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The p23 co-chaperone protein is a novel substrate of CK2 in *Arabidopsis*

Kendra Tosoni · Alex Costa · Stefania Sarno · Stefano D'Alessandro · Francesca Sparla · Lorenzo A. Pinna · Michela Zottini · Maria Ruzzene

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Abstract The ubiquitous Ser/Thr protein kinase CK2, which phosphorylates hundreds of substrates and is essential for cell life, plays important roles also in plants; however, only few plant substrates have been identified so far. During a study aimed at identifying proteins targeted by CK2 in plant response to salicylic acid (SA), we found that the *Arabidopsis* co-chaperone protein p23 is a CK2 target, readily phosphorylated in vitro by human and maize CK2, being also a substrate for an endogenous casein kinase activity present in *Arabidopsis* extracts, which displays distinctive characteristics of protein kinase CK2. We also demonstrated that p23 and the catalytic subunit of CK2 interact in vitro and possibly in *Arabidopsis* mesophyll protoplasts, where they colocalize in the cytosol and in the nucleus. Although its exact function is presently unknown, p23 is considered a co-chaperone because of its ability to associate to the chaperone protein Hsp90; therefore, an involvement of p23 in plant signal transduction pathways, such as SA signaling, is highly conceivable, and its phosphorylation may represent a fine mechanism for the regulation of cellular responses.

Kendra Tosoni and Alex Costa equally contributed to this work.

K. Tosoni · S. Sarno · L. A. Pinna · M. Ruzzene (✉)
Department of Biological Chemistry and Venetian Institute of Molecular Medicine (VIMM), University of Padova, Viale G. Colombo, 3, 35131 Padova, Italy
e-mail: maria.ruzzene@unipd.it

A. Costa · S. D'Alessandro · M. Zottini
Department of Biology, University of Padova, Padova, Italy

F. Sparla
Department of Experimental Evolutionary Biology, University of Bologna, Bologna, Italy

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Introduction

Protein kinase CK2 [1, 2] is a ubiquitous and constitutively active Ser/Thr protein kinase, usually organized as a tetrameric complex, consisting of two catalytic and two regulatory subunits. It is essential for cell viability and it plays a special function in tumor cells as a pro-proliferative and anti-apoptotic kinase [3–5]. It phosphorylates many proteins: more than 300 substrates [6] are known so far, most from mammals, with a strict site specificity requiring a precise consensus including acidic residues, in particular at the $n + 3$ position downstream from the target Ser/Thr [7]. Among the CK2 numerous substrates, several chaperone proteins have been identified which deserve special attention: in fact, it has been demonstrated [8] that CK2, by phosphorylating this class of proteins, can regulate the levels and the functions of many other proteins, thus controlling different cellular processes.

CK2 is well conserved in all eukaryotes, and it has been studied also in plants, where multiple forms of CK2 subunits exist: indeed, while in mammals only two catalytic (α and α') and one regulatory (β) subunits are present, in *Arabidopsis* four genes coding for α (denoted as A, B, C, and D) and four for β (1, 2, 3, and 4) have been described [9].

CK2 is essential for cell viability also in plants [10], where it is involved in several crucial processes, including cell cycle and proliferation [11], circadian rhythm [12–15], auxin signaling pathways [16], dark/light-dependent enzyme regulation [17], translation [18], and the salicylic acid (SA)-mediated defense response [19, 20].

We have recently investigated the SA signaling pathway, and, in contrast to what observed by others [19], we did not find any change in CK2 catalytic activity induced by SA [20]; we therefore reasoned that CK2 intervenes in SA signaling by specifically modulating the phosphorylation level of only one or few proteins among its substrates, which become available as targets only upon an SA-mediated event, such as changing in protein expression, cellular translocations, or post-translational modifications catalyzed by other enzymes. Based on this premise, we performed a study where a proteomic approach was exploited for the identification of SA-dependent CK2 substrates (manuscript in preparation). During this study, we identified a novel CK2 substrate in *Arabidopsis*, the co-chaperone protein p23, whose phosphorylation was investigated in detail.

Experimental procedures

Materials

The CK2 inhibitor TBB (4,5,6,7-tetrabrome benzotriazole) was synthesized as in [21], Quinalizarin was provided by Produits Chimiques ACP Chemicals. Staurosporine and K252a were from Sigma-Aldrich. Recombinant human and maize CK2 were produced and purified as described in [22]. Purified proteins were dialyzed against 25 mM Tris pH 7.5 and 50% of glycerol and stored at −20°C. Radioactive ATP was from PerkinElmer.

Plant material and growth conditions

Arabidopsis thaliana Columbia ecotype plants were used in this study. Plants were grown on MS/2 0.8% agar medium [23] with 16/8 h cycles of light (70 μE m^{-2} s^{-1}) at 22°C and 75% RH. Seeds of *Arabidopsis* lines overexpressing the CK2B3 subunit (ox18 and ox41 [12]) were kindly provided by Prof. Tobin from UCLA (Los Angeles, CA). For the treatment, *Arabidopsis* 8-day-old seedlings were transferred into 5 cm Petri dishes and washed with LB buffer (5 mM MES-KOH, pH 5.7, 1 mM $CaCl_2$, 0.25 mM KCl). The LB buffer was then replaced with the same buffer supplemented with 30 μM TBB or DMSO as control. The proteins were then extracted as described in [20].

Cloning, expression, and purification of recombinant p23-2

The coding sequence of *Arabidopsis* p23-2 protein (At3g 03773) was amplified by PCR using the following primers: p23-2-For-NdeI CATGCATATGAGTCGTAATCCGGAG GTTCTT (forward primer), p23-2-Rev-NdeI CATGCATA TGCTACTTGTTTCTTGCCTTTTCCA (reverse primer). The template for PCR reactions was cDNA obtained by the retrotranscription of total RNA extracted from 8-day-old *Arabidopsis* seedlings as previously described [24]. The PCR was performed by using the Phusion® DNA Polymerase (Finnzymes). The amplified fragment was inserted into NdeI pre-digested expression vector pET28a(+) (Novagen). The p23 cDNA was placed in frame with a His-tag and a cleavable thrombin site at the 5′. The recombinant plasmid was amplified into *E. coli* XL1Blue (Stratagene) cells and sequenced before the transfer into BL21(DE3) *E. coli* cells for expression. Heterologous expression, purification of the recombinant protein, and removal of the His-tag were performed as in [25]. The purification grade of recombinant proteins was checked by SDS-PAGE and Coomassie staining.

Vector construction for subcellular localization and BiFC analyses

For the subcellular localization analyses of p23-2 (At3g 03773.1) and CK2αC (At2g23080) the coding sequences of the two genes were cloned in front of YFP and GFP in a modified pGreen 0029 vector [26] and pSAT-EGFP-N1 vector, respectively [27] by using the following primers: p23-2-For-NcoI CATGCCATGGCCATGAGTCGTAATC CGGAGGTTCTT and p23-2-Rev-NcoI CATGCCATG GCCGCCTTGTTTCTTGCCTTTTCCA; CK2αC-For-SacI CATGGAGCTCAATGTCGAAAGCTAGGGTTTATAC AGAT and CK2αC-Rev-BamHI CATGGGATCCCT GCCTGAGTTCGTAGTCTGCTGCT. For the BiFC analysis the p23-2 coding sequence was subcloned in front of the splitted N-terminal part of the Venus in the pSAT1A-nVenus-N pE3231 vector, whereas the CK2αC was inserted in front of the splitted C-terminal part of the CFP in the pSAT1-cCFP-N pE3449 vector [28]. The p23-2 coding sequence was amplified by PCR using the following primers: p23-2-For-SacI CATGGAGCTCATGAGTCGTAATCCGGAGG TTCTT and p23-2-Rev-BamHI CATGGGATCCCCGCC TTGTTTCTTGCCTTTTCCA; the CK2αC was directly subcloned from the pSAT-CK2αC-EGFP-N1 vector. In all the constructs made the coding sequences were amplified by using as template the same cDNA described above by using the Phusion® DNA Polymerase (Finnzymes). The vectors were then sequenced to verify that no mistakes were introduced. All the cloned genes were under control of a double CaMV35S promoter.

Protoplasts transformation and confocal analyses

The *Arabidopsis* mesophyll protoplasts were isolated and transformed following the Jen Sheen's protocol [29]. Briefly, 20 leaves from 4-week-old *Arabidopsis* plants grown in Giffy pots [24] were cut in thin slices and placed

in enzymatic solution. Protoplasts were then isolated at a density of 1–2 $\times$ 10^5/ml and a PEG-calcium transfection was performed. The protoplasts were then maintained at 22°C for 16 h in the dark before the analyses.

The Confocal microscopy analyses were performed using a Nikon PCM2000 (Bio-Rad, Germany) laser scanning confocal imaging system. For the green fluorescence protein (GFP) detection the excitation was at 488 nm and emission between 515 and 530 nm, for yellow fluorescence protein (YFP), and reconstituted nVenus, detection, the excitation was still at 488 nm but the emission between 530 and 560 nm. For the chlorophyll detection, excitation was at 488 nm and detection over 600 nm. Image analyses were done with the IMAGEJ BUNDLE software (http://rsb.info.nih.gov/ij/).

In vitro phosphorylation assays

Protein substrates (p23 or β-casein) were incubated at 30°C with recombinant human monomeric (α) or tetrameric ($\alpha_2\beta_2$) CK2 or maize CK2 α, in a phosphorylation mixture containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 20 μM [γ-^{33}P] ATP (or GTP) (1000–2000 cpm/pmol) according to the K_m value for ATP of human CK2 (10 μM) in a total volume of 20 μl. 100 mM NaCl was added when tetrameric CK2 was used. Further details are specified in the figure legends. After incubation, samples were loaded on a SDS-PAGE, which was stained with Coomassie blue, and analyzed by autoradiography with the Cyclone Plus Storage Phosphor System (PerkinElmer). When quantization was required, [γ-^{32}P] ATP instead of [γ-^{33}P] ATP was used, radioactive bands were excised and counted in a scintillation counter. For the calculation of kinetic values, initial rate data were fitted to the Michaelis–Menten equation with the program Prism (GraphPad Software).

Recombinant p23 phosphorylation by *Arabidopsis* lysates was performed as described above, but without the addition of any kinase.

In-gel kinase assay

For this assay, a protein substrate (500 μg/ml β-casein or 10 μg/ml p23) was included into a 11% SDS-PAGE where cytosolic proteins (5–20 μg) from *Arabidopsis* seedling extracts were separated according to Laemmli [30]. After the electrophoresis, SDS was removed and protein renatured, as elsewhere described [31]. Then gel was incubated with a phosphorylation mixture containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 20 μM ATP, [γ-^{33}P]ATP (specific radioactivity $\sim$1000–5000 cpm/pmol). After Coomassie blue staining, the gel was analyzed by autoradiography for the detection of radioactive bands.

BIAcore experiments

For the surface plasmon resonance (SPR) analysis, a BIAcore X system (GE Healthcare) was used, as described in [32]. Human CK2α was covalently coupled to a CM5 sensor chip (carboxymethylated dextran surface), by the amine-coupling chemistry, to a final density of 1600 RU (resonance units); a flow cell with no immobilized protein was used as a control. His-tagged p23 solutions were injected at the indicated concentrations in HBS-EP buffer (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) P20) at a flow rate of 10 μl/min; the same buffer flowed before injections and during the dissociation phase; after each injection the surface was regenerated by injecting 1 M NaCl for 1 min; this treatment restored the baseline to the initial resonance unit value. Each sensorgram (time course of the SPR signal) was corrected for the response obtained in the control flow cell, and normalized to baseline.

Results

Identification of p23 as a CK2 substrate in *Arabidopsis*

During a proteomic study where extracts from 8-old-day *Arabidopsis* seedlings treated with SA were analyzed for their CK2-dependent phosphorylations (manuscript in preparation), we found a major protein identified as the *Arabidopsis* p23. This protein is defined as a co-chaperone protein, since, similarly to its animal homologous [33], it has been demonstrated to associate to Hsp90 [34]. Two isoforms of p23 are expressed in *Arabidopsis*, denoted as p23-1 (At4g02450.1) and p23-2 (At3g03773.1). Their sequences were analyzed looking for CK2 consensus sites [7], and revealed the presence of putative CK2 targets, more numerous in the p23-2 isoform (Fig. 1). We therefore decided to verify and characterize the p23-2 phosphorylation by CK2 in vitro. We cloned the coding sequence of At3g03773.1 locus (whose product will be hereafter denoted as p23) into a prokaryotic expression vector to produce a His-p23 protein, which was then purified and subjected to thrombin digestion to cleave the His-tag and obtain a 17.4 kDa p23 recombinant protein (Fig. 2). Although preliminary experiments showed that the His-tag did not affect the phosphorylation degree, we preferred to use this thrombin-digested protein as kinase substrate; we performed a first set of experiments with human CK2, using the model substrate β-casein as a comparison: we found that indeed human CK2 phosphorylates the recombinant p23 in vitro, with an efficiency similar to that obtained with a sixfold higher concentration of β-casein (Fig. 3a). The stoichiometry of phosphorylation reached

1.5 mol Pi/mol protein, with the monomeric CK2α (not shown). Since most of the CK2 substrates are better phosphorylated by tetrameric CK2, while few of them are instead preferred by monomeric CK2 [2], we analyzed the efficiency of the two CK2 forms on p23: we found that an amount of the tetrameric CK2 ($\alpha_2\beta_2$) sufficient to produce a phosphorylation degree similar to CK2α on β-casein was slightly less efficient on p23. Consistently, when CK2β was added to CK2α during the phosphorylation assay, while a significant increase was observed on β-casein phosphorylation, no appreciable effect was induced on p23 (Fig. 3a). We also checked the effect of polylysine, an in vitro CK2 activator absolutely required by those substrates whose phosphorylation is prevented by the β subunit [2]; however, only a slight improvement of p23 phosphorylation was induced by polylysine, while a strong activation occurred towards β-casein (Fig. 3a). The kinetics of p23 by CK2 are shown in Fig. 3b, where the K_m and V_{max} values are also indicated for the two CK2 isoforms.

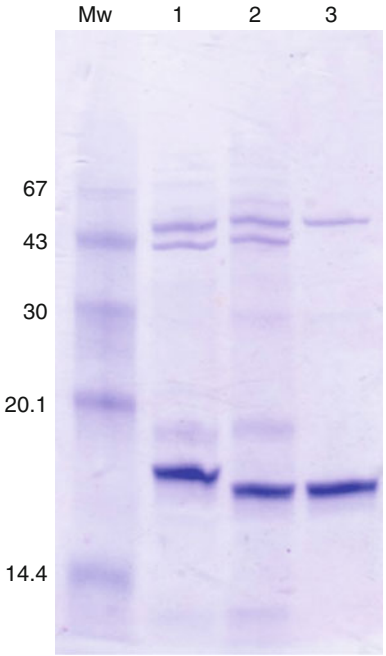

Fig. 2 Purification of *Arabidopsis* p23 protein. The purified recombinant p23 protein was loaded on a 15% SDS-PAGE, whose Coomassie blue staining is shown. *Lane 1* His-p23 protein purified by metal affinity chromatography (NiNTA) purification system, *lane 2* p23 protein after thrombin digestion, *lane 3* thrombin-digested p23 eluted from NiNTA. Electrophoretic mobility of molecular mass markers (Mw) in kDa is indicated on the *left*

Then we confirmed our data with a plant CK2, evolutionary more similar to the *Arabidopsis* enzyme than human one; the available recombinant and purified enzyme was CK2 α from maize [35], and the results, very similar to those obtained with human CK2α, are shown in Fig. 3c.

p23 phosphorylation by an *Arabidopsis* CK2-like activity

Next we wanted to verify if a CK2-like activity able to phosphorylate the recombinant p23 was present in *Arabidopsis* protein extracts. To this purpose, we performed in vitro radioactive phosphorylation using the soluble fraction from 8-day-old *Arabidopsis* seedling extracts as source of kinases, and recombinant p23 as added substrate. The results (Fig. 4a) clearly show that p23 could be phosphorylated by a kinase present in *Arabidopsis* extract, and that this phosphorylation was sensitive to the CK2 inhibitor TBB [36]; notably, a very similar effect was observed

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p23-1   1       MSRHPEVKWAETTEKIFLTVVLADTKDTKVNLDPEGVFDFSAKVGPENHVYELKLELADK 60
p23-2   1       MSRNPEVLWAQRSDKVYLTVALPDAKDISVKCEPQGLFSFSA-LGAQGERFEFSLELYGK 59
                ***:*** **: ::*::***.*.*:** .*: :*:*:*.*** :*.:.. :*:.*** .*

p23-1   61      VNVEESKINIGERSIFCIIEKAEPERWNKLLRVK-KPPHYVKVDWDKWVDEDDEG-SAGA 118
p23-2   60      IMTEYRKN-VGLRNIIFSIQKEERSWWTRLLKSEEKPAPYIKVDWNKWCDEDEEVNSETA 118
                : .*  *  :* *.*:  *:* * . *.:**: : **. *:****:** ***:*  *  *

p23-1   119     ADMDMAGMEGMGGMGGMGGMGGMGGMGGMGGMGGMEGMDFSKLMGGMGGMGGMGGLEGLG 178
p23-2   119     SDDESA---------------------------------------------------- 124
                :* : *

p23-1   179     GMGGMGGMGGMGGMGGMEEFEDSDDEEETAKSGDKKDDAVKEEGLATEKAPAAEETTSVK 238
p23-2   125     ------------------FVNQDSESS--------SDDDGLLYLPDLEKARNK------- 150
                              * :.*.*.         .**          ***

p23-1   239     EDK                                                         241
p23-2           ---                                                         150
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Fig. 1 Sequence of the p23 proteins expressed in *Arabidopsis*. The alignment between the two p23 isoforms (p23-1 and p23-2) was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The output is labeled with *symbols* (bottom row of alignment) indicating the degree of sequence similarity (* = exact matches, : = strong similarity, . = weak similarity) (http://www.yeastgenome.org/help/SeqSimQuery.html). Putative CK2 consensus sites, identified on the basis of CK2 specificity as in [7], are *bold underlined*. The residue denoted by italic type indicates a putative hierarchical phosphorylation site, which becomes a consensus sequence only when a downstream phosphorylation (in $n + 3$ position) occurs [7]

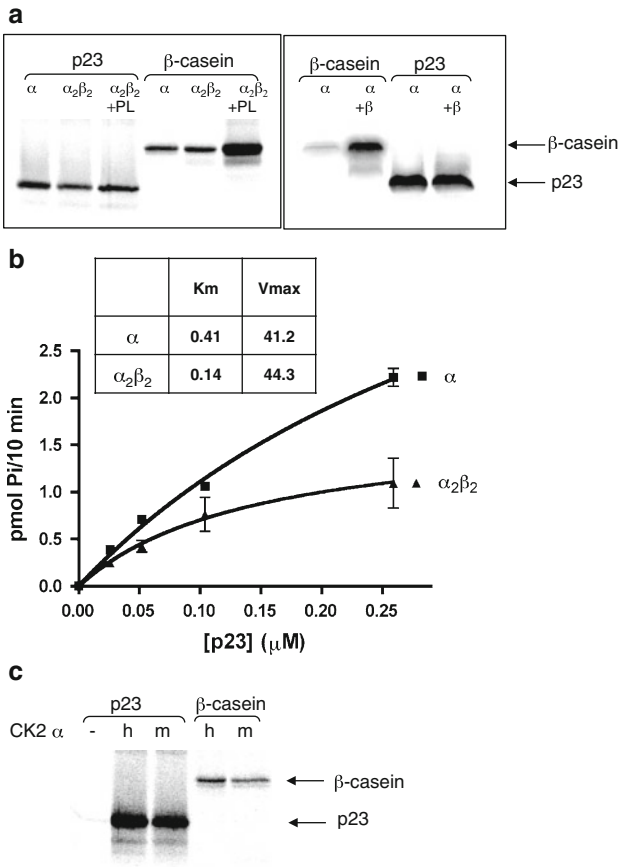

Fig. 3 Phosphorylation of p23 by recombinant CK2. **a** Recombinant *Arabidopsis* p23 (0.1 μg, 0.29 μM) was incubate with monomeric α (15 ng in the *left panel*, 100 ng in the *right panel*) or tetrameric $\alpha_2\beta_2$ (4.4 ng) human CK2, as indicated, for 10 min in a radioactive phosphorylation mixture (total volume 20 μl). Where present, polylysine (PL, 400 μg) and human CK2*β* subunit (100 ng), were added at the beginning of the incubation time. Where indicated, *β*-casein (1 μg, 1.72 μM) replaced p23. After incubation, samples were analyzed by SDS-PAGE and autoradiography. **b** The kinetics is shown, obtained by phosphorylating increasing amounts of recombinant p23 with α (15 ng) or $\alpha_2\beta_2$ (4.4 ng) human CK2, as described above. The kinetic values are also shown: K_m is expressed in μM, V_{max} in pmol of phosphate (Pi)/min/μg enzyme. **c** Recombinant *Arabidopsis* p23 (0.29 μM) or *β*-casein (1.72 μM) were phosphorylated for 10 min by human (*h*) or maize (*m*) CK2 α (40 or 20 ng, respectively, to ensure a similar degree of *β*-casein phosphorylation), as described above. The *first lane* corresponds to p23 incubated in the absence of any enzyme

when TBB was added in vitro during the phosphorylation assay, or in vivo to the *Arabidopsis* seedlings. A corroborating experiment was performed by incubating p23 with extracts from two transgenic *Arabidopsis* lines overexpressing CK2*β*3 and reported to display a higher CK2 activity [12]: as shown in Fig. 4a, indeed, a higher p23 phosphorylation degree was observed in both transgenic lines compared to wt *Arabidopsis* line. These data strongly suggest that p23 phosphorylation was catalyzed by endogenous CK2 in *Arabidopsis* lysates. To further confirm these results, we compared the efficacy of different protein kinase inhibitors and effectors on this reaction: as shown in Fig. 4b, the phosphorylation level of p23 was decreased by two different CK2 inhibitors (TBB and quinalizarin [37]), but very weakly by general protein kinase inhibitors not effective on CK2 (staurosporine and K252a) [20, 38] or by calcium deprivation by EGTA. Moreover, the phosphorylation of p23 was also observed when ATP was replaced by GTP, a phosphate donor that can be used by CK2, but not by the majority of the other protein kinases [39].

Next, to assess if the main endogenous enzyme responsible for p23 phosphorylation was able to phosphorylate casein and displays a size consistent with that of CK2 catalytic subunit, we performed a set of in-gel kinase assays, including either casein or p23 in the gel, and performing the radioactive phosphorylation of these substrates by enzymes present at certain migration positions, after SDS-PAGE and protein renaturing. These experiments (Fig. 4c) demonstrated that the only kinase phosphorylating p23 in *Arabidopsis* migrates at about 39 kDa; at the same size, also a band able to phosphorylate casein is present, strongly suggesting that the major p23 kinase in *Arabidopsis* is a casein kinase with the size expected for CK2αC (At2g23080.1). Interestingly, a higher Mw band phosphorylating casein is also present in the *Arabidopsis* extract (Fig. 4c, right panel), whose size roughly corresponds to that expected for the other CK2 α isoforms expressed in *Arabidopsis* (At5g67380 and At3g50000); however, this activity towards casein is not accompanied by activity towards p23, indicating that CK2αC is the major CK2 isoform responsible for the p23 phosphorylation.

Physical association between CK2 and p23

Considering that p23 is phosphorylated in vitro by CK2, we wondered if also a stable association can occur, in vitro and in vivo, between the two proteins. To assess this point, we first performed BIAcore experiments, where recombinant p23 was flowed over a surface where human CK2α was immobilized. As shown in Fig. 5, a concentration-dependent signal was observed, corresponding to p23 binding to CK2α.

In order to test if an in vivo association of the *Arabidopsis* CK2 and p23 proteins also occurs, we first assessed the subcellular localization of these two proteins. In *Arabidopsis*, three non plastidial CK2 α subunits have been identified (A, B, C) [9] and among them the CK2 αC (At2g23080.1) is the one expected to phosphorylate p23, as judged from the size displayed in the in-gel kinase assay (see Fig. 4c). We then fused the GFP at the C-terminal end of the CK2αC and expressed it in *Arabidopsis* mesophyll protoplasts. The confocal microscopy analyses showed that the GFP signal was clearly detectable in the cytoplasm and nucleus (Fig. 6a–c), confirming published data [9]. We then tested the subcellular localization of p23 by fusing it to the

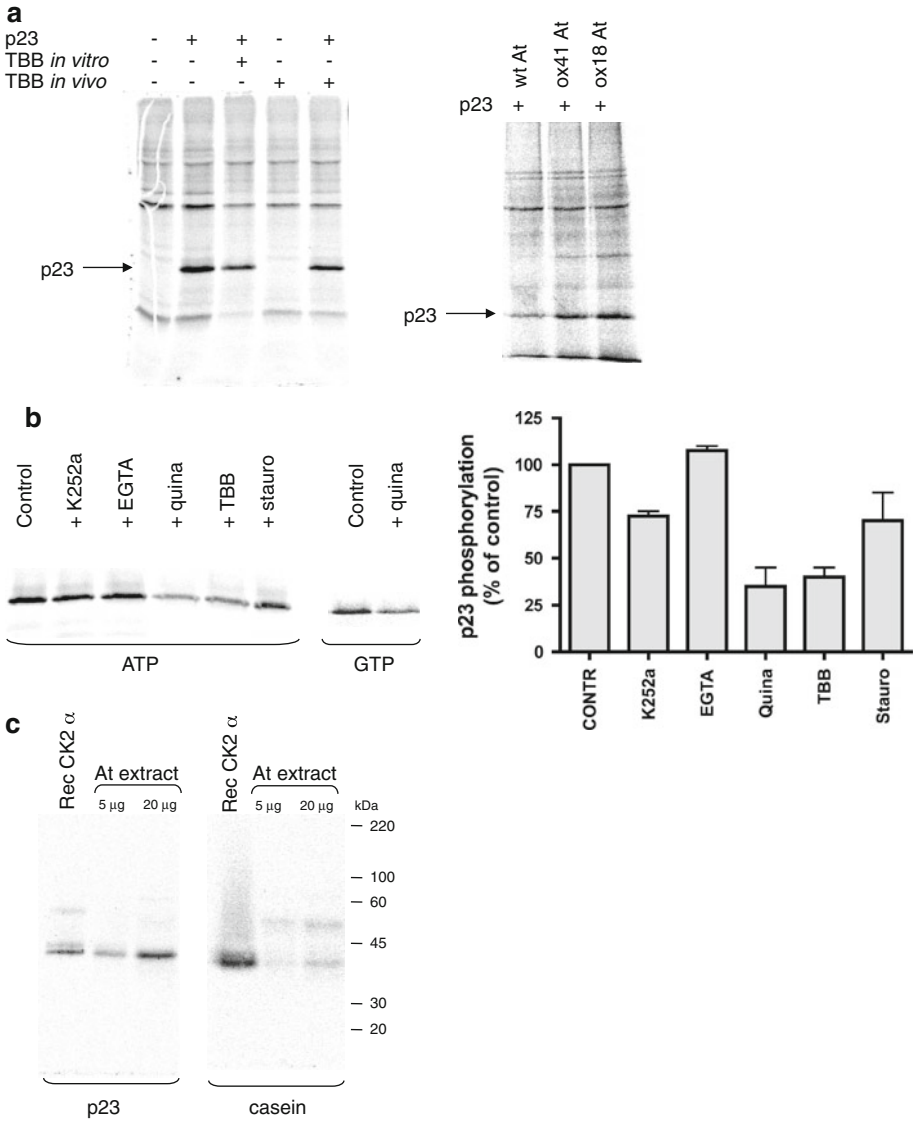

Fig. 4 Phosphorylation of p23 by *Arabidopsis* cytosolic extracts. **a** 10 µg of proteins from cytosolic extracts of *Arabidopsis* seedlings (previously treated with 30 µM TBB, where indicated as in vivo), were incubated for 10 min at 30°C with a radioactive phosphorylation mixture, in the presence, where indicate (+), of recombinant p23 (0.1 µg). If added during the phosphorylation reaction (in vitro), TBB was 2 µM. Proteins were separated by SDS-PAGE and radioactivity detected by autoradiography. On the *right panel*, phosphorylations obtained with cytosolic extracts from wt *Arabidopsis* and from transgenic plants overexpressing CK2β3, indicated as ox41At and ox18At as in [12], are compared. **b** Recombinant p23 (0.1 µg) was phosphorylated by proteins from *Arabidopsis* extracts as in **a**, with the addition of the indicated protein kinase effectors as follows: 0.1 µM K252a, 500 µM EGTA, 2 µM quinalizarin (Quina), 2 µM TBB, 1 µM staurosporine (Stauro). Where indicated, radioactive GTP replaced ATP as phosphate donor. The autoradiography corresponding to the migration of p23 is shown on the *left*, while quantification of p23 phosphorylation (expressed as percentage of control, obtained without any effector) is shown by the graph on the right; *vertical bars* indicate the standard deviation to the mean obtained from three separated experiments. **c** For the in-gel kinase assay, 5 or 20 µg of proteins from *Arabidopsis* cytosolic extracts were loaded on a gel containing 10 µg/ml p23 (*left*) or 500 µg/ml β-casein (*right*). The gels, after incubation with a radioactive phosphorylation mixture, were analyzed by autoradiography. 10 ng of recombinant human CK2α, truncated at the C-terminus (1-336 sequence), were loaded as a positive control. The migration positions of Mw markers are shown on the *right*. At, *Arabidopsis thaliana*

YFP and expressed it in *Arabidopsis* mesophyll protoplast, and, also in this case, the confocal microscopy analyses show its presence in cytoplasm and nucleus (Fig. 6d–f).

Having demonstrated that CK2αC and p23 localize in the same subcellular compartments (cytoplasm and nucleus) we then wanted to assess their in vivo interaction by means of bimolecular fluorescence complementation technique (BiFC) [40]. To this purpose, the CK2αC protein was fused upstream to the C-terminal portion of the cyan fluorescence protein (cCFP) and the p23 upstream the N-terminal portions of Venus (nVenus) [28]. The two constructs were then introduced in *Arabidopsis* mesophyll

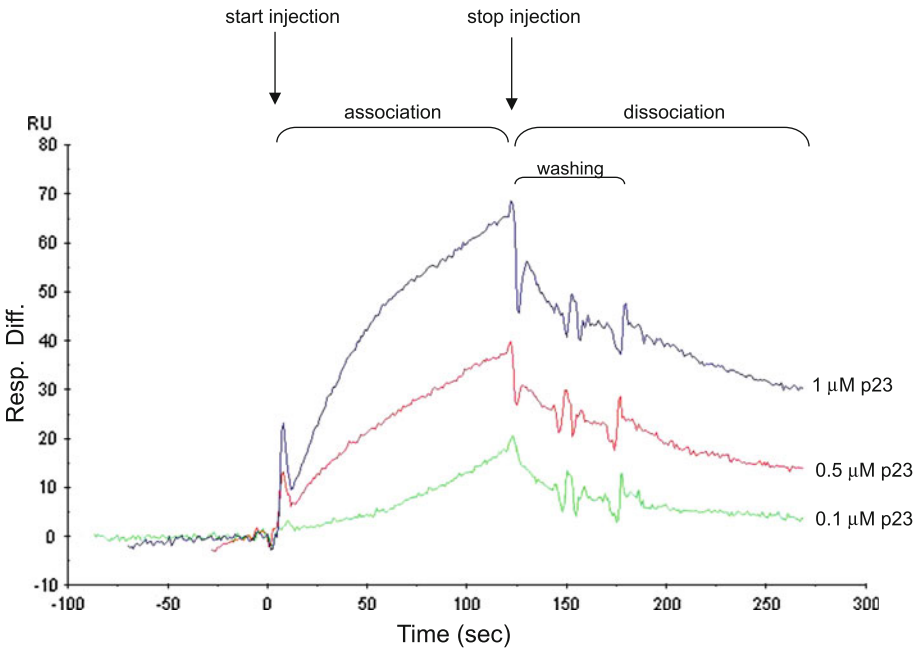

Fig. 5 Detection of CK2 α/p23 interaction by means of SPR. On a BIAcore X system, p23 solutions were injected at the indicated concentrations in HBS buffer, over a surface where human CK2 α was covalently coupled. Injection time was 2 min (association phase), then HBS buffer started to flow (dissociation phase). The variation of the response in the SPR signal is shown as response difference (Resp. diff.), after subtraction of the signal of the control cell. For this kind of experiments, His-tagged p23 solutions were used, since they were available at higher concentrations than thrombin-cleaved p23 solutions; however, single concentration experiments performed with p23 devoid of His-tag confirmed the binding (not shown)

protoplasts and analyzed by means of confocal microscopy. The results presented in Fig. 6g–i show that indeed we were able to recover a fluorescence signal, denoting the reconstruction of a functional fluorophore generated by the interaction between the cCFP and nVenus portions. In accordance with the subcellular localization analyses of CK2αC and p23, the signal was present in the cytoplasm and nucleus. However, when we tried to obtain negative controls by co-expressing p23-nVenus with the cCFP alone, a fluorescence signal was recovered as well (not shown), possibly due to system pitfalls [40]. Therefore, at present, our results are not conclusive for an in vivo interaction between CK2αC and p23; however, they clearly confirm the presence of the two proteins in the same subcellular compartments.

Discussion

CK2 is a highly pleiotropic kinase whose importance has been extensively described also in plant physiology; however, despite the huge number of substrates reported so far, only a minority of these are from plants [6].

In this study we identify the *Arabidopsis* p23 protein as a novel substrate for CK2. p23 is a co-chaperone protein homologous to the human p23 protein, which was first identified as a Hsp90 partner [41], with the role of assisting it in the chaperoning of steroid receptors [42, 43]. The molecular characterization of the *Arabidopsis* p23 has been recently published [34], showing that it binds to Hsp90 in its ATP-bound conformation; however, p23 has no effect on the Hsp90 ATPase activity, and, since the Hsp90/p23 client proteins in plants are unknown, no evident function of p23 has been described so far. Human p23, which shares only a 25–27% sequence identity with the *Arabidopsis* protein [34], was already reported as a CK2 substrate, in a paper where it was considered a prostaglandin E synthase [44]; in that work, the phosphorylated sites were also identified on Ser113 and Ser118.

Here we show that *Arabidopsis* p23 is phosphorylated by human and maize recombinant CK2, with high stoichiometry and favorable kinetic values; at difference to what observed for most of the CK2 substrates, the β regulatory subunit does not have a significant stimulatory effect on the p23 phosphorylation; on the other hand, this substrate does not match the features of the class II CK2 substrates, whose phosphorylation in the presence of the β subunit is inhibited, but completely rescued by the addition of polycationic effectors such as polylysine [2], which instead has no effect on p23 phosphorylation.

Our investigation was mainly performed in vitro with recombinant proteins, but we have also demonstrated that

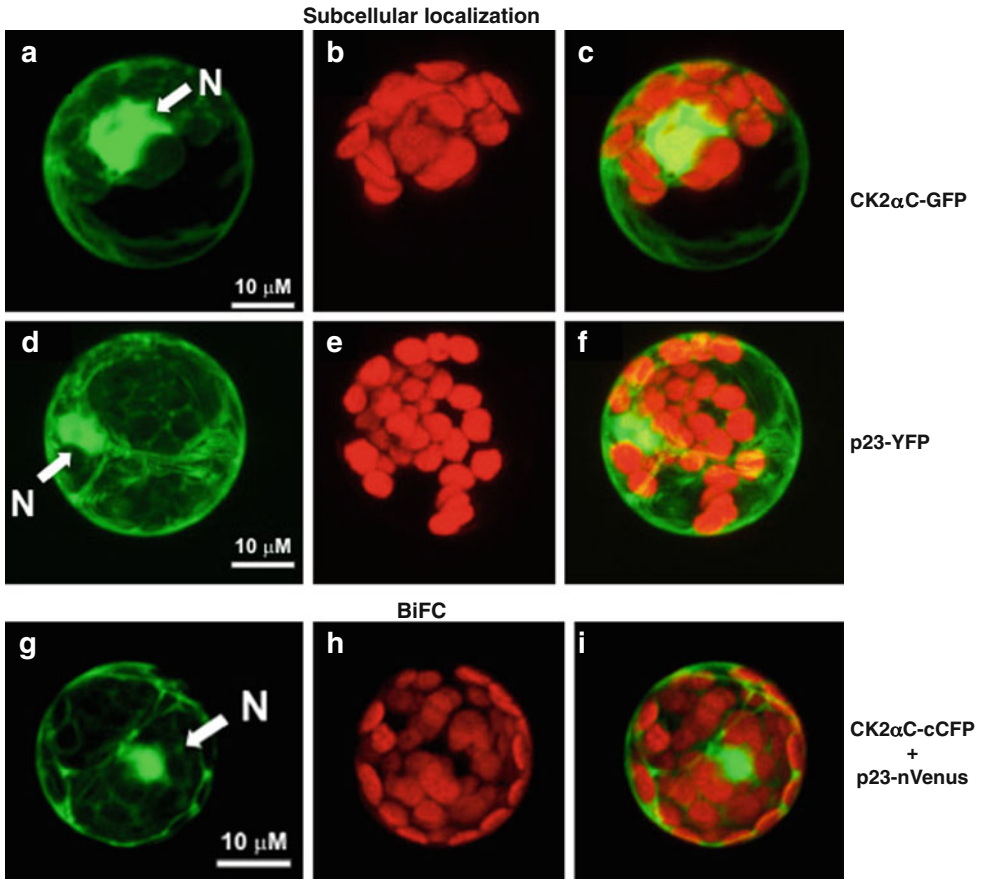

Fig. 6 Subcellular localization of CK2αC-GFP and p23-YFP and BiFC analysis in *Arabidopsis* mesophyll protoplasts. **a–c** Confocal 3D-reconstruction of a representative *Arabidopsis* mesophyll protoplast expressing the chimeric CK2αC-GFP protein: **a** GFP fluorescence present in the cytoplasm and nucleus (*N*) of the protoplast; **b** Chlorophyll autofluorescence of the same protoplast shown in a; **c** Overlay image of **a** and **b**. **d–f** Confocal 3D-reconstruction of a representative *Arabidopsis* mesophyll protoplast expressing the chimeric p23-YFP protein: **d** YFP fluorescence present in the cytoplasm and nucleus of the protoplast; **e** Chlorophyll autofluorescence of the same protoplast shown in **d**; **f** Overlay image of **d** and **e**. **g–i** Confocal 3D-reconstruction of a representative *Arabidopsis* mesophyll protoplast co-expressing the chimeric CK2αC-cCFP and p23-nVenus proteins: **g** reconstituted nVenus fluorescence present in the cytoplasm and nucleus of the protoplast; **h** chlorophyll autofluorescence of the same protoplast shown in **g**; **i** Overlay image of **g** and **h**

in *Arabidopsis* cytosol a kinase responsible for the p23 phosphorylation exists; this displays typical properties of CK2, being able to phosphorylate also casein and to use GTP as phosphate donor, having the size expected for *Arabidopsis* CK2αC, and being susceptible to inhibition by well-known CK2 inhibitors. Although an unequivocal demonstration of the in vivo association of p23 and CK2 was not achieved, we clearly showed that they colocalize in *Arabidopsis* mesophyll protoplasts, rendering their interaction quite conceivable.

This study originated from the observation that CK2 is required for the *Arabidopsis* response to SA treatment [20], and from the idea that only some specific substrates of CK2 change their phosphorylation state when SA is applied. Here we propose that p23 is a candidate for this role; however, for the time being, we can not say which isoform of p23 is really involved in the SA signaling, having considered, in this work, only p23-2; since the other isoform, p23-1, also displays CK2 consensus sites, its phosphorylation will be also investigated in the next future. Further studies will be also required to assess if (and which) SA-dependent events make p23 protein more readily accessible to CK2-dependent phosphorylation.

In conclusion, we can presently say that the p23 co-chaperone protein is a newly identified substrate of CK2 in plants, and that, despite no specific function of this protein is known so far, its involvement in the chaperone machinery makes it quite attractive, being a potential upstream regulator of numerous client proteins, including other protein kinases as well.

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