AACT2i-1 plants exhibited sterility phenotype with plenty of aborted siliques and substantial number of smaller buds remained closing during the whole life span (Figure 8f). In contrast with the wild type plants, these AACT2i-1 plants displayed reduced fertility with lower seed yield and only produced two-third the amount of seeds produced by wild type sibling plants (Figure 8i). AACT2i-1 plants typically had shorter siliques compared with wild type siliques (Figure 8j). Siliques of the AACT2i-1 plants produced on average 28 seeds per silique relative to those of wild type plants, which generated on average 43 seeds per silique (3 siliques per plants, 6 plants for each genotype) (Figure 8h). These AACT2i-1 plants produced smaller seed size, 6% shorter in length than those of wild type plants (Figure 8m). In addition, a lot
of deformed seeds with variable sizes were found in AACT2i-1 plants, whereas the size of seeds from wild type siblings was uniform (Figure 8k and l). However, it should be mentioned that similar phenotypes, as described above, were also observed in other independent RNAi lines showing reduced AACT2 protein level generated from the same pAGRIKOLA-based construct. In addition, these phenotypes were inheritable and maintained in following generation T3 plants.

The altered morphological phenotype cosegregates with the AACT2 RNAi transgene

To further demonstrate whether the pleiotropic morphological phenotype in the AACT2-RNAi lines was caused by AACT2-RNAi transgene, PCR was used to track the inheritance of the transgene. Out of resulting 118 siblings resulting from self-pollination of heterozygous AACT2i-1 plants, 83 showing characteristic AACT2-RNAi phenotype were PCR-positive and none of the resting 35 showing wild type phenotype was PCR-positive, determined by PCR using transgene-specific primers. Out of these 83 plants displaying typical AACT2-RNAi phenotype, only 6 homozygous plants have been identified in the T2 progeny by screening T3 progeny with glufosinate ammonium from each T2 plants, implying that the transgene was poorly transmitted through Arabidopsis gametophytic phase of the life cycle. These data clearly show that presence of AACT2-RNAi transgene was responsible for the abnormal morphological changes in the RNAi line and decrease in AACT2 protein level are sufficient to explain the defective phenotype observed in AACT2-RNAi plants. Because the RNAi construct harbors an herbicide resistance gene, its segregation pattern can easily be followed by analyzing its progeny. The segregation ratio was significantly deviated from 1:2:1, as would be expected for an allele of a normal Mendelian segregation ratio (p-value<0.05), consistent with our data from study of T-DNA insertion aact2-1 mutant that
showed reduced \textit{aact2-1} allele transmission through male gametophyte and \textit{aact2-1} was homozygous lethal.

\textbf{Cell size and numbers are reduced in leaf and stems in plants with knocking-down the expression levels of AACT2}

To further investigate the cellular basis for the remarkably change in leaf morphology associated with the AACT2i-1 plants and study if the reduction in leaf size and plant height were caused by decrease in cell number or cell size, stems of the first internodes from 8-week-old sibling wild type plants and AACT2i-1 plants and fully expanded rosette leaves from 6-week-old plants were microscopically examined (Figure 9a-d). In order to avoid potential effect of stem bolting length on stem cell sizes and numbers, we labeled each plants bolting day and chose the same bolting day plants for microsocpic analysis. AACT2i-1 plants showed dwarfing phenotype and thinner stems. In order to quantify the cell number in different stem tissue, we drew a 60° angle radiating from the stem center, including one vascular bundle. Microscopic analysis of stem cross sections revealed that there was considerably less cell number in stem of AACT2i-1 plants than that of wild type siblings (Figure 9e). In particular, cell number of phloem and xylem was dramatically reduced, only 60% and 50% that of wild-type, respectively. Moreover, cell length was reduced in AACT2i-1 plants, compared to wild type siblings. The cell length of epidermis, cortex, phloem, xylem and pith were 71%, 74%, 78%, 76% and 63% that of wild type-cells (Figure 9f). Leaves from AACT2i-1 plants were thinner and smaller than wild-type leaves (Figure 9f). This decrease in leaf thickness was the result of reduced cell size and apoplastic space (Figure 9c, d and f). Measurement of the dimensions of epidermal, spongy and palisade mesophyll cells indicated that all cell types were about 50% smaller than the corresponding
wild-type cells (Figure 9f). Despite the reduction in cell and leaf size, the overall cellular organization within the leaf was retained. These data indicate that dwarfing phenotype was caused by reduced cell elongation, less cell number and reduced apoplastic space.

**RNAi knocking-down AACT2 expression results in faster degeneration of tapetum cells and loss of pollen coat**

AACT2i-1 displayed male sterile phenotype. To study the underlying mechanism of this phenotype, we analyzed the pollen grains from the oldest bud (just before opening) in the inflorescence by transmission electron microscope. Electron microscopy revealed that the pollen coat from AACT2i-1 plants was lost (Figure 10b) and the pollen coat from wild type sibling plants was still intact (Figure 10a), which is consistent with the results of AACT2 promoter::GUS fusion that highly expressed in anther. In addition, we also analyzed three stages of buds (three oldest buds) from an inflorescence branch. Light microscopic analysis showed that tapetum cells of AACT2i-1 plants were completely degenerated in stage two, whereas the corresponding tapetum cells of wild type plants was degenerated in stage three (Figure 11).

**Reduction of AACT2 expression decreases sterol content**

AACT catalyzes the first step of acetyl-CoA condensation pathway that generates isoprenoids including sterol. To study the role of AACT2 in sterol biosynthesis and the effect of a reduction in AACT2 expression level on sterol content, we analyzed the sterol contents and composition of wild type and RNAi knock-down AACT2i-1 plants via gas chromatography-mass spectrometry. Roots of 16-day-old seedlings were used for extracting total sterol. The total sterol contents of AACT2i-1 are 40% lower than that of wild type siblings (p-value<0.05) (Figure 12a and b). In addition, the sterol composition of AACT2i-1
was significantly altered (Figure 12a). Moreover, the ratio of campesterol to sitosterol and stigmasterol, which plays a critical role in balancing plant growth requirements and membrane integrity, was increased by 24% in AACT2i-1, compared to that of wild type plants (Figure 12c).

**Mevalonate rescues the morphological and chemical phenotype of plants with knock-dwon AACT2 expression**

To test the hypothesis that reduction of acetoacetyl-CoA derived compounds in RNAi-AACT2 plants resulted in the pleiotropic phenotype, aceto-acetyl-CoA derived compounds was used for complementation including mevalonate, cycloartenol, squalene, sterol-PEG600 and mixture of sitosterol, stigmasterol and campesterol (1:7:2). Among these chemicals, only mevalonate restored the AACT2i-1 abnormal phenotype to near wild type phenotype (Figure 12a-d). Mevalonate treatment did not have visible effect on the growth of wild-type plants. The other chemicals did not rescue these pleiotropic phenotypes (data not shown). The restoration of sterol content was revealed by analysis of roots of 16-day-old seedlings via GC-MS (Figure 13a). The sterol content of AACT2i-1 increases to levels similar to those of wild type plants (Figure 13b). In addition, the bulk sterol ratio (campesterol/sitosterol and stigmasterol) was also reverted to wild-type ratio (Figure 13c). These data show that exogenous mevalonate revert the morphological and chemical phenotype associated with the reduction in AACT2 expression level.
DISCUSSION

Acetoacetyl-CoA thiolase (AACT) catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA in cytosol, which is first step of isoprenoids biosynthesis. Genome analysis revealed two genes coding for AACT in Arabidopsis. In this study, we characterize these two AACT genes and their functions in plant growth and development using T-DNA knock-out mutants and RNAi knocking-down lines. Since acetoacetyl-CoA is precursor of many important compounds including membrane sterols, and some plant hormones, it is expected that knock-out of AACTs will result in severe effect on plant growth and development. Indeed, we have showed that the aact2-1 allele is recessive embryo lethal. This finding is consistent with the idea that isopenoids biosynthesis is an indispensable pathway.

The occurrence of two AACT paralogs raises the question about their individual function for plant growth and development and the resulting effect on isoprenoids biosynthesis. To address this question, we analyzed the expression levels of the AACT1 and AACT2 genes in different plant organs by western blot and expression patterns of these two genes at different growth stages by promoter::GUS histochemical analysis, respectively. Western blot result showed that the expression level of AACT2 was much higher than that of AACT1. In addition, promoter::GUS analysis showed that the two AACTs displayed different spatial and temporal expression patterns during different plant development stages. This suggests that these two genes have different functionalities. Surprisingly, the two AACT1 mutants aact1-1 and aact1-2 were indistinguishable from wild type plants under standard growth conditions. Although several stress conditions were performed to treat aact1-1 and aact1-2, both mutants
did not show any visible phenotype, too. Moreover, the amount and composition of sterol were not affected by disruption of AACT1, regardless of both genes complementing yeast AACT mutant. In addition, the GFP fusion experiments of previous study have shown that two of three isoforms of predicted proteins encoded by AACT1 are located in cytosol and one of them are located in peroxisome. Both two isoforms encoded by AACT2 are located in cytosol (Carrie et al., 2007). Furthermore, previous studies have shown that isopentenyl diphosphate isomerase, which is an enzyme of cytosolic MVA pathway, are targeted to multiple subcellular compartments including cytosol and peroxisome (Phillips et al., 2008; Sapir-Mir et al., 2008). It is possible that AACT1 is involved in producing some specific isoprenoids for plants under some growth conditions. Therefore, it is postulated that AACT1 is involved in enhancing plant growth under some suboptimal growth conditions. However, we were unable to find out the specific growth conditions that affect these mutants growth. In contrast, AACT2 was found to be essential for plant growth and development and aact2-1 mutant is homozygous lethal. One possible interpretation of the above findings is that the lower expression level and different expression patterns of AACT1 might not be sufficient to replace the functions provided by AACT2.

In higher plants, there are two independent biochemical isoprenoid pathways: one residue in plastids and the other in the cytosol. In spite of cross-talk between two pathways demonstrated by feeding experiments with stable isotopes and chemically blocking MVA or MEP pathways (Arigoni et al., 1997; Kasahara et al., 2002; De-Eknamkul and Potduang, 2003), our results are consistent with previous studies that MEP pathway can not complement the defective MVA pathway (Rodriguez-Concepcion and Boronat, 2002; Hsieh and Goodman, 2005).
The generation of transgenic \textit{AACT2-RNAi} plants with reduced level of \textit{AACT2} enabled us to further study the physiological function of \textit{AACT2}. \textit{AACT2-RNAi} plants showed pleiotropic phenotype. Plant hormone brassinosteroid is a derivative of acetoacetyl-CoA. However, \textit{AACT2-RNAi} plants did not exhibit typical defective brassinosteroid phenotype. This observation is consistent with our phytosterol analysis of \textit{AACT2-RNAi} plants exhibiting high campesterol, which is the precursor of brassinosteroid. Several mutants defective in sterol biosynthesis and have been reported (Diener et al., 2000; Jang et al., 2000; Schrick et al., 2000; Souter et al., 2002). Most of these mutants exhibited similar phenotype including dwarf, reduced fertility and abnormal embryogenesis. The similarity in phenotype between \textit{AACT2-RNAi} and the sterol mutants may indicate that a reduction in the availability of sterols and altered sterol composition have a severe effect on the plant growth and development. The availability of sterols and ratio of bulk sterol campesterol to sitosterol and stigmasterol plays a critical role in balancing plant growth requirements and membrane integrity (Schaeffer et al., 2001). The altered sterol of \textit{AACT2-RNAi} plants modified the membrane properties in terms of membrane fluidity, protein-membrane interacting environments and limits the sterol availability, which may be responsible for the pleiotropic phenotype of \textit{AACT2-RNAi} plants. However, to our knowledge, no mutant defective in isoprenoids pathway that has been characterized is detrimental to male gametophyte. Our data clearly demonstrated that the disruption of \textit{AACT2} gene results in the defective transmission efficiency of \textit{aact2-1} allele revealed by reciprocal crossing and affects pollen tube elongation. Maybe the fast elongation of pollen grains requires some specific isoprenoid compounds such as sterol during germination.
Sterol is required for normal functions of cell membrane, functioning as membrane reinforcer and interacting with lipids and proteins within the membrane (Bloch, 1983). The most likely explanation for the miniature phenotype related with reduced AACT2 is that reduction of AACT2 activity limits the availability of phytosterols for membrane biogenesis. In response to the reduction of sterol availability, the AACT2-RNAi plants adapt to the limitation of sterol availability for growth requirements and membrane integrity by reducing the cell size and membrane area to conserve membrane sterol. Consequently, reduction of cell size and less cell number resulted in thinner and smaller leaves and stems of the AACT2-RNAi plants, which exhibited miniature phenotype. This observation is consistent with promoter::GUS staining that AACT2 was highly expressed in young tissue that needs sterol for rapid cell division and elongation. In spite of the reduction in cell size and number, the overall cellular organization in rosette leaves and stems of the AACT2-RNAi plants remained unchanged. Furthermore, regardless of the total sterol content of AACT2-RNAi seedling roots was considerably less than that of wild-type seedling roots, we were unable to detect a significant decrease in the sterol content of the AACT2-RNAi plant aerial parts when quantified on the basis of weight. This observation is mainly attributable to the smaller cell sizes, less cell numbers and reduced apoplastic spaces of aerial part of AACT2-RNAi plant.

Our data revealed that reduction of AACT2 activity changed the sterol composition and reduced sterol content. Previous studies have shown that balanced sterol composition is essential for keeping cell polarity and auxin efflux in Arabidopsis (Willemsen et al., 2003). Considering that auxin gradient established by PIN is essential for proper embryogenesis, it is possible that altered sterol affected the role of membrane sterols in regulating PIN
endocytosis (Pan et al., 2009), resulting in embryo lethality. In addition, previous studies have proposed that certain sterol or intermediates of the isoprenoid pathway may play a signaling role in embryogenesis (Jang et al., 2000; Schrick et al., 2000). Therefore, it is also possible that the embryo lethality resulted from reduction of these signaling molecules.

It is believed that extracellular lipids in pollen coat were deposited on the pollen surface upon degeneration of tapetal cells, while intracellular pollen lipids were determined by expression of the haploid pollen genome (Piffanelli et al., 1998). Various nutrients are released from tapetal cells are transported to pollen surface and sustain pollen development. Lipids including sterol esters are essential for pollen viability and germination (Wolters-Arts et al., 1998). Tapetum cells contain lipidic organelle, such as elaioplasts and tapetosomes, which accumulate sterol esters that deposit to pollen surface and become components of pollen coats upon degeneration of tapetum cells (Piffanellia, 1998; Hernandez-Pinzon et al., 1999). Therefore, the reduced availability of sterols in tapetum cells resulted in the missing pollen coat of AACT2-RNAi lines. In addition, microscopic analysis revealed faster degeneration of tapetum cells in AACT2-RNAi lines. It is possible that reduction of sterols in elaioplasts and tapetosomes was responsible for faster degeneration of tapetum cells.

Our data revealed that sterols and sterol precursors were unable to rescue the pleiotropic phenotype. We analyzed the AACT2i-1 line grown in sterol precursor cycloartenol via GC-MS. However, the sterol level of AACT2i-1 line remained similar to that of AACT2i-1 plants when grown on MS medium with cycloartenol and did not restore to the level of wild type plants. One possible explanation is that plants absorb exogenous sterols very inefficiently (Hartmann, 1998).
In the western blot analysis of AACT protein using AACT1 antibody, 3 protein bands were detected. AACT1 and AACT2 share 78.4% identity at the protein level. The middle band was missing in both aact1-1 and aact1-2 homozygous mutants. In another experiment, when we added purified AACT2 protein with the first antibody in order to decrease AACT2 band, the intensity of bottom bands was strongly decreased whereas the middle band remained the same intensity (data not shown). This result suggests that the bottom band is AACT2. In addition, the bottom band is remarkably decreased in AACT2 RNAi lines. Based on these data, we concluded that the middle band was AACT1 and the bottom band was AACT2. The top band maybe was KATs since they share approxiamtely one third identity with AACT1 and AACT2 at the protein level.

In an attempt to complement the aact2-1 mutant embryo lethal phenotype, we failed to recover aact2-1 homozygote progeny of aact2-1 heterogynous plants transformed with the pMDC32-based plasmid harboring the full-length AACT2 cDNA under the control of 35S promoter after PCR-screening 61 independent T1 transgenic plants and 96 T2 progeny from 8 independent T1 plants. One possible explanation is that 35S promoter was not active during the early stages of embryogenesis in Arabidopsis, which was proved by fusing 35S promoter to the GUS reporter gene in tobacco and Brassica napus (Custers et al., 1999). Another possible explanation is that the regulatory elements of AACT2 gene are essential for its precise spatial and temporal expression, which is critical for AACT2 functioning during the early stages of embryogenesis.
METHODS AND MATERIALS

Plant growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) and T-DNA insertion mutants were acquired from ABRC (Arabidopsis Biological Resource Center, Columbus, OH). Seeds were sterilized with 70% ethanol for 3 min and subjected to 50% bleach, 0.1% tween-20 for 5 min and then washed with sterilized water three times before sowing on Murashige and Skoog agar medium (Invitrogen, Carlsbad, CA) with 1% sucrose or seeds can be directly sowed in LC1 Sunshine Mix soil (Sun Gro Horticulture, Bellevue, WA). After cold treatment for 4 days at 4°C in the dark, plants were grown at 23±2°C under continuous illumination (100 µmol m⁻² s⁻¹). After 10-day-growth on plates, plants were transferred onto soil and cover with saran wrap for 2-3 days.

Recombinant DNA construction

A 5 kb *Arabidopsis* genomic fragment containing the AACT2 gene was cloned into pCAMBIA 1300 for genetic complementation of aact2-1 (*emb1276*) allele. The pair of primers P22 and P23 (see Supplemental table 1 for primer sequences) was used to amplify this fragment from *Arabidopsis thaliana* ecotype Columbia. A full length AACT2 cDNA was cloned into pENTR/SD/D-TOPO and then subcloned into pMDC32 (Curtis and Grossniklaus, 2003) by LR recombination (Invitrogen, Carlsbad, CA). The promoters region including 5’UTRs of the AACT1 and AACT2 genes (1.5kb and 0.8kb, respectively) were amplified by PCR using the pair of primers P11 and P12 and the pair of P13 and P14 from genomic DNA from *Arabidopsis thaliana* ecotype Columbia and cloned into pENTR/D-TOPO and then subcloned into pBGWFS7 (Karimi et al., 2002), respectively. The
vectors were electroporated into *Agrobacterium tumefaciens* strain C58C1 and used to transform *Arabidopsis* plants. The full length *AACT1* and *AACT2* cDNA were cloned into pENTR/D-TOPO and then subcloned into pDEST52, respectively. The vectors were electroporated into yeast BY4743 YPL028W from ATCC. The pair of primers P7 and P8 was used to amplify *AACT1* cDNA. The pair of primers P9 and P10 was used to amplify *AACT2* cDNA. The resulting PCR products were cloned into pENTR/SD/D-TOPO vector (Invitrogen Corporation, Carlsbad, CA), respectively. Then the AACT1 and AACT2 cDNA fragments were subcloned into pDEST15 expression vector by LR recombination (Invitrogen Corporation, Carlsbad, CA), respectively. The two vectors were electroporated into *E. coli* BL21-AI strains.

**Isolation of T-DNA mutants for *AACT1* (At5g47720) and *AACT2* (At5g48230)**

The T-DNA insertion alleles *aact1-1* and *aact1-2* (Sail_448_H10 and Salk_008505) for *AACT1* (At5g47720) and the allele *aact2-1* (*emb1276*) for *AACT2* (At5g48230) were acquired from the Arabidopsis Biological Resource Center. Primers for identifying T-DNA insertion were designed by the T-DNA Express Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). To verify each T-DNA insertion, both sides of genomic DNA sequences flanking the T-DNA were PCR-amplified and sequenced (see Supplemental table 1 for primer sequences). The structures of these T-DNA-tagged alleles were obtained by aligning these flanking DNA sequences against the *Arabidopsis* genome. The same pairs of primers were performed to genotype a plant. A T-DNA border primer and a gene-specific primer were used to detect the T-DNA tagged allele and a pair of gene specific primers that flank the T-DNA insertion site to detect the wild-type allele.
Plant transformation and selection

Transformation was conducted by Agrobacterium tumefaciens-mediated infiltration method (Clough and Bent, 1998). T1 seeds were collected from dipped plants and selected on MS medium containing 25 µg/ml hygromycin for screening genetic complementation of emb1276 mutant transformants of the AACT2 cDNA construct or genomic AACT2 construct. After 10 days, the hygromycin-resistant seedlings were transferred into soil. For screening the transformants of promoter::GUS fusion and RNAi constructs, T1 seeds were directly sowed on the soil. After 14-day-growth, seedlings were sprayed with the herbicide BASTA (20µg/ml glufosinate ammonium, AgrRvo, Montvale, NJ) once a day for at least three times in total. Seeds from T1 plants were harvested individually and T2 plants were used for further characterization. To confirm the presence of the 35S::AACT2-RNAi transgene in the genome, primers P26 and P27 were designed to amplify a 250-bp sequence of this construct. Forward primer was designed to anneal to sequence of the 35S promoter and reverse primer annealed to 180-bp gene-specific from AACT2.

GUS Staining

Transgenic T2 plants were used for GUS staining and tissues were harvested at different stages of development. Histochemical staining for detecting GUS expression was performed as described (Jefferson, 1987). After staining, chlorophyll was extracted with 70% ethanol and tissues can be kept in 70% ethanol at 4°C. Ten transgenic independent lines and three plants for each line were analyzed for each construct and one representative from each construct transformat was selected for photographing.

PCR and RT-PCR analysis
RNA samples were extracted from freshly collected leaves of 6-week-old plants using the RNease plant mini Kit (Qiagen Inc., Valencia, CA). One microgram of total RNA was treated with RNase-free DNase I (Invitrogen Corporation, Carlsbad, CA) followed by inactivation of the DNase I. Reverse transcription was conducted using Supertranscriptase II and random hexamer (Invitrogen Corporation, Carlsbad, CA). Genomic DNA was extracted from the leaves of 3-week-old plants using shorty buffer (0.2M pH 9.0 Tris-HCl, 0.4M LiCl, 25mM EDTA, 1% SDS). PCR amplification was performed with Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA). PCR condition is 95°C for 2 min, followed by 25-35 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72°C for 1-5 min. The resulting PCR products were separated and analyzed by agarose gel electrophoresis.

**Protein purification and preparation of AACT1 and AACT2 antibodies**

Recombinant AACT1 and AACT2 His-tag fusion proteins were affinity-purified via their tags from *E. coli* BL21-AI strains harboring pDEST15-(AACT), grown in the presence of 0.2% L-arabinose. Recovered proteins further purified via SDS-PAGE. The recombinant protein bands was excised from gels, crushed in PBS solution and used to immunize mice to generate antibodies against each protein.

**Protein gel blot analysis**

Total protein was extracted in a buffer consisting of 01.M Hepes-KOH pH 7.0, 20 mM 2-mercaptoethanol. 0.1% (v/v) Triton X-100 1mM EDTA 20% glycerol and plant protease inhibitor (Sigma, St. Louis, MO) added just before use. 30 µg of total protein per lane (Bradford, 1976) were electrophoresed by 10% SDS-PAGE (Laemmli, 1970) and subsequently electrotransferred to a nitrocellulose membrane using Mini-PROTEAN II Electrophoresis (Bio-Rad Laboratories, Hercules, CA). AACT proteins were identified with
antibody raised in mice against recombinant *Arabidopsis* AACT1 protein, using a HRP-conjugated secondary antibody (Biorad Laboratories, Hercules, CA) and the chemiluminescence immunodetection detection kit (Pierce, Rockford, IL).

**Cytological analyses of pollen**

For *in vivo* germination assay, limited pollens were applied to *ms1-1* stigmas as described by Eric Lalanne (Lalanne et al., 2004). 18 hours after pollination, stigmas were excised and transferred onto microscope slides and stained with Alexander staining. More than 200 pollens on 20 stigmas were analyzed for pollens from 6 wild type and 6 *aact2-1* heterozygous plants, respectively. Pollen germination was scored under bright field microscopy. For *in vitro* germination, pollens from fully open flowers were directly released on plates with 30ml pollen germination medium (pH 7.0) containing 18% sucrose, 0.01% H$_3$BO$_3$, 1mM CaCl$_2$, 1mM Ca(NO$_3$)$_2$, 1mM MgSO$_4$ and 0.5% agar by gently vibrating the forceps that holds a flower (Li et al., 1999). Then the plates were sealed and incubated for 6 hours at 22°C under continuous illumination. Germination was directly scored on plates under a Zeiss Axiovert inverted microscope.

Alexander and DAPI staining were used to assay pollen viability. Fully open flowers were collected from heterozygous *aact2-1* plants and wild-type siblings. 6 flowers were collected in an eppendorf tube with 200 µl of staining solution. After Alexander’s staining for 10 min, viable pollens stained red to purple and nonviable pollen stained light blue. Pollens were stained for 30 min in the dark with 1 µg/ml DAPI solution to view nuclei by UV epi-illumination. After briefly vortexing and centrifugation, 20µl solution with the pollen grains were transferred to a microscope glass slide and observed with bright field microscopy (Alexander's stain) and UV epi-illumination (DAPI). 100 to 200 pollens stained by DAPI
were examined and about 1000 pollens stained by Alexander stain were scored from 6 heterozygous *act2-1* plants and 6 wild-type siblings, respectively.

Light microscopy was performed on a Zeiss Axioplan 2 light microscope under bright field or UV epi-illumination with a UV filter set consisting of an excitation filter (BP 365/12 nm), a beam splitter (395 nm), and an emission filter (LP 397 nm) for observing the DAPI fluorescent stains. Digital images were acquired using a Zeiss AxioCamHRc digital camera (Carl Zeiss, Inc., Thornwood, NY) and AxioVision 4.3 software.

**Light and electron microscopy**

Plant tissues were fixed in a solution composed of 1% paraformaldehyde and 2% glutaraldehyde in a 65 mM cacodylate buffer at pH 7.2. After 4-hour-infiltration under low vacuum, samples were fixed at 4°C for 48 more hours. Samples were washed three times with 65 mM Cacodylate buffer, pH 7.2, then post-fixed in 1% buffered OsO₄ for 1 hour. Post-fixed samples were washed with Cacodylate buffer overnight and then en bloc stained by 3% uranyl acetate for 1 hour. Following ethanol series dehydration (25, 50, 70, 90, 100), then samples were serially infiltrated with acetone, acetone: Spurr’s resin (3:1), acetone: Spurr’s resin (1:1), acetone: Spurr’s resin (1:3), finally embedded in 100% Spurr’s resin (EM Sciences, Fort Washington, PA) and polymerized by incubation at 60 °C for 24 hours and then 70 °C for 24 hours.

For light microscopy analysis, samples were sectioned with a Reichert Ultracut S Ultramicrotome (North Central Instruments, Minneapolis, MN), one µm-thick sections were stained with 1% toluidine blue O, and observed under bright-field optics on a Leitz orthoplan light microscope (Sciscope; Leica, Iowa City, IA).
For transmission electron microscopy analysis, silver or golden sections (40-80nm thickness) were obtained with the same ultramicrotome and placed onto 200 mesh cooper grids, stained with 5% uranyl acetate in methanol for 15 minutes and Sato’s lead stain for an additional 15-minumte (Sato, 1967) and observed and images were taken with a JEOL 2100 scanning transmission electron microscope (Japan Electron Optics Laboratory, Peabody, MA) at 200KV accelerating voltage.

For scanning electron microscopy analysis of pollen, fully open flowers were processed in the same procedure that was used for fixation, post-fixation, en bloc staining and dehydration as described above. Then samples were critical point-dried in a DCP-1 Denton critical point-drying apparatus (Denton Vacuum Inc., Cherry Hill NJ) with liquid CO₂. After the samples were mounted on aluminum stubs with double-sided sticky tape and silver cement and sputter-coated with the 20/80 gold-palladium alloy in a Denton Vacuum LLC Desk II Cold Sputter Unit (Denton Vacuum Inc., Moorestown, NJ) for 120 seconds, they were viewed under SEI mode on a JEOL 5800 LV scanning electron microscope at 10KV (Japan Electron Optics Laboratory, Peabody, MA).

Olympus SZH10 research stereomicroscope mounting with an AxioCam HRC digital camera was used for capturing digital stereimages of Arabidopsis seeds.

**Sterol extraction and analysis**

50mg Plant material was homogenized in the presence of liquid nitrogen and was extracted at 75°C for 60 min with 4 mL solution consisting of chloroform:methanol (2:1, v/v, containing 1.0 µg epi-cholestanol as an internal standard). Solvents were dried under nitrogen stream, and the residue was saponified at 90°C for 60 min in 2 mL 10% (w/v) KOH in methanol. After cooling, 1 mL H₂O and 3 mL hexanes were added and the mixture was vortexed
vigorously for 20 seconds. After centrifugation, the hexane phase was transferred to a glass test tube, and the aqueous phase was re-extracted with 3 mL hexane two more times. The combined hexane phase were evaporated to 1 mL and then was derivatized using 100 µL of N,O-Bis (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA/TMCS) at 65 °C for 30 minutes. Solution was dried under nitrogen stream and the residue was reconstituted in 200 µL chloroform. The sample was subjected to GC/MS analysis. GC-MS analyses were performed on an Agilent 6890N GC coupled to an Agilent 5973 MSD detector equipped with a HP-5 MS fused silica column (30 m×250 µm; 0.25 µm film thickness) (Agilent Technologies, Santa Clara, CA). The temperatures of the injector and MSD interface were both set to 280 °C. Helium (1.0ml/min) was used as carrier gas. The temperature gradient was programmed from 150 to 280 °C at 10 °C/min, and then to 300°C at 20 °C /min, isothermal at 300 °C for 5 min. Peaks were deconvoluted using AMDIS software. Analytes were identified based on their mass fragmentation patterns by comparison with those of authentic standards using the NIST Mass Spectral Search Program.

**Biochemical complementation**

To complement AACT2 RNAi phenotype, wild-type and RNAi plants were grown on 30ml of MS media in plate or 60ml of MS media in magenta boxes with different chemicals. At 16 and 42 DAI, plants were collected for phenotype analysis. Biochemicals that were used for complementation include the following: 1mM Mevalonate, 5-30µM cycloartenol, 1µM squalene, 5µM sitosterol, stigmasterol, campesterol mixture, 5µM sterol-PEG600.

**Complementation of Yeast \*Aerg10\* mutant**

Transformed heterozygous diploid yeast cells were forced to sporulate to obtain transformed haploid cells by dissecting tetrads on minimal medium SC (-Ura, 2% galactose instead of
glucose). Haploid yeast transformants of mutants harboring the AACT genes were streaked out on complete YPD induction medium and YPG non-induction medium at 30°C to test functional complementation of the Arabidopsis AACT genes.

**Stress test**

For NaCl and mannitol treatment, 1-week-old seedlings were transferred to MS agar medium without or with 100 mM NaCl or 300 mM mannitol and incubated for 14 more days until collecting for scoring the phenotype. For cold treatment, 1-week-old seedlings were transferred to the cold room and keep them at 4°C until harvesting. For heat treatment, 1-week-old seedlings were exposed to 37°C for 2 hours and then returned to the standard growth conditions. For drought stress, 3-week-old plants lasted for one week without irrigation. For UV stress, 1-week-old seedlings were irradiated for 20 min with 4 florescent tubes of 0.15 W m⁻² and then returned to the standard growth conditions.

**Cell size measurement and cell number counting**

Three images of cross sections of the fully expanded rosette leaves from 6-week-old plants and three images of cross sections of the first internode stems from 8-week-old wild-type plants and AACT2-RNAi siblings were used for cell size measurement, respectively. For each leaf cross section micrograph, 10 upper epidermal, 15 spongy, 20 palisade and lower epidermal cells were measured; for each stem cross section micrograph, 15 epidermal, 50 cortex, 30 phloem, 50 xylem and 40 pith cells were measured with software Analysis. For counting cell number of stem cross section, a 60° angle was drawn from the center of stem, including one vascular bundle and the cells were counted inside this angle.
ACKNOWLEDGMENTS

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REFERENCES

Carrie, C., Murcha, M.W., Millar, A.H., Smith, S.M., and Whelan, J. (2007). Nine 3-ketoacyl-CoA thiolases (KATs) and acetoacetyl-CoA thiolases (ACATs) encoded by five genes in Arabidopsis thaliana are targeted either to peroxisomes or cytosol but not to mitochondria. Plant Mol Biol 63, 97-108.


### TABLES AND FIGURES

Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Fragments amplified or purpose</th>
<th>Primer name and sequence</th>
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<tr>
<td>To amplify different portion of At5g47720 mRNA fragment</td>
<td>P1: TCAAGCATAAGTGATGGTGCG</td>
</tr>
<tr>
<td></td>
<td>P2: TTGAGCCGTTCCAGGATCTAATC</td>
</tr>
<tr>
<td></td>
<td>P3: GTCCCTGGATTTGCTGAGAG</td>
</tr>
<tr>
<td></td>
<td>P4: GAAATCTCCGCTCATTTTCTAAC</td>
</tr>
<tr>
<td>To amplify 18S rRNA as total RNA control</td>
<td>P5: CATGCAATGTGTAAGTATGAAC</td>
</tr>
<tr>
<td></td>
<td>P6: AGAGGTCAGTCTGTCATACAT</td>
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<tr>
<td>To amplify AACT1 cDNA for pENTR vector cloning</td>
<td>P7: CACCAGGAATGTGTAAGTATGAAC</td>
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<tr>
<td></td>
<td>P8: TCAGAGGTCAGGATGAGGAC</td>
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<tr>
<td>To amplify AACT2 cDNA for pENTR vector cloning</td>
<td>P9: CACCAGGAATGTGTAAGTATGAAC</td>
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<tr>
<td></td>
<td>P10: TTTACCAACCTGGATTCATAAT</td>
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<td>To amplify AACT1 promoter</td>
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<tr>
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<td>P12: GTTTTCGAGGAGGAGGAG</td>
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<tr>
<td>To amplify AACT2 promoter</td>
<td>P13: CACCAGGAATGTGTAAGTATGAAC</td>
</tr>
<tr>
<td></td>
<td>P14: TTTTTCGAGGAGGAGGAG</td>
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<td>To amplify wild-type allele for AACT1</td>
<td>P15: AAGCTATAAAGCGGGCTGG</td>
</tr>
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<td></td>
<td>P16: TCAAGGACAAGTGCTGAGG</td>
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<tr>
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<td>P17: TTTTGCGGGATATCTGTTTC</td>
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<tr>
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<td>P18: TCAGGTCATAAATGAGGAGG</td>
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<td>To amplify wild-type allele for AACT2</td>
<td>P19: ACTCCAATGGGTGCTCTTC</td>
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<td></td>
<td>P20: CACGCTCAAACGCTGAAGC</td>
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<td>P21: TCAAGGACAAGTGCTGAGG</td>
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<tr>
<td>To amplify AACT2 5-kb- AT5g48230-genomic DNA</td>
<td>P22: GGATCCTACTTGAGGGAGG</td>
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<tr>
<td></td>
<td>P23: GGATCCTACTTGAGGGAGG</td>
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<tr>
<td>To amplify wild type AACT2 allele from plants transformed with 5-kb-AT5g48230-genomic DNA</td>
<td>P24: CCCTTATGGAATTCTTTC</td>
</tr>
<tr>
<td></td>
<td>P25: GCCTTATGGAATTCTTTC</td>
</tr>
<tr>
<td>To identify At5g48230 RNAi transgene</td>
<td>P26: CGCAATTCCTACCACAT</td>
</tr>
<tr>
<td></td>
<td>P27: CACTCCTCCCACACGGA</td>
</tr>
</tbody>
</table>

Figure 1. Alignment of AACT2 (AT5g48230) and AACT1 (AT5g47720). Black shading indicates identical residues and gray shading indicates similar residues.

| AACT2 | 1  ------  NNMECDVCQGVARALCPIGFLGSLSLPAKLCSAAALK |
| AACT1 | 1  MAPPVSSDDSSQPRDVCQGVARALCPIGFLGSLSLPAKLCSAAALK |
| AACT2 | 44 RAYVDPALVECVFVFNVLVNLGQAPARKQALGAGLPVSICTEKNVCA |
| AACT1 | 51 RAYVDPALVECVFVFNVLVNLGQAPARKQALGAGLPVSICTEKNVCA |
| AACT2 | 94 SGKIANMVAAOSIQVGDVQAVAGMSMSNTPKVLAAKRGSRCHDSL |
| AACT1 | 101 AGKIANMVAAOSIQVGDVQAVAGMSMSNTPKVLAAKRGSRCHDSL |
| AACT2 | 144 VDCMHDGLWDYNGDCGMSCABECAKEQITRECDAYAVSFERGIA |
| AACT1 | 151 VDCMHDGLWDYNGDCGMSCABECAKEQITRECDAYAVSFERGIA |
| AACT2 | 194 CEAGNAFTVETVPEVSCGGRPSGTIDKDBGLKPKDAALKKLRPSFEN |
| AACT1 | 201 CMQKEMARTVETVPEVSCGGRPSGTIDKDBGLKPKDAALKKLRPSFEN |
| AACT2 | 244 GCGVTAGNASSISDGAALVVLVSHEQCLGLVIAKKGYDAAQPEF |
| AACT1 | 251 GCGVTAGNASSISDGAALVVLVSHEQCLGLVIAKKGYDAAQPEF |
| AACT2 | 294 FTRPPALAPDKAHCEGSQVYRINEAPVVALANQKLLCAPEKV |
| AACT1 | 301 FTRPPALAPDKAHCEGSQVYRINEAPVVALANQKLLCAPEKV |
| AACT2 | 344 NUNGAVSGLHFPLGCSARILTLGLLXENKGYGVCWCGNGGGSASAL |
| AACT1 | 351 NUNGAVSGLHFPLGCSARILTLGLLXENKGYGVCWCGNGGGSASAL |
| AACT2 | 394 VLLEL  ------  |
| AACT1 | 401 VLLFSEKTI GYAS |

Figure 2. Growth of wild-type yeast and \(\Delta erg10\) mutants with AACT1 and AACT2 individually on induction medium (YPG) and non-induction medium (YPD).
Figure 3. Representative staining patterns of transgenic plants expressing promoter::GUS fusions for each of AACTs. 8-day-old seedling for AACT1 (a) and AACT2 (b). Roots of 8-day-old seedling for AACT1 (c) and AACT2 (d). GUS staining for AACT1 and AACT2 in inflorescences (e) and (f), buds (g) and (h), flowers (i) and (j), siliques (k) and (l) and whole siliques of different growth stages (m) and (n), top stems (o) and (q), stems of the first internode (p) and (r), young leaves (s) and (u) and fully expanded leaves (t) and (v) from 6-week-old plants, respectively.
Figure 4. Protein levels of *AACT1* and *AACT2*.
(a) Western analysis of AACT1 and AACT2 proteins in different organs with AACT1 antibody. (b) Total protein loading amount control stained with Coomassie blue. (c) Western results of purified recombinant AACT1 and AACT2 protein.
Figure 5. Gene structure of AACT1 and characterization of aact1-1 and aact1-2 alleles.
(a) The predicted AACT1 structure is composed of 13 exons (black boxes) and 13 introns (black lines). 5' and 3' UTRs are shown by white boxes. T-DNA insertions of both alleles are indicated by the gray triangles. The primers shown were used to amplify the different portion of AACT1 mRNA. (b) RT-PCR analysis of both aact1 T-DNA insertion alleles (WT: wild-type; Hm: homozygote). 18S rRNA was used as an internal control. (c) Western analysis of both alleles. Total protein was extracted from 6-week-old plants. (d) Total protein loading amount (30 µg/lane) stained with Coomassie blue.
Figure 6. Gene structure of AACT2 and characterization of acct2-1 allele.
(a) The predicted AACT2 structure is composed of 11 exons (black boxes) and 11 introns (black lines). 5’ and 3’ UTRs are shown by white boxes. T-DNA insertion ofacct2-1 allele is indicated by the gray triangles. (b) and (c) Representative siliques from wild-type (b) and from acct2-1 heterozygous plants (c). Arrows show aborted brown seeds. (d) and (e) Isolated seeds from wild-type and from acct2-1 heterozygous plants. Arrows show aborted seeds. (f) Percentage of aborted seeds from wild-type and acct2-1 heterozygous plants. (g) and (h) Germination of pollen grains from wild-type and acct2-1/+ heterozygotes on pistils of ms1-1/ms1-1 plants, stained with Alexander’s staining 18 hours later after pollination, respectively. Blue and purple arrows show germinated and non-germinated pollen, respectively. (i) The frequency of germinated and non-germinated pollen from wild-type and acct2-1 heterozygous plants. Bar represent ±S.E..
Figure 7. AACT2 expression is reduced in AACT2-RNAi plants that show a defective phenotype. (a) Representative 6-week-old wild-type and AACT2-RNAi plants of mild, moderate, severe and very severe phenotype. (b) Western analysis of AACT2 protein level in wild-type and AACT2-RNAi plants. Total protein was extracted from inflorescence of 6-week-old plants. (c) Total protein loading amount (30 µg/lane) stained with Coomassie blue.
Figure 8. AACT2-RNAi line AACT2i-1 exhibits pleiotropic phenotypes. (a) 6-week-old wild-type and AACT2i-1 plants. (b) 4-week-old wild-type and AACT2i-1 plants. (c) Bolt height of 20-week-old plants (n=8 for wild-type and n=39 for AACT2i-1). (d) Arrangement of all leaves from 4-week-old plants. (e) The first internode stems from 6-week-old plants. (f) Inflorescences from 6-week-old plants. White arrow shows aborted bud and red arrow shows aborted silique. (g) Flowering length (n=8 for wild-type and n=39 for AACT2i-1). (h) Seed number per silique. (i) Flowering length (n=8 for wild-type and n=39 for AACT2i-1). (j) Representative siliques from 6-week-old plants. (k) Isolated seeds from wild-type plants. Arrows show deformed seeds. (l) Isolated seeds from AACT2i-1 plants. Bar represents ±S.E..
Figure 9. Reduction in AACT2 results in smaller cell size and reduced cell number. (a) and (b) Micrographs of cross sections of the first internode stem from 8-week-old plants. (c) and (d) Micrographs of cross sections of fully expanded leaves from 6-week-old plants. (e) Cell number of a 60° angle of stem cross section. (f) Cell length of different tissue cells. Bar represents ±S.E.
Figure 10. Reduction of AACT2 expression leads to faster degeneration of tapetum cells. (a), (c) and (e) Three oldest buds from wild-type plant inflorescence. Arrow shows tapetum cells still present in stage 2 anther. (b), (d) and (f) Three oldest buds from AACT2i-1 plant inflorescence. Arrow shows tapetum cells are fully degenerated in anther of stage 2. Three buds from each stage were examined and nine buds from each genotype were analyzed.
Figure 11. Suppression of AACT2 expression results in loss of pollen coat. (a) and (b) Transmission electron micrographs of pollens from wild-type and AACT2i-1 plants. White arrow shows pollen coat from wild-type plant and black arrow indicates the pollen coat is missing in AACT2i-1 plant.
Figure 12. Mevalonate complements the altered phenotype associated with reduced AACT2. (a) and (b) 16-day-old wild-type and AACT2i-1 seedling grown without 1mM mevaloante (a) or with 1mM mevaloante (b). (c) and (d) 6-week-old wild-type and AACT2i-1 plants grown without 1mM mevaloante (c) or with 1mM mevaloante (d).
Figure 13. Mevalonate treatment results in the restoration of altered sterol accumulation on AACT2i-1. (a) Sterol composition, (b) Total sterol content and (c) Ratio of campesterol:(stigmasterol and sitosterol) from 16-day-old seedling roots of wild-type and AACT2i-1 grown without 1mM mevalonate or with 1mM mevalonate. Bar represents ±S.E.
CHAPTER V. GENERAL CONCLUSIONS

Acetyl-CoA is metabolized via one of three mechanisms, carboxylation, acetylation and condensation. Acetoacetyl-CoA thiolase (AACT) catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. The fate of acetoacetyl-CoA depends on the biological context in which it is generated. In microbes, such as *Rhodospirillum rubrum*, acetoacetyl-CoA is the precursor of the storage polymer polyhydroxyalkanoates (PHA). In the cytosol of plant cells, it is the precursor of mevalonate-derived isoprenoids.

In *R. rubrum*, the AACT enzyme is encoded within the *phaABC1* operon, which is responsible for PHA biosynthesis. Furthermore, *R. rubrum* contains one *phaJ* gene encoding (R)-specific 2-enoyl-CoA hydratase and two additional *phaC1*-like genes, called *phaC2* and *phaC3*. To characterize the roles of these genes in acetyl-CoA metabolism, we have developed an inducible-expression system for individually over-expressing each *pha* gene. Our data clearly revealed that over-expressing *phaC1* and *phaC2* alone significantly enhanced PHA production, whereas over-expressing *phaC3* and *phaJ* alone did not have significant effect on PHA production when *R. rubrum* grew on nitrogen-limiting RRNCO medium. Our data also showed that over-expressing each gene of operon *phaC1AB* alone significantly increased PHA production and over-expressing *phaB* resulted in the increasing of the PHA content the most. Furthermore, we found that the expression level of *phaB* controlled PHA production and that PhaB was the key enzyme of the operon *phaC1AB* PHA biosynthetic pathway. In addition, our data suggested that *phaB* was subject to post-transcriptional regulation when transcribed in the whole operon form. Under these conditions, strain AB-9 over-expressing *phaA* and *phaB* accumulated 30% of cellular dry weight, 2.5 times of control strain’s PHA content.
In addition, we investigated the redundancy of three paralogous \textit{phaC} genes and the function of \textit{phaJ}. We found that PHA was required for \textit{R. rubrum} optimal growth when grown on acetate as carbon source and that PHA enhanced bacteria growth in the stationary phase when grown on hexanoate as carbon source. It is found that PhaC2 is the major enzymes for synthesize PHA \textit{in vivo} when grown in either carbon source and responsible for integrating 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) monomers into polymer and that PhaC1 encoded by \textit{phaCAB} operon locus and PhaC3 are specific for integrating 3HB into polymer. In addition, our data indicated that PhaC1 and PhaC3 interacted with each other. We demonstrated that PhaJ was responsible for converting trans-2, 3-enoylacyl-CoA into (R)-3-hydroxyacyl-CoA \textit{in vivo}. Furthermore, our results revealed that presence of 3HHx monomer in PHA results in continuous accumulation of PHA in \textit{R. rubrum}.

Genomic analyses revealed two \textit{AACT} genes in the \textit{Arabidopsis} genome, At5g47720 (\textit{AACT1}) and At5g48230 (\textit{AACT2}). These two genes code for proteins that share 78.4\% sequence identity. To study the physiology function of each AACT, two T-DNA insertion alleles \textit{aact1-1} and \textit{aact1-2} at \textit{AACT1} gene and one T-DNA insertion allele \textit{aact2-1} at \textit{AACT2} have been isolated and characterized. RT-PCR and western blot analyses showed that both \textit{aact1-1} and \textit{aact1-2} alleles were null alleles. To test whether mutation of \textit{AACT1} affects transmission efficiency of both alleles, segregation analysis was performed by PCR-genotyping the progenitors from one heterozygous plant. Both \textit{aact1-1} and \textit{aact1-2} alleles exhibited normal Mendelian ratio inheritance. In addition, we were unable to observe visible phenotype of both \textit{aact1-1} and \textit{aact1-2} mutants under the growth conditions and stress conditions used in this study. Moreover, analysis of phytosterol showed no significant
phytosterol changes were detected in the both \textit{aact1-1} and \textit{aact1-2} mutants. In contrast, we found that the disruption of \textit{AACT2} resulted in embryo lethality and reduced transmission efficiency of \textit{aact2-1} allele through male gametophyte result from lower \textit{in vivo} mutant pollen germination rate by characterizing the T-DNA insertion allele \textit{aact2-1} and complementation of heterozygous plants with a 5 kb genomic DNA containing \textit{AACT2} with its native promoter. To address the question that \textit{AACT2} is an essential and cannot be replaced by \textit{AACT1}, we generated promoter::GUS fusion transgenic plants for each gene to examine the spatial and temporal expression pattern of the two \textit{AACT} genes in tissues and organs, assessed the expression patterns of two \textit{AACT} genes by western blot using total protein from different organs (leaves, roots, stems, inflorescences and siliques) and examine both AACTs activities at enzymatic level by complementation of yeast \textit{Δerg10} (ortholog of \textit{Arabidopsis} AACT) mutant with each AACT open reading frame. Our data showed that both AACTs were active at enzymatic level. The AACT1 promoter::GUS construct transformants showed strong GUS staining in vascular system of various organs. In contrast, AACT2 is mainly expressed in root tips of seedlings, the aerial parts of seedlings, new emerging leaves of seedlings, young leaves and stems of adult plants and exclusively expressed in anther of the bud at the microspore stage. Furthermore, the expression levels of AACT2 were much higher than those of AACT1 in leaves, stems, inflorescences and siliques, whereas in roots, both genes show similar expression levels. The different expression pattern of these two genes and higher expression level of AACT2 explains the nonequivalent roles of these two genes in plant growth and development.

Because the mutation of AACT2 gene resulted in embryo lethality, were generated transgenic RNAi lines to further study the role of AACT2 in plant growth and development.
AACT2-RNAi lines showed pleiotropic phenotypes, including reduced apical dominance, early senescence, elongated life span and flowering duration, sterility, dwarfing, reduced seed yield and shorter root length. We found that dwarfing was caused by smaller cell size and loss of pollen coat resulted in sterility. The cosegregation of the altered phenotype and the AACT2-RNAi transgene and strongly reduced AACT2 protein in AACT2-RNAi lines confirmed that the morphological change resulted from reduced AACT2 activity. The results were further confirmed by the chemical complementation. These phenotypes were rescued when they were grown in the presence of mevalonate, which is a derivative of acetoacetyl-CoA. These phenotypic changes of AACT2-RNAi lines can be explained by reduced supply of membrane sterols and alternation of sterol composition revealed by phytosterol analysis.

In combination, these studies provide the new insight of the role of AACT in the acetyl-CoA metabolic network.
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