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Research paper

Family-wide expression characterization of *Arabidopsis* beta-carbonic anhydrase genes using qRT-PCR and Promoter::GUS fusions

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Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes found throughout the phylogenetic tree. The β-class carbonic anhydrases (β-CAs) are the predominating class of CAs in plants. Growing evidence underscores the importance of β-CAs in plant immunity and environmental adaptation in addition to their roles in photosynthesis. However, many fundamental problems in *Arabidopsis βCAs* expression remain unsolved. Here we examined the transcript abundance of *AtβCAs* in different tissues of *Arabidopsis thaliana*, and the accumulation of mRNA in response to CO_2 and darkness. Histochemical analysis was performed to study the promoter activity of *AtβCAs* during post-germination seedling growth and in mature plants. All six members of the *AtβCA* subfamily showed a response to changed CO_2 level and darkness, but each member showed a specific dynamic pattern. Although expression of each *AtβCA* was unique, in general most *AtβCAs* were synchronously expressed in green leaves since 5 days after germination until flowering. *AtβCA1* and *AtβCA2* were most highly expressed in leaves but *AtβCA2* displayed weaker expression in roots. The level of *AtβCA3* transcripts was highest in flowers, while *AtβCA5* was most widely expressed and might be involved in more processes than other members. *AtβCA6* was unique for increased expression in darkness and no expression in either the anther or pistil. The present study provides useful information for further functional investigation.

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1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) catalyze, with high efficiency, the reversible hydration of carbon dioxide (CO_2), a reaction underlying many diverse physiological processes in animals, plants, archaebacteria, and eubacteria [1]. Based on sequence comparison, CA proteins were grouped into four distinct classes: α, β, γ or δ, that share no sequence similarities and appear to have independent origins but have experienced functional convergent evolution [2,3]. To date, the δ class is restricted to marine diatoms. The α, β, and γ CAs are the three main and ubiquitous classes [4,5].The ubiquity of the distribution of CAs implies that they play diverse but essential roles in many biological processes. Several important physiological functions played by CA isozymes, are related to respiration and transport of CO_2/bicarbonate between tissues, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, various biosynthetic reactions, and CO_2 fixation [6–12]. In addition, CA is a plausible source of H_2S (hydrogen sulfide) within the plant leaf via catalyzing the conversion of COS (carbonyl sulfide) to CO_2 and H_2S [13].

βCAs have been found in all life kingdoms [2,14,15] and are the predominant class of CAs in plants [16]. βCA catalyzes the first reaction in the C4 photosynthetic pathway: the hydration of atmospheric CO_2-producing bicarbonate for *phosphoenol* pyruvate carboxylase (PEPC), the primary carboxylase of C4 plants [17]. Solid evidence is lacking that βCA plays a direct role in photosynthesis of C3 plants. C3 plastidial βCA has been reported to play a role in stomatal closure [18]. Cytosolic βCAs have been implicated in maintaining adequate levels of CO_2 for ribulose bisphosphate carboxylase (Rubisco), and in the provision of bicarbonate for non-

Abbreviations: CA, carbonic anhydrase; CO_2, carbon dioxide; DAG, days after germination; PEPC, phosphoenol pyruvate carboxylase; qRT-PCR, quantitative reverse transcription PCR; UTR, untranslated region.

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photosynthetic forms of PEPC for anaplerotic roles such as replenishment of tricarboxylic acid intermediates, amino acid synthesis, and maintenance of cellular pH [19–21]. βCAs have also been reported to play other non-photosynthetic functions, in such as lipid synthesis [7], disease resistance [9,22], nodule development [23], and tolerance to abiotic stresses [24,25].

Available information about βCA in the model plant *Arabidopsis* originated largely from molecular, biochemical, and genetic studies on the role of these CA isoforms in plant responses to environmental CO_2 and light, and in plant resistance. The genome of *Arabidopsis* contains six *βCA* genes (*AtβCA1-6*) [4].Raines et al. [26] reported that the *CA* gene (*AtβCA1*) is responsive to changes in environmental CO_2 concentrations and Fett and Coleman [19] reported that *CA1* and *CA2* (*AtβCA1* and *AtβCA2*) are light-regulated in *Arabidopsis*. Biochemical studies on variants of *Arabidopsis β*-CA revealed that several sites are crucial for CA enzyme activity [27–29]. All members of the *AtβCA* subfamily display tissue-specific expression and appear to be present in different subcellular locations [4]. RNA antisense and gene knockout studies demonstrated that *AtβCA1* deficiency reduced the establishment of seedlings [30]. The CA function of AtSABP3 (*Arabidopsis thaliana* salicylic acid-binding protein 3, which corresponds to AtβCA1) was required for the expression of resistance in the host against pathogen infection, indicating that the inhibition of AtSABP3 CA function could contribute to a negative feedback loop that modulates the plant defense response [22]. *Arabidopsis* plants mutated in both *AtβCA1* and *AtβCA4* showed impaired CO_2-regulation of stomatal movements and increased stomatal density, demonstrating the importance of carbonic anhydrase catalysis in βCA-mediated CO_2-induced stomatal closure [18]. Proteomic analysis showed that AtβCA1 and AtβCA2 were abundant proteins in herbivore-resistant recombinant inbred lines, correlating with the enhanced production of reactive oxygen species (ROS) for resistance [31].

All these studies underlined the importance of AtβCAs in plant immunity and environmental adaptation in addition to their roles in photosynthesis. Global climate change is resulting in elevation of air temperature and CO2 level, together with a decrease in water availability. Plants need to adapt to the dilemma of both high CO2 and low CO2 conditions that result from stomatal closure under unfavorable conditions. Are AtβCA members important for the acclimation of plants to high CO2 or CO2-limiting conditions? The expression responses of *AtβCA* genes to changes of CO_2 in environment have been largely undefined. Using semi-quantitative RT-PCR analysis of all *AtβCAs* at one time-point under abnormal CO_2 environments, Fabre et al. [4] found some discrepancy with the previous report of Raines et al. [26]. Given the possibility that new functions for this subfamily may emerge from in-depth investigations, it is in great need of addressing the fundamental problems in molecular and expression features of *AtβCAs*. In this study, the predominant transcripts for *AtβCA*s were proposed through molecular characterization in combination with RT-PCR analysis and sequencing. Quantitative RT-PCR was performed to reveal the dynamic expression responses of *AtβCA*s to abnormal CO_2 concentrations and darkness. In addition, the promoter activities of *AtβCA*s were investigated using GUS staining in transgenic plants throughout the plant cycle, from seed germination to silique maturation. Our work provides a full overview of molecular features and expression patterns of *AtβCAs* for future functional studies.

2. Materials and methods

2.1. Structural analysis of AtβCAs

For the six *AtβCA* genes, different transcripts resulted from alternative splicing were retrieved from the TAIR database (http://www.arabidopsis.org/). Size information of exons and introns were further obtained from the TAIR database and preliminarily confirmed by cDNA and DNA sequences alignment for each gene. Those conserved exons were identified and structural diagram for each gene was constructed. Later, amino acid sequences were obtained for all alternative *AtβCA* transcripts. Conserved domains were identified at http://pfam.sanger.ac.uk and http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml. The alternative splicing mechanisms for AtβCAs were summarized in Appendix S1 table. Specific primers for each transcript were designed and RT-PCR method combined with cloning and sequencing were performed to further confirm these gene models.

2.2. Plant growth conditions and treatments

Arabidopsis thaliana (*Col-0*) was used in this study. Plants were grown in a greenhouse under a 12 h photoperiod of 120 μmol m^{-2} s^{-1} photon flux density, at a constant temperature of 22 °C and a humidity of 60–70%. For expression analysis, different tissues were harvested from 30-d-old flowering plants. For transcription analysis in response to CO_2, 20-d-old plants were transferred (at am 8:30) from greenhouse (ambient CO_2 level, 393 ppm ± 18 ppm) to an environmental chamber with CO_2 concentration regulated. CO_2 concentration in the chamber was monitored using an infrared gas analyzer (TES, Taiwan, TES-1370). CO_2 concentration was maintained in the chamber at desired levels of 150 ± 30 ppm or 900 ppm ± 100 ppm. In CO2 study, the second and third rosette leaves from at least five independent plants were harvested for RNA extraction at 0, 1, 6, 12, 24, and 48 h after transfer, respectively. For expression analysis in response to darkness, seeds were first germinated in an environmental chamber under unremitting light conditions at 22 °C. After 21 days, the chamber was set to dark. Then, the second and third rosette leaves from six to eight independent plants were collected for RNA extraction at each time point in darkness (0, 1, 6, 12, 24, and 48 h, respectively). Two biological repeats were independently conducted.

2.3. RNA isolation and reverse-transcription PCR

Total RNA was extracted from frozen samples using EASYspin RNA isolation kit (YuanPingHao, Beijing, China, DR103-02) following the manufacturer's instructions. The quality and concentration of RNA samples were examined through Goldview-stained agarose gel electrophoresis and spectrophotometric analysis. After 42 °C treatment for 2 min to remove genomic DNA contamination, total RNA samples were used to synthesize the first-strand cDNAs at 37 °C for 15 min and followed by 85 °C for 5 s using PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Dalian, China, DRR047A). Relative expression of *AtβCA* genes in different tissues were first analyzed by semi-quantitative RT-PCR on PTC-100 Programmable Thermal Controller (BIO-RAD, USA). Quantitative real-time PCR was performed on ABI 7300 Real-Time Cycler (Applied Biosystems, USA) with Premix Ex Taq™ (Perfect Real Time) kit (Takara, Dalian, China, DRR041A). The PCR conditions were set as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 52–59 °C for 31 s, 72 °C for 31 s. Fluorescence data collected during the 72 °C step were analyzed with ABI 7300 Analysis Software by creating a new relative quantitative study. In qRT-PCR analysis, quantification is based on Ct values. The Ct values for each qRT-PCR reaction were normalized in relation to the Ct value corresponding to *Actin* (At5g09810) amplified with primer pair 5′-TCTCTATGCCAGTGGT CGTA-3′ and 5′-CCTCAGGACAACGGAATC-3′. mRNA abundance for each gene is relative to its level in roots or in rosette leaves before transferring (0 h). RT-PCR primers for *AtβCA* genes were shown in Appendix S2 table. All reactions were performed in triplicates in one

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