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The 5th IBC 2012

Santosa Hotel, Senggigi Beach
Mataram, Lombok Island
Province of West Nusa Tenggara, Indonesia
July 4th -7th 2012

The 5th Indonesia Biotechnology Conference
An International Forum

"Green Industrial Innovation through Biotechnology"

PROCEEDINGS

published by:
Indonesian Biotechnology Consortium
2012

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2012

Series Description

Indonesia Biotechnology Conference is an annual conference held by Indonesian Biotechnology Consortium. This year is the 5th year the conference being held by Indonesian Biotechnology Consortium. The fifth conference's theme is "Green Industrial Innovation through Biotechnology" and the topics are Agriculture & Forestry Biotechnology, Health & Medical Biotechnology, Energy and Environmental Biotechnology, Marine Biotechnology and Industrial Biotechnology. The proceeding book will consist of academic papers presented in the conference.

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- Agriculture & Forestry Biotechnology
- Health & Medical Biotechnology
- Energy and Environmental Biotechnology
- Marine Biotechnology
- Industrial Biotechnology

September 2012

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Province of West Nusa Tenggara, Indonesia

Indonesian Biotechnology Consortium (KBI)

September 2012

The 5th
Indonesia Biotechnology Conference
An International Forum

"Green Industrial Innovation through Biotechnology"

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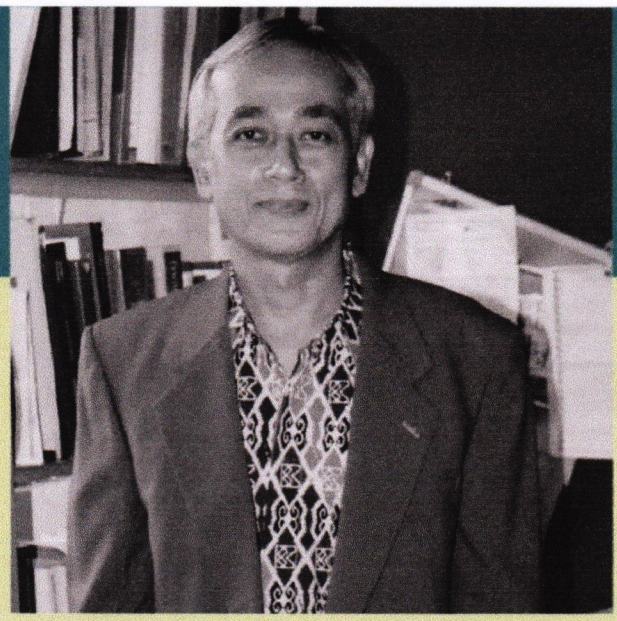


Indonesia has an important position in terms of global biodiversity, since it is one of the ten countries with the richest biodiversity, often is known as *mega diversity country*.

If the diversity of coral reef species Indonesia, or the soil and cave biota as well as their associated organisms are taken into account, Indonesia could well be on the top of the list in terms of biodiversity richness. The geological history and topography supports its biological diversity and uniqueness. For instance, Indonesia is an archipelagic country located in the biodiversity distribution path of the Asian continent (Java, Sumatera and Kalimantan islands) and Australia (Papua), and in the transitional zone of the wallace line (Sulawesi, Maluku and Nusa Tenggara islands), and therefore harbors the biological richness of Asia, Australia and the transitional zone of the two continents. The geological history of each island in Indonesia gave rise to the climate variations, which is wet in the western part and drier in the eastern part, thus influencing the ecosystem formations and flora and fauna distribution.

With the microbial diversity richness and molecular biology tools will be known the potency for conserve in the future. However, further study, assessment and application are still required to develop the potency of microbial diversity for the industrial application.

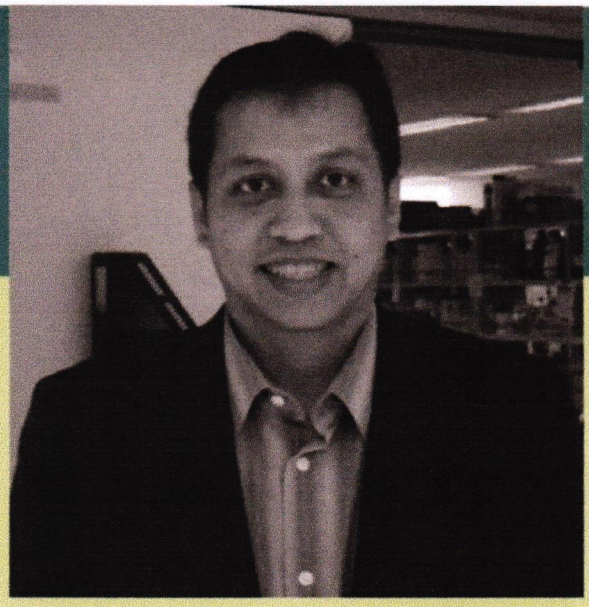
Biotechnology refers to the large and growing array of scientific tools that use living cells and their molecules to make biological products for many different industries. Human and animal health care, agriculture, forestry, environment, and specialty chemicals are among the industries that have benefited most from biotechnology. The economic promise of biotechnology is extraordinary. At present a $60 billion sector worldwide, it is estimated to become a market of at least $120 billion annually within 10 years. Although this is a high-growth sector, moving a promising research discovery to market is a complex, costly and challenging undertaking.

In this proceeding we have identified and addressed challenges that are unique to a biotechnology startup. The approach used to collect information on biotechnology research involving biotechnologiest which includes government, businessmen, researchers and industry.

Hopefully this proceedings can be useful for the development of science and technology and as a means of of effective information dissemination of biotechnology development.

Siswa Setyahadi
Chief Editor

Chairman of Organizing Committee
Misri Gozan



Dear distinguished guests and participants,

We cordially welcome you to the 5th Indonesia Biotechnology Conference (IBC V) in Senggigi Beach, Lombok Island. This IBC V is organized by the Konsorsium Bioteknologi Indonesia (Indonesian Biotechnology Consortium) supported by Universitas Mataram (UNRAM), Universitas Indonesia (UI), LIPI, and all 50 KBI's members. We are sincerely grateful to welcome honorable keynote speakers, distinguished invited speakers and excellent all participants. Today, more than 300 participants from Sumatra, Jawa, Bali, Kalimantan, Sulawesi, Nusa Tenggara Barat and many more gather in this place. We are also having scientists from several different countries, among them are from Japan, Korea, India, Pakistan, Bangladesh, Srilanka, Germany, the United States, Australia and others. We believe that IBC V has become truly a melting pot of scientists and engineers to nurture the Biotechnology development for the benefit of humankind.

As global warming unavoidably touches various aspects of human life immensely, managing earth resources in sustainable manner has never been more important. The wide spectrum of green, red, blue and white biotechnology has been providing answers to many industrial problems. Therefore, the theme "Green Industrial Innovation through Biotechnology" was selected for this conference.

We hope IBC V will be a journey through many inovations in the field of agricultural, health, medical, energy, enviromental, food, and industrial biotechnology. Through green industrial inovation, biotechnology will keep playing important roles in positive ways for the future.

On behalf of the organizing committee, I gratefully thanks many sponsors for supporting the 5th Indonesia Biotechnology Conference. We thank all KBI members that make this event happens and meaningfull. In this ocassion, let me express my special thanks to UNRAM which is one of the youngest member of KBI but has been giving a tremendous support to this event. Most importantly and above all, we thank God for giving us this earth, life and blessing us with everything on them.

I hope we all have fruitfull discussion while sharing knowledge, trends, and products during the conference, exhibition, and meeting occasions. To all participants, please also enjoy the venue, the food, the tradition, the trip and the hospitality of people in Lombok Island!

Through the 5th Indonesia Biotechnology Conference, let us genetically engineer the prosperity of future generations!

Assoc. Prof. Misri Gozan
Chairman of Organizing Committee

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President of KBI

Bambang Prasetya

First of all, let us pray to God the Almighty for His blessings bestowed on us so that we can be here to attend the 5th Indonesia Biotechnology Conference, an International Forum "Green Industrial Innovation Through Biotechnology", in Mataram 4th-7th July 2012 Lombok Island Indonesia. On behalf of the chairman of the Indonesian Biotechnology Consortium (KBI), I am delighted to convey my warmest welcome to foreign delegates, speakers and participants.

As we are all aware we are living in the era, which we have to increase our effort to make significantly contribution to emerging and reemerging problem in related environment, food security, health and energy security, and green technology as well. Many scientific of the world are confident that biotechnology are one of powerfull tools facing these issues and move forward to achieve for a better live and prosperity. Success stories that in many countries biotechnology have been a driving factor to accelerate the sustainable development and achieve the Millenium Development Goals.

Based on this fact, the theme in this conference is focused on "Green Industrial Innovation Through Biotechnology", This theme is directly related to the goals of KBI, such as the establishment of a Consortium of Biotechnology to accelerate meeting the basic human needs in Indonesia. The availability and also the accessibility of health care services and adequate food supply are very important and critical in achieving human prosperity. It is recognized that food and health is essential, not only for survival, but more important, for achieving quality of life.

Due to their priority in human needs, research and study in the field of food and medical care has to be increased and improved. To ensure the availability and accessibility of food and health services, the role of biotechnology must be enhanced.

Therefore, some research fields are the focus of the Indonesian Consortium of Biotechnology, such as food and medical biotechnology, forestry and agricultural biotechnology, environmental and industrial biotechnology.

In Indonesia, there are so many cities which are surrounded by industrial activities. At any time the development of industrial company can not be prevented, but we can try to give as much beneficial effect as possible to human beings. Application of Green Industrial system is one method for improving the ecosystem. Therefore, the green industrial innovation through Biotechnology is important to be discussed and hence to be implemented in Indonesia.

This conference is important since it has provided us, specifically for scientists, and biotechnologists a medium for exchanging ideas to study recent developments in biotechnology from a local, regional and international perspective. Also, we have chance to align our perceptions on the direction and main priorities of biotechnology development. Just for your information that one of the important missions of KBI is to strengthen the development of biotechnology in Indonesia through the strengthening of collaboration, networking, information sharing, exchanging of scientific resources, human resource development, facilitation as well as coordination of collaboration between members and institutions from overseas.

In this meeting, we are happy to have an opportunity to listen to and discuss with the group of experts in the area of biotechnology. I would like to congratulate the Organizing Committee for holding such an important conference. To the distinguished participants from overseas, I wish to welcome you all to Indonesia, and I hope you all, including the domestic participants, have a fruitful conference. To conclude my remarks, on behalf of the chairman of Indonesian Biotechnology Consortium, I hope the seminar and conference run effectively in achieving all their goals.

Finally, a hearty welcome awaits all of you to explore the beauty nature and exotic culture in Lombok Island.

Prof. Dr. Ir. Bambang Prasetya
President of KBI
(Indonesian Biotechnology Consortium)
Board of 2010-2014

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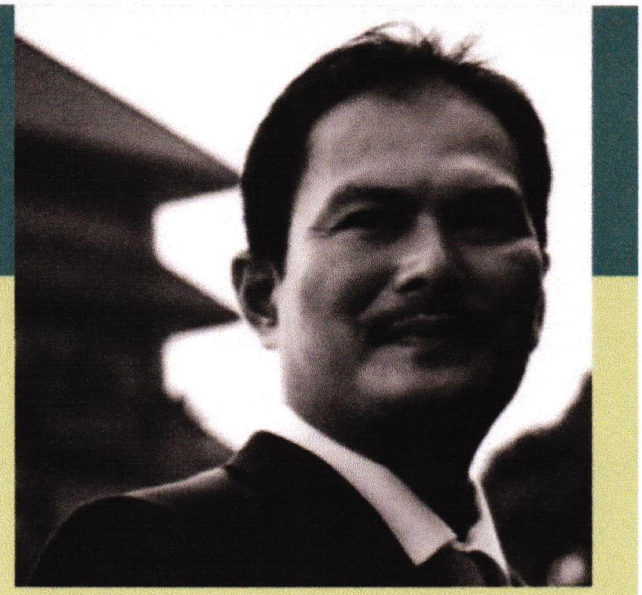
Rector of Universitas Indonesia
Gumilar R. Somantri



The world is facing numerous challenges that will likely continue to happen in the future. First, the demographics of the world are alarming because of human population growth is uncontrolled. Second, the imbalance between supply and demand for food and agricultural products would continue to increase. Third, the energy crisis caused by the consumption of renewable energy in large quantities. Then, last but not least, is the impact of technological and industrial development on the environment.

In answer to these global challenges, biotechnology has a strategic role and position. This is evidenced by the rapid growth in biotechnology research, and many products of biotechnology that have been benefitting mankind in various aspects of life, such as agriculture, food, health, environmental, and other industries.

Universitas Indonesia is becoming a world-class research university. Since the year 2010 UI form clusters of 3 disciplines, namely cluster of Health Sciences, cluster of Science and Technology, as well as cluster of Social Sciences and Humanities. Development and utilization of biotechnology researches at the Universitas Indonesia, which is a multidisciplinary activity, are currently running in an encouraging atmosphere. Faculty of Medicine, Faculty of Natural Sciences, Faculty of Pharmacy and Faculty of Engineering have research centers of their superior research-based biotechnology, such as: leading genome research, bioenergy, pharmaceuticals and cosmeceuticals. Universitas Indonesia have been through the process of internationalization by increasing its funding especially on research cooperation and international publication.

Therefore, Universitas Indonesia gives it's full support for the 5th Indonesia Biotechnology Conference organized by the Indonesian Biotechnology Consortium, where we are a member of it.

We sincerely expect this event to generate more cooperation in research and in education. Such cooperation can lead to progress in all areas of Biotechnology for the welfare of mankind.

My warmest greetings to you all who have the spirit of research. Congratulations and enjoy the conference in the atmosphere of both local and global communities.

Prof. Dr. der Soz. Drs. Gumilar Rusliwa Somantri
Rector of Universitas Indonesia

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Rector of Universitas Mataram
Sunarpi

Distinguished Ministry of Science and Technology - Republic of Indonesia, Governor West Nusa Tenggara, Respected Guests,Keynote speakers, Conference Participants, and all other participants.

It is a great honor for me to address the opening of the 5th Indonesian Biotechnology Conference (IBC), here in Lombok. I would like to take this opportunity to cordially welcome you all to Lombok, an Island in West Nusa Tenggara where the University of Mataram is. Lombok is known for its natural and cultural diversity with beaches, waterfalls, mountain, traditional villages and handicraft of many ethnics including Sasak, Samawa, Mbojo, Balinese, Chinese, Arabic and many others. Therefore, while presenting and sharing ideas in the Conference, participats could experience Lombok and its unique nature and culture.

The IBC Conference is held regularly by the Indonesian Consortium of Biotechnology (KBI). The current event is organized in collaboration with Universitas Indonesia (UI) and University of Mataram (Unram). On behalf of Unram, I would like to express my appreciation to the Steering and Organizing Committees from KBI, UI and Unram for the excellent work and collaboration. I wish such excellent collaboration could be maintained and improved in the future.

My appeciation also goes to the Governoer of West Nusa Tenggara, PT Newmont Nusa Tenggara, NTB Branch of BI, BTN, Bank Mandiri and all others sponsors for their willingness to ssupport this event.

Ladies and Gentlemen, as we are aware that in the scientific conference, keynotes and invited speakers are very important, and I am very pleased that the committee were able to invite and bring knowledgeable keynotes and invited speakers from Indonesia and overseas. Herewith, I would like to acknowledge all of National and International invited speakers for their willingness to come to Lombok, and present their acknowledged works. I understand the time, and efforts, given for this conference, and therefore I would like to express my high appreciation to all keynote and invited speakers. Thanks also to all participants for the high interest in attending this conference. I hope that this conference will be a good forum, not only in communicating and sharing ideas and knowledge in Biotchnology, but also in building and enhancing network of collaboration amongs academia, students and industry.

Finally, I wish you most successful conference, and hope that this may provide new ideas and strategies in biotechnology innovation towards green industry.

Prof. Ir. Sunarpi, Ph.D.
Rector of Universitas Mataram

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O 3

Isolation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase (ACS) Genes from White Clover (*Trifolium repens* L.) Subjected to a Water Deficit Condition

Aluh Nikmatullah

University of Mataram, Jl. Majapahit 62 Mataram, Indonesia
aluhnikma@hotmail.com

Many reports suggest that ethylene is an important hormone regulates various physiologycal proses during plant development as well as a mediator of responses to different environmental cues. The biosynthesis of the hormone involves two key enzymes, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO). ACS is suggested to be the rate limiting enzyme in ethylene biosynthesis. This presentation reports isolation of orthologous as well as a novel, water-deficit associate, ACS genes isolated from white clover. Three of four isolated TR-ACS genes were similar from the previously isolated TR-ACS from Grasslands Challenge genotype 10F while the fourth was a novel gene designated TR-ACS4. All of the TR-ACS genes isolated, including previously reported inactive TR-ACS3, contains 20 amino acids corresponding to the active site of the ACC synthase enzyme, including three conserved amino acid residues (one of which is the catalytic lysine residue where binding to the pyridoxal phosphate is proposed to be occurred). The novel TR-ACS4 is 64%, 64% and 63% homologous to TR-ACS1, TR-ACS2 and TR-ACS3, respectively in terms of nucleotide sequence. In the GeneBank database, TR-ACS4 shares highly homology to ACC synthase sequences including those induced by auxin-, wounding- and ethylene-treated.

Keywords: *isolation, ACS gene, white clover, water deficit*

1. Introduction

Ethylene is an important gaseous plant hormone regulating many processes of plant growth and development [1][2]. It is synthesis *via* two committed steps comprise of the conversion of *S*-adenosyl-$_L$-methionine (SAM) into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS) and the oxidative cleavage of ACC by the enzyme ACC oxidase (ACO) into ethylene. ACS is considered as the rate-limiting enzyme while ACO catalyses the last step of ethylene biosynthesis in plants [2],[3].

It is reported that ACS is encoded by a multigene family [4],[5]. Although various ACS isoenzymes catalyse the same chemical reaction, but each member of the gene family is differentially expressed and regulated during plant growth and development, and in response to different hormonal and environmental cues. For example, nine ACS genes in *Arabidopsis thaliana* are differentially expressed during plant development [4], [6], where all genes, except *ACS9* (which is expressed in mature plants), are expressed in the seedling particularly in the epidermal cell layer, guard cells and the vascular tissue. Similarly, amongst three different *ACS* genes in rose (*Rosa hybrida),* only *Rh-ACS3* is differentially expressed during flower maturation in ethylene sensitive and insensitive cultivars, whilst expression of *Rh-ACS1* and *Rh-ACS2* is similar in both cultivars [7]. Published report on differential *ACS* expression by various environmental and hormonal cues is also accumulating. *Arabidopsis ACS2* and *ACS6,* for example, are induced by various biotic and abiotic stresses [8] while cabbage (*Bra5ssica oleracea*) A*CS2, ACS6* and *ACS7* are induced by ozone stress [9]. In addition,it is reported that promoter activity of *Arabidopsis ACS4* is induced by ethylene but not the *ACS5* and *ACS7*, whilst exogenous application of IAA and jasmonic acid (JA) increased the promoter activity of *Arabidopsis ACS4* and *ACS7,* but not *ACS5*[5]. Moreover, expression of *ACS7* is also induced by Gibberellic acid (GA_3), abscisic acid (AbA), salicylic acid (SA) and brassinosteroid (BR) [10]. Those evidence further supports the suggestion that certain members of the *ACS* gene families are

differentially regulated during plant growth and development as well as in response to different environmental and hormonal cues.

In white clover, three different *ACS* genes have been cloned and the transcripts were shown to be expressed in different developmental stages of the leaves5 [11]. *Tr-ACS1* is specifically expressed in the apical structure of the stolon, in mature green leaf tissues and in leaf tissue at the onset of senescence. *Tr-ACS2* is expressed in the apical structure of stolon and in newly initiated leaves, whilst *Tr-ACS3* is only expressed in the senescent leaf (Murray and McManus, 2005). In this paper, we report the isolation and characterisation of orthologous *Tr-AcS1, Tr-ACS* and *Tr-ACS3* as well as *a* novel *Tr-ACS4* from white clover ecotype of Tienshan. Due to the low abundance of ACS transcripts, water deficit treatment was used to induce expression of *Tr-ACS* allowing isolating the novel *Tr-ACS4* gene from the apical structures of Tienshan ecotype of white clover.

2. Methods.

Plant Materials and Water Deficit Treatment to Isolate Novel *TR-ACS* gene.

Sets of four weeks old clonally-propagated white clover plants of Chinese ecotype, Tienshan, grown in a 10-L capacity pot (2 plants per pot) were transferred into climate rooms in the New Zealand Climate Environment Laboratory (NZCEL), Plant and Food, Palmerston North and acclimated for 1 week in the NZCEL before being exposed to water deficit treatments. The Climate Room conditions were maintained at constant temperatures of 21°C (day) and 14°C (night), constant relative humidity (RH) of 75% and CO_2 at 350 $\mu L\ L^{-1}$ and equipped with 4 x Metal Halide (1.0 kW) and 4 x Tungsten Halogen (1.0 kW) lights providing 650 $\mu mm^{-2}s^{-1}$ PFD with 14 hours photoperiod. which was achieved by complete withholding of water and plants were maintained until petiole elongation rate (PER) in the first fully-expanded leaves ceased. Isolation of *TR-ACS* gene were undertaken at five key points: at fully hydrated, before PER was declined, when PER was declined, at the time before PER was ceased and at the time when PER was

ceased. The PER was measured in the first-fully expanded leaves as the rate of petiole elongation per day. All measurements were conducted daily at 3 to 5 hours after down.

RNA Extraction.

Total RNA was isolated using the Hot-Borate method (Hunter and Reid, 2001) with some modifications. All centrifugation was carried out with speed of 10 800 x g at 4°C. To isolate the RNA, grounded-frozen tissues were transferred into a microtube containing five volumes (w/v) of warm (*ca.* 85°C) extraction buffer [200mM di-sodium tetraborate decahydrate, 500 mM EDTA, 10% (w/v) sodium deoxycholate, 1% (w/v) SDS, 100 mM DTT containing 2% (w/v) PVP-40, 1 % (w/v) IGEPAL CA-630), pH 9.0], mixed Proteinase-K (0.75 %; w/v) added, and incubated at 42°C, with shaking, for 90 min. Immediately after incubation, KCl (total concentration of 160 mM) was added, mixed and incubated in an ice bath and shaken horizontally at *ca.* 100 rpm for 30 min. The extraction mix was then centrifuged and the supernatants transferred to a fresh tube and LiCl (to final concentration of 2 M) added and the RNA was precipitated overnight at 4°C. RNA was pelleted by centrifugation and resuspended in 500 µl of DEPC-treated water prior, sodium acetate (a final concentration of 0.3 M) and 550 µL [1: 1 (v/v)] of chloroform/isoamyalcohol was added. The aqueous and organic phases were separated by centrifugation, the upper aqueous phase was transferred into a fresh sterile microfuge tube and RNA precipated with isopropanol (1:1; v/v) by incubating on ice for 1 h. The RNA was then pelletted by centrifugation, the pellet washed with 500 µL of 80 % (v/v) ice-cold ethanol, air dried for 5 min and resuspended in 500 µL of DEPC-water. To remove genomic DNA contamination, the RNA was routinely precipitated with 4 M LiCl (added to a final concentration of 2 M) and incubated either overnight at 4°C or 1 h on ice. The RNA was pelletted by centrifugation, then washed with ice-cold 80% (v/v) ethanol, air dried, then resuspended in 30 to 50 µL of DEPC-water. The RNA was used immediately or stored at -80°C until required.

Amplification of *ACC Synthase* transcript by RT-PCR.

Reverse transcription of RNA was carried out using total RNA (4 µg) and oligo (dT) or random hexamer primers (ThermoScript™ RT-PCR system, Invitrogen), according to manufacture instruction. Nested degenerate oligonucleotide primers were design based on conserve sequence of many ACS genes obtained from GeneBank database. The first round primers generated cDNA transcripts of approximately 780 bp from the total cDNA pool and an aliquot of the first round PCR products were then used as templates for the second round of PCR amplification.

Cloning of PCR Products into Plasmid Vector

DNA fragments were either purified by column purification (High Pure PCR Product Purification Kit, Roche Diagnostic GmbH, Germany) or recovered from the agarose gel using the QIAquick gel extraction kit (Qiagen, Australia) according to the manufacturer's instructions. Purified DNA sequences were ligated into the pGEM®-T Easy vector (Promega) according to the protocol supplied with the cloning kit.

Characterisation and Sequencing of Cloned DNA in *E. Coli*.

Plasmid DNA was isolated using the ChargeSwitch®-Pro Plasmid Miniprep Kit (Invitrogen) according to the agarose gel electrophoresis. DNA was then automatically sequenced using Big Dye Terminator method. DNA was sequenced based on the standard protocol of automated ABI PRISM™ 3730 DNA Capillary Sequencer (Applied Biosystems).

DNA Sequence Analysis.

A BLAST search based of the nucleotide collection (nt/nr) of the plant genomes in the Genebank database was used to search for nucleotide sequences of interest. The sequences were aligned using ClustalW (EMBL-European Bioinformatic Institute, Cambridge, UK, http://www.ebi.ac.uk/Tools/clustalw2/index.html).

3. Results and Discussion

RT-PCR Amplification of Putative ACC Synthase Gene Transcripts

Previously, RT-PCR had been used to isolate and clone three *Tr-ACS* genes from the white clover genotype 10F of cultivar Grasslands Challenge [11]. Similar procedures were then used to isolate orthologues and novel *Tr-ACS* genes from the white clover ecotype Tienshan. Initially, ACC synthase cDNAs were amplified by RT-PCR using nested degenerate oligonucleotide primers corresponding to conserved sequences within the *ACS* genes (boxes 1 to 6, Figure 1.).

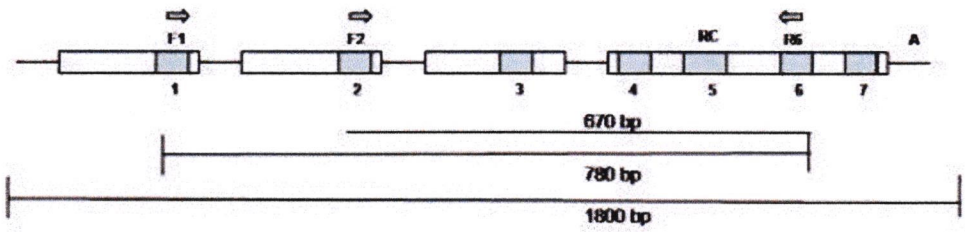

Figure 1 Diagrammatic representation of an ACC synthase gene from *Arabidopsis thaliana* (modified from Murray, 2001). The boxed regions denote the exons and lines denote the introns; conserved regions are denoted by the dark boxes; RC, the reactive centre; A, polyadenylation signal. The position of degenerate primers or the amplification of ACC synthase genes in white clover are shown as: first round forward primer (F1), second round forward primer (F2) and reverse primer (R6).

The first round primers generated cDNA transcripts of approximately 780 bp from the total cDNA pool, which represented *ACS* transcripts within the conserved boxes 1 to 6. An aliquot of the first round PCR products were then used as templates for the second round of PCR amplification usingthe second primer sets. The amplified first round PCR products (*ca.* 780 bp) were unable to be detected after electrophoresis on a 1% (w/v) agarose gel and ethidium bromide staining (data not shown). However, amplified products from the second round PCR (*ca.* 670 bp) were detected (Figure 2).

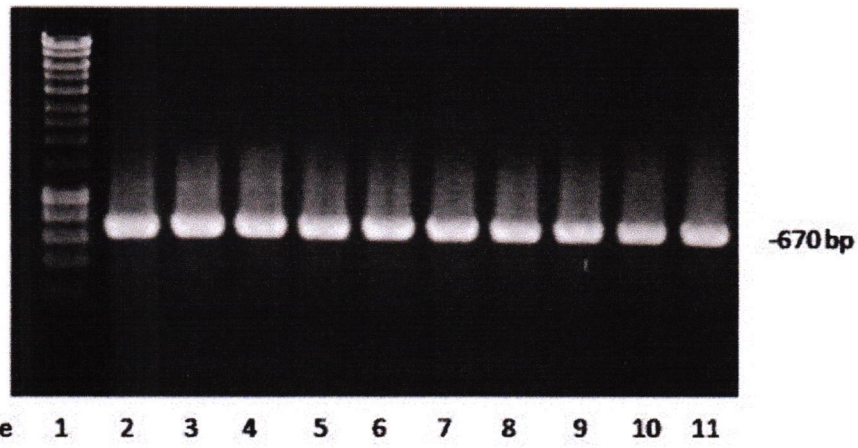

Figure 2. RT-PCR using RNA isolated from apical structures and first-fully expanded leaves of the Tienshan ecotype harvested at different soil water contents. Nested degenerate primers were used for two rounds of PCR amplification. The approximate size of the amplified cDNA is indicated. Lane 1. DNA Ladder. Lanes 2 to 6. RT-PCR products amplified from RNA isolated from apical structures at *ca.* 28%, 20%, 18%, 8% and 6% SWC respectively.

The second round PCR products (Figure 2) were TA-cloned into the pGEM T-easy vector and transformed into the *E. coli* strain DH5α, and putative inserts detected by blue/white screening (Figure 3). Initially, only white colonies were selected and cultured in LB Amp^{100} broth, plasmids isolated and the presence of inserts confirmed by PCR, using M13 primers, and the DNA sequences then obtained. All of the DNA sequences obtained from this protocol belonged to two genes, which have high sequence identity to the *Tr-ACS1* and *Tr-ACS2* sequences of white clover genotype 10F, Cultivar Grasslands Challenge [13]. These results suggested that *Tr-ACS3* and any novel *Tr-ACS* genes may not occur frequently (or at all) in apical tissues, or they might have been present in the blue or pale blue colonies. Since blue colonies could also contain the insert, as a result of in-frame cloning into the *LacZ* gene or the introduction of a mutation during the amplification process, no further blue/white colony selection was carried out.

For subsequent clones, the presence of inserts of approximately 670 bp was determined by PCR from all colonies without selection. To perform

this, PCR was carried out directly from the colonies (without prior plasmid isolation) using the second round *ACS* degenerate primer sets, prior to separation by 1% (w/v) agarose gel electrophoresis and visualization following ethidium bromide staining (Figure 4). For further identification and screening, the clones were rescued by sub-culturing onto a new LB Amp^{100} plate and numbered. Clones containing inserts were further screened for the presence of *Tr-ACS1* or *Tr-ACS2,* using gene-specific primers. Positive clones which were not amplified by the gene-specific *Tr-ACS1* or *Tr-ACS2* primers could therefore contain either the *Tr-ACS3* gene or a novel *Tr-ACS* gene not previously identified by Murray (2001). Those clones were subjected to seqeuncing.

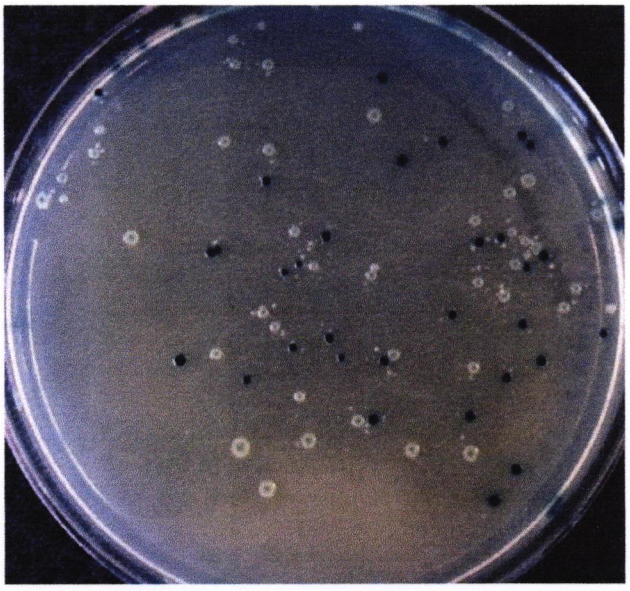

Figure 3 White-blue colony selection of sub-libraries generated from the apical structures of the Tienshan ecotype harvested at 8% SWC. Colonies were grown in LB media supplemented with IPTG and X-Gal.

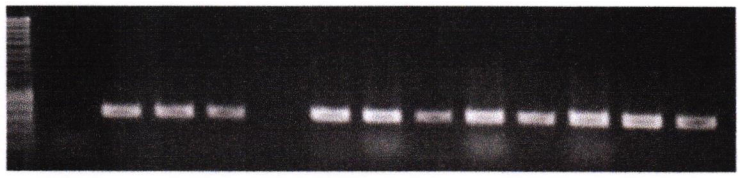

Figure 4. PCR selection of putative ACC synthase gene fragments from sub-libraries generated from RNA isolated from apical structures of Tienshan ecotype at *ca.* 8% SWC. PCR products were separated on a 1% (w/v) agarose gel and visulalised with ethidium bromide. The molecular weights of standards are indicated on the left (line 1). The size of PCR products (lanes 2 to 14) is indicated on the right.

Confirmation of Putative ACC Synthase Gene Transcripts by Sequence Analysis.

2346 clones were screened by colony PCR and a total of sixty clones were then subjected to DNA sequencing. The BLAST analysis in GeneBank indicated that all of these sequences displayed high sequence homology to ACC synthase gene sequences. The sequences obtained were aligned using ClustalW, which revealed that all of the sequences could be separated into four groups with each of the sequences within a group showing greater than 98% homology The consensus sequences between these groups were generated and then compared for homology at the nucleotide level, and identity at the amino acid level (Table 1). The four cDNA sequences share between 60% to 70% nucleotide homology. The low homology between these four cDNA sequences suggested that the four groups represented four distinct ACC synthase cDNAs, and so the genes from Tienshan ecotype were designated as *Tr-ACS1, Tr-ACS2, Tr-ACS3* and *Tr-ACS4*.

Table 1. Comparison of the percentage of nucleotide homology and percentage of amino acid identity (in parenthesis) between the four ACC synthase consensus sequences amplified from tissues of the Tienshan ecotype by RT-PCR

	Tr-ACS1	*Tr-ACS2*	*Tr-ACS3*	*Tr-ACS4*
Tr-ACS1	-	61 (50)	70 (65)	64 (56)
Tr-ACS2	61 (50)	-	60 (55)	64 (59)
Tr-ACS3	70 (65)	60 (55)	-	63 (55)
Tr-ACS4	64 (56)	64 (59)	63 (55)	-

The consensus sequences of these four ACC synthase gene and their derived amino acid sequences were aligned (Figures 5 and 6). The partial coding regions consist of between 622 to 642 bp, and sequence comparison of their derived amino acid sequences reveal that the four ACC synthase proteins are similar in the conserved domain three, four, five and six. BLAST analysis of the NCBI reference protein sequences (Figure 7.) suggested that all these sequences contain the conserved catalytic amino acid residue in the active site (lysine correspond to position 182 or L^{182} in Tr-ACS1). ACS belongs to the Aspartate aminotransferase enzyme family and this enzyme requires pyridoxal-5'-phosphate (PLP) as a cofactor. The four ACS proteins isolated from Tienshan ecotype contained 8 or 9 (out of 11 known) conserved amino acid residues involved in the binding of substrate to pyridoxal 5'phosphate (Figure 7).

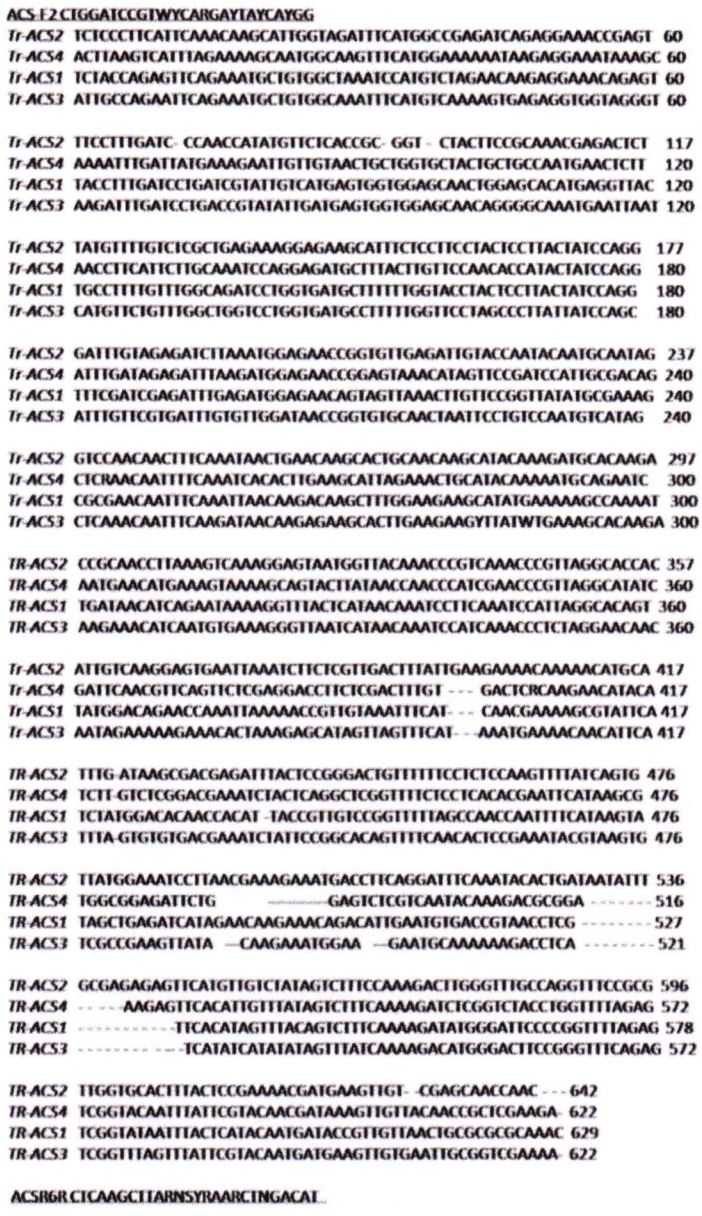

Figure 5. Alignment of coding frame region of *Tr-ACS1, Tr-ACS2, Tr-ACS3, and Tr-ACS4* consensus sequences isolated from the Tienshan ecotype. Degenerate primer sequences are underlined and gene specific primer sequences are shaded.

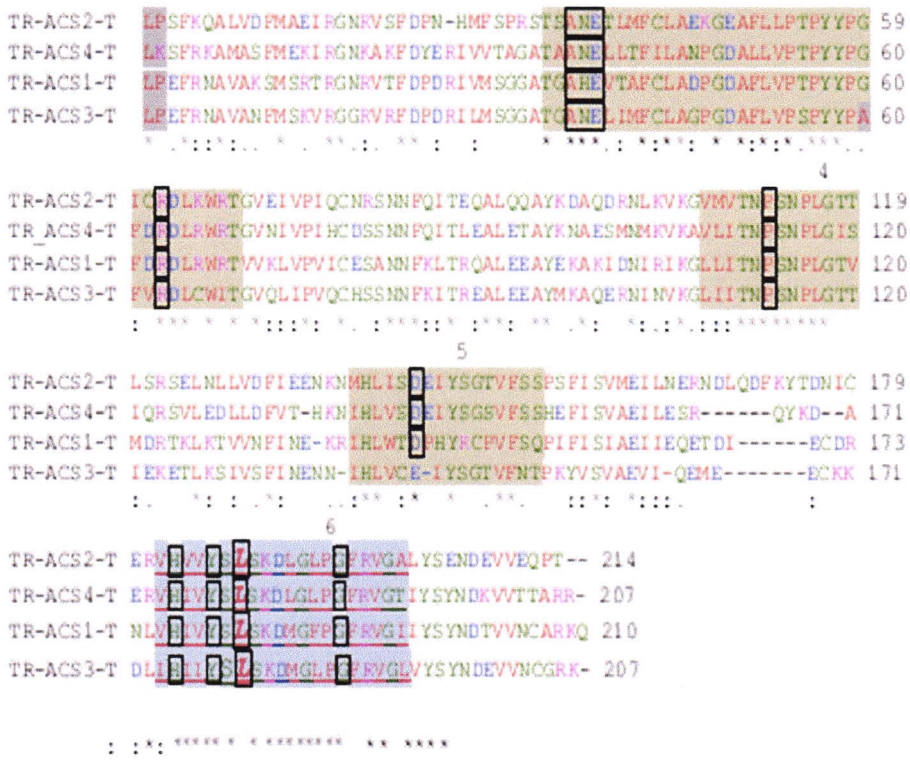

Figure 6. Alignment of deduced amino acid sequences of the Tr-ACS1, Tr-ACS2, Tr-ACS3 and Tr-ACS4 proteins.

(*) represents identical residue, (.) represents semi-conserved substitution sequence, (:) represents conserved substitution (-) represents no sequence, boxes represent conserved residues in Aspartate aminotransferases and other ACC synthase proteins and boxed-*italics* represent the catalytic residue of the enzyme. Shaded-numbered sequences represent the conserved regions of ACC synthase, while the shaded underlined sequences represent the active site of the enzyme.

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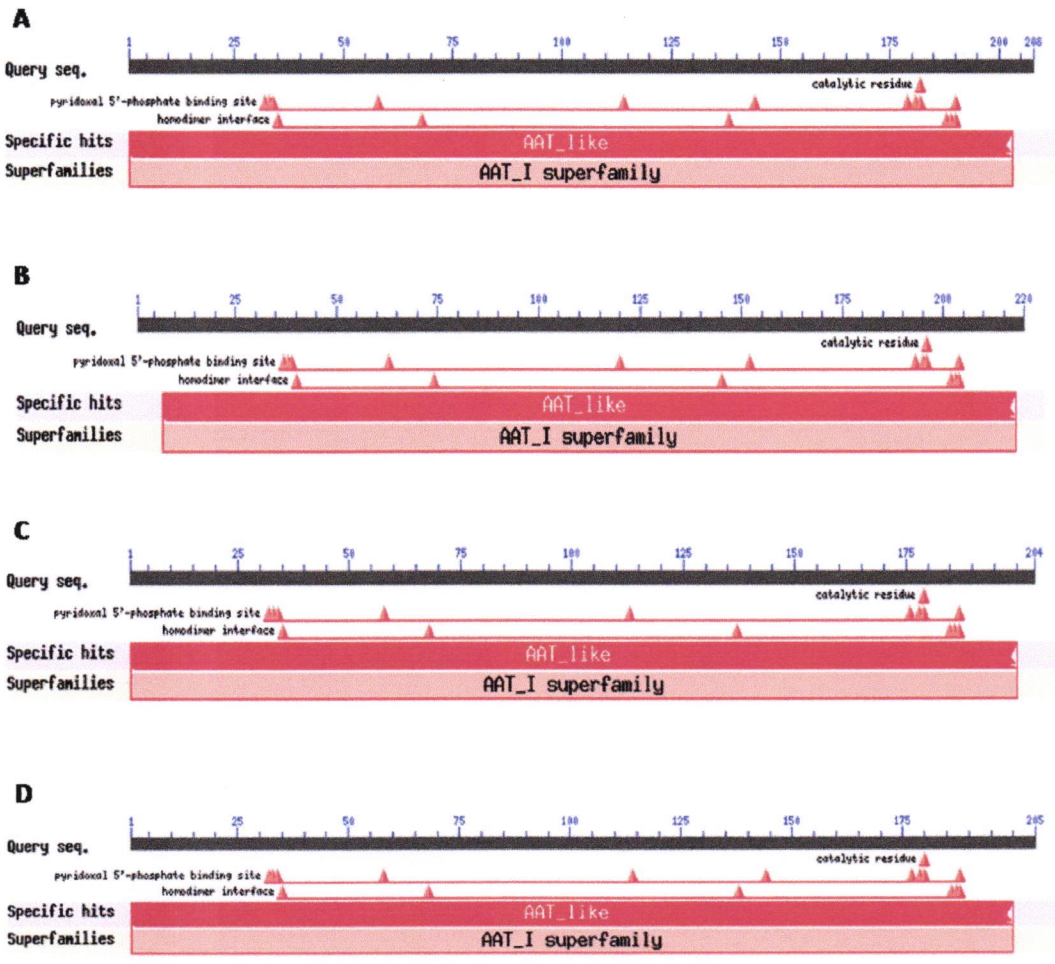

Figure 7 Conserved amino acid residues detected in the Tr-ACS1 (A), Tr-ACS2 (B), Tr-ACS3 (C) and Tr-ACS4 (D) when compared to an aminotransferase-I enzyme super family in the NCBI database.

Ten of the 11 amino acid residues required for the binding of the substrate to pyridoxal-5'-phosphate (PLP) are present in the TR-ACS1, TR-ACS2 and TR-ACS4 and 9 of these are present in the TR-ACS3.

Tr-ACS4 was amplified only from RNA isolated from the apical structures of Tienshan grown at less than 10% SWC. Only two *Tr-ACS4-T* clones were obtained suggesting a low abundance of this transcript in the total RNA pool. Thus, *Tr-ACS4-T* could be a water deficit associated ACC synthase in white clover. The expression of *Tr-ACS-4* in response to a water deficit was examined in the Tienshan ecotype using first fully-expanded leaf material (Data not shown). However, no evidence for a consistent change in expression was observed.

It is common for many plant species. In *Arabidopsis*, for example, eight of nine functional *ACS* genes are expressed in the seedlings while one, *ACS9,* is not [6]. In flower, these authors found that only *ACS11* was expressed in the trichomes of the sepals, whilst *ACS1* was specifically expressed in the replum. A *PnACS* isolated from the cotyledon *of Pharbitis nil* is expressed constitutively in cotyledons, petioles, hypotocyls, roots and shoot apices of both light- and dark-grown seedlings with the highest expression level in the roots [14]. In addition, only certain members of the *ACS* gene family are reported to be induced by environmental cues. For example, *ACS2, ACS4, ACS5, ACS6* and *ACS7* in *Arabidopsis* are induced by various biotic and abiotic stresses [5],[15], *ACS1* and *ACS2* expression is induced by the pathogen *Penicillium digitatum* in citrus fruit [16] and *NT-ACS2* in tobacco is induced by cold and light but not by wounding [17].

Phylogenetic Analysis of TR-ACS1, TR-ACS2, TR-ACS3 and TR-ACS4

A phylogenetic tree was constructed, using ClustalW, from the alignment of derived amino acid sequences of Tr-ACS1, Tr-ACS2, Tr-ACS3 and Tr-ACS4 with twenty other ACC synthase sequences obtained from GenBank database that have high identity with the ACC synthase genes from white clover (Figure 8).

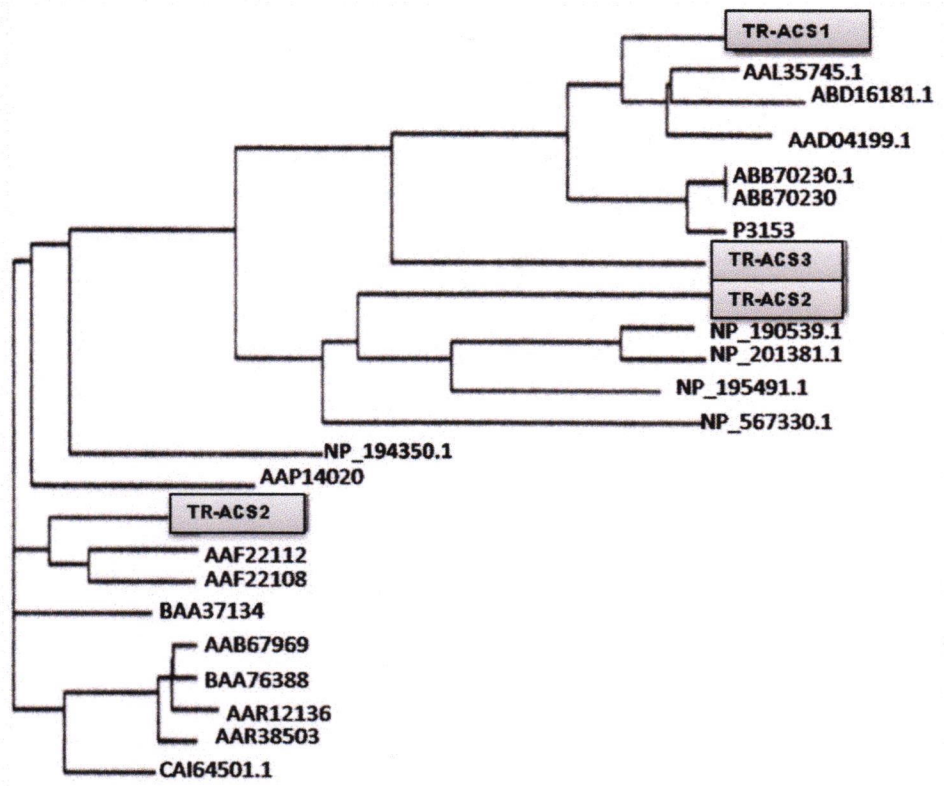

Figure 8. Amino acid sequence based phylogeny of the four ACC synthase family. The phylogenetic tree was generated using ClustalW and constructed from alignment of deduced amino acid sequences of Tr-ACS1, Tr-ACS2, Tr-ACS3 and Tr-ACS4 with amino acid sequences of other ACC synthases from the GenBank database.

The four ACC synthase genes isolated from white clover were more closely related to other ACC synthase sequences in the phylogenetic tree rather than to each other. The Tr-ACS1 was most closely related to ACC synthases isolated from barrel medic (accession number AAL35745.1), chickpea seeds (accession number ABD16181) and an ACC synthase isolated from IAA-treated etiolated pea seedlings (55AAD04199). The neighbouring sequences of Tr-ACS2 were the four of type-2 ACC synthase proteins from Arabidopsis including the ETO3 (ETHYLENE OVERPRODUCING) ACC synthase (accession number NP_190539.1) or ACS9, ACS5 (accession

number NP_201381.1), ACS8 (accession number NP_195491), ACS11 (accession number NP_567330.1) and ACS7 (accession number NP_194350.1). Interestingly, there was no neighboring sequence to Tr-ACS3 seen in the phylogenetic tree. The Tr-ACS4 was most closely related to ACS5 and ACS4 isolated from IAA- and wound-treated white lupin seedlings (accession numbers AAF221112 and AAF22108) and an ACC synthase protein from ripening fruit of *Passiflora edulis* (accession number BAA37134).

Comparison of each of the *Tr-ACS* sequences with ACS nucleotide sequences in the GeneBank determined that both *Tr-ACS1* and *TR-ACS2* share highest homology with ACC synthases isolated from leaves, etiolated or IAA-treated seedlings, while *TR-ACS3* shares highest homology to *ACS* genes induced by wounding or ageing. Thus these results suggest that the *Tr-ACS* gene family is developmentally regulated. Many papers have described differential regulation of ACC synthase during plant development. Nine functional *AtACS* transcripts in *Arabidopsis* has been isolated and their expression has been reported to be either tissues-specific or some overlap in different tissues [6]. Similar to this, three *ZmACS* genes were also found to be specifically expressed in different tissues, with some overlapping or some expressed in specific tissues. Of those, *ZmACS6* was specifically expressed in the root cap and *ZmACS6* expression was found to be important for root development in impeded soil [18]. These results suggest that the overlapping expression of *ACS* genes is not unusual. In the case of novel, *TR-ACS4* gene, it has wide range of similarities with other *ACS* genes isolated from many different developmental stages. However, generally the results showed that *Tr-ACS4* was closely related to other *ACS* genes that are induced by a variety of hormonal cues. It has been well documented for many plant species that not all member of ACS gene family is responsive to hormonal cues. In *Arabidopsis* roots, expression of 7 functional *ACS* genes (*ACS2*, *4*, *5*, *6*, *7*, *8*, and *11*) are induced by IAA [4] while *ACS4, ACS5* and *ACS7* expression is induced by exogenously applied ethylene [5]. However, only *ACS7* is also

responsive to Gibberellic acid (GA_3), abscisic acid (ABA), salicylic acid (SA) and brassinosteroid (BR) application [10]. Therefore, it seems that *Arabidopsis ACS7* is responsive to a variety of hormonal cues, and *Tr-ACS4* may be those responsive to hormonal cues in white clover. Investigation is now underway to charachterize expression of *Tr-ACS4* under different hormonal and abiotic cues as well 55as to isolate full-length sequence of any *Tr-ACS* gene family from white clover.

4. Conclusion

Three *Tr-ACS-like* transcripts and a novel *Tr-ACS* gene, designated *Tr-ACS4*, were isolated from the Tienshan ecotype following a water deficit treatment. All of the *Tr-ACS* genes isolated from the Tienshan ecotype encoded functional enzymes and contained the conserved amino acid residues in the active site which are required for binding of the substrate to the PLP co-factor.

The novel *Tr-ACS4* gene was isolated from the apical structures of Tienshan grown at less than 10% SWC, but expression analysis did not indicate that expression of the gene was influenced by water deficit. Bioinformatic analysis indicated a wide range of similarities of *Tr-ACS4* with other *ACS* genes isolated from various developmental stages of plants, and also those induced by hormonal cues.

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