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Arabidopsis casein kinase 2 α4 subunit regulates various developmental pathways in a functionally overlapping manner

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ABSTRACT

Casein kinase 2 (CK2) is an essential and well-conserved Ser/Thr kinase that regulates proteins in a post-translational manner. CK2 has been shown to affect a large number of developmental processes across eukaryotes. It is a tetrameric protein composed of a dimer of alpha (catalytic) and beta (regulatory) subunit each. In our previous study we showed that three of the four CK2 α subunits in Arabidopsis act in a functionally redundant manner to regulate various developmental pathways. In this study we constructed two independent CK2 α4 RNAi lines in the CK2 alpha triple mutant background. Through functional characterization of these RNAi lines we show that the fourth α subunit in Arabidopsis also functions redundantly in regulating ABA response, lateral root formation and flowering time. CK2 α4-GFP localizes to the chloroplast in transgenic Arabidopsis seedlings, consistent with the presence of a chloroplast localization signal at the amino-terminus of CK2 α4 subunit. Taken together, our results suggest a functionally overlapping role for the CK2 α4 subunit in regulating various developmental processes in plants.

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1. Introduction

The group of enzymes termed protein kinases comprises an essential set of enzymes for all living organisms. Kinases add one/more phosphate groups to their substrate protein which often regulates its cellular function and/or abundance. The phosphate group can be removed by an oppositely acting enzyme called phosphatase. The reversibility of this posttranslational modification allows cells to rapidly respond to a stimulus. Casein kinase 2 (CK2) is an essential and ubiquitous Ser/Thr kinase present in all eukaryotes. The enzyme is a tetramer composed of one dimmer of regulatory (beta) and catalytic (alpha) subunits each [1]. Although, CK2 is primarily a Ser/Thr kinase, it has been shown to phosphorylate Tyrosine residues in a few cases [2,3]. CK2 recognizes S/TXXD/E/Yp/Sp as a consensus sequence and is one of the few kinases that can utilize both ATP and GTP as phosphate source [4]. In mammals, CK2 has been extensively studied as a potential target for anticancer drugs due to its role in the cell cycle regulation controlling both cell division as well as apoptosis [5].

CK2 has been shown to regulate various developmental and stress response pathways through functional studies from various species in plants (Arabidopsis, maize, rice, tobacco, wheat and mustard) [6–11]. Compared to over 300 targets in animal system, there is less number of known CK2 substrates in plants; however, the list is steadily growing [1,12]. In Arabidopsis, both alpha and beta subunits are redundantly encoded by four genes each and are ubiquitously expressed spatially and developmentally. All the CK2 alpha subunits except α4 are nuclear localized. CK2 α4 subunit has an N-terminal chloroplast localization signal; however, it also contains the internal conserved Nuclear Localization Signal (NLS) [10]. This subunit also has the highest transcript level spatially and developmentally amongst the four alpha subunits [13]. The regulatory beta subunits are found both in the nucleus and cytoplasm. Both the subunits exist in a complex and as well as monomers in the cell [10].

This genetic and functional redundancy in both the catalytic and regulatory subunits has long hindered identification of the roles of CK2 in plant development. In the past few years, two approaches, creating an inducible dominant negative catalytic subunit mutant and creating higher order catalytic subunit knock out mutants have given important insights into involvement of CK2 in various pathways in Arabidopsis. The study carried out using an inducible dominant negative alpha subunit mutant showed that CK2 positively regulated the development of lateral roots in Arabidopsis.

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Also, the dominant negative mutant was defective in cell cycle and cell expansion and root and shoot growth. However, prolonged induction of defective alpha subunit was lethal supporting essential nature of the enzyme [14]. Two independent studies using different CK2 alpha subunit T-DNA insertion lines to create higher order mutants have shown involvement of nuclear CK2 alpha subunits in various pathways in Arabidopsis [13,15]. CK2 alpha subunits positively regulate response to hormone ABA, Salt (NaCl) stress and lateral root development in an overlapping manner. They also redundantly regulate the flowering time under both short and long day conditions [13,16].

A recent study reported the characterization of T-DNA insertion knock out lines of the chloroplast localized CK2 α4 subunit. The CK2 α4 single knock out mutant was found to be hyposensitive to ABA and salt stress similar to CK2 alpha triple mutant. The CK2 α4 knockout mutant was also hypersensitive to heat stress and showed reduced expression of genes involved in retrograde signaling [17]. Identification and characterization of a chloroplastic CK2 in rice has shown that the phosphorylation sites for target proteins in monocots and dicotyledonous plants have evolved to be different suggesting plastid CK2 regulation has changed over time in monocots and dicotyledonous plants [18].

In this study we characterized the function of the α4 subunit of CK2 in *Arabidopsis thaliana* using RNAi approach. CK2 is an essential enzyme and a quadruple alpha subunit mutant is likely to be lethal. Previous studies have also shown that the CK2 alpha subunits are functionally redundant and the single mutants did not exhibit any noticeable phenotype. Hence, we developed constitutive RNAi lines to down regulate CK2 α4 gene in both the wild type (Col-0) and CK2 alpha triple (*α123*) mutant background to better understand the function of α4 subunit.

2. Materials and methods

2.1. Plant growth conditions and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, http://www.sungro.com/) under constant light at 24 ± 0.5 °C. Seeds were surface sterilized and plated on Murashige–Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described [19]. After stratification at 4 °C in the dark, seeds were transferred to constant white light chamber at 22 ± 0.5 °C for respective number of days. For lateral root number, seedlings were grown under white light at 22 °C for 9 days on a vertical plate. The number of lateral roots visible to the naked eye was counted. The experiments were repeated at least three times.

2.2. Construction of CK2 α4 RNAi and CK2 α4-GFP over expression transgenic lines

Q6 A 200 bp region starting from the stop codon in the 3' UTR of Arabidopsis CK2 α4 gene (TAIR: AT2G23070) was cloned into pENTR vector (Invitrogen Inc., Carlsbad, CA) and subsequently recombined into GATEWAY cloning vector pB7GWIWG2(II),0 [20]. The recombinant construct was introduced into Wild type (Col-0) and CK2 alpha triple mutant plants via Agrobacterium mediated transformation [21]. The reduction in the transcript level of CK2 α4 in the homozygous RNAi lines was confirmed through quantitative real time PCR. Q7 Q8 The list of primers is used in Table 1. Full length open reading frame encoding CK2 α4 gene was cloned into pENTR vector (Invitrogen Inc., Carlsbad, CA) and subsequently recombined into GATEWAY cloning vector called pB7FWG2 [20]. The recombinant vector was introduced into wild type Col-0 plants via Agrobacterium mediated transformation as described [21].

2.3. Protein extraction and CK2 kinase assays

Protein extraction and CK2 kinase assays were performed as described [22–24]. Briefly, crude extracts were prepared by homogenizing 2-week-old green seedlings in buffer [40 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM sodium orthovanadate, 40 mM β-glycerophosphate and 10% glycerol]. The homogenate was centrifuged at 16 000 × g for 15 min at 4 °C. Protein concentration was determined using the Bradford protein assay method (Bio-Rad Laboratories, http://www.bio-rad.com/). For the CK2 phosphorylation assays, 25 μl kinase assay mixtures contained 50 mM HEPES–KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM γ-[32P]ATP (~250 c.p.m. per pmol), 100 mM KCl, ~1 pmol CK2 and ~10–20 pmol rWheIF4B. For the extracts from wild-type, triple mutant plants and homozygous RNAi lines in both wild type and triple mutant background, 0.5 μg of total protein was added for each reaction. The reaction was incubated at 30 °C for 10 min and terminated by the addition of 6× SDS loading buffer. Samples were boiled for 3 min and separated on an 8% SDS–PAGE gel. The gels were dried and exposed to a PhosphorImager.

2.4. Quantitative RT-PCR assays for floral integrator genes

Quantitative RT-PCR was performed as previously described [25]. Briefly, samples were collected every 4 h starting at ZT0 from 12-day-old wild-type, CK2 α triple mutant and one of the two RNAi lines in the triple mutant background seedlings grown under SD and LD conditions. Total RNA was extracted using a Spectrum plant total RNA kit (Sigma–Aldrich, http://www.sigmaaldrich.com/) and reverse transcribed using MMLV (Invitrogen, http://www.invitrogen.com/) as per the manufacturer's protocol. The qRT-PCR assays used the Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems, http://www.appliedbiosystems.com/). Real-time PCR was performed on a 7900HT Fast Real-time PCR system (Applied Biosystems). PP2A (TAIR: At1g13320) was used as a control for normalization of the expression data. The resulting cycle threshold (Ct) values were used for calculation of the levels of expression of different genes relative to PP2A as follows: $2^{\Delta Ct}$ where $\Delta Ct = Ct(PP2A) - Ct(\text{specific gene})$. Primer sequences used for qRT-PCR are listed in Table 1.

2.5. Abscisic acid response assays

Seeds were surface sterilized and plated on MS GM-Suc supplemented with different concentrations of hormone (ABA). After stratification at 4 °C in the dark, seeds were placed under continuous light at 22 °C. The numbers of germinated seeds and open, green cotyledons were recorded by observation under a microscope. At least 50 seeds for each genotype were plated for each experiment and the experiment was repeated at least three times.

2.6. Light fluorescence microscopy

Arabidopsis transgenic seedlings expressing CK2 α4-GFP were grown on MS plates for 2 weeks under white light. Leaf and root sections from these seedlings were observed under 100× magnification using Zeiss Axiovert fluorescent light microscope.

3. Results

3.1. Development of CK2 α4 RNAi lines in wild type and alpha triple mutant background

In our previous study, we showed that the three nuclear subunits are functionally redundant and hence the single subunit

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