PLANT SIGNALING & BEHAVIOR
2020, VOL. 15, NO. 2, e1714189 (5 pages)
https://doi.org/10.1080/15592324.2020.1714189

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SHORT COMMUNICATION

Arabidopsis mtHSC70-1 physically interacts with the Cox2 subunit of cytochrome *c* oxidase

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ABSTRACT

The 70-kD heat shock proteins (HSP70s or HSC70s) function as molecular chaperones and are involved in diverse cellular processes. We recently demonstrated the roles of mitochondrial HSC70-1 (mtHSC70-1) in the establishment of cytochrome *c* oxidase (COX)-dependent respiration and redox homeostasis in *Arabidopsis thaliana*. Defects in COX assembly were observed in the *mtHSC70-1* knockout lines. The levels of Cox2 (COX subunit 2) proteins in COX complex were markedly lower in the mutants than in wild-type plants; however, the levels of total Cox2 proteins in the mutants were not obviously different from those in wild-type plants, suggesting that the stability of COX or the availability of Cox2 was impaired in the *mtHSC70-1* mutants. Here, we further detected the interaction between mtHSC70-1 and Cox2 proteins through co-immunoprecipitation, pull-down and firefly luciferase complementation imaging assays. The results showed that mtHSC70-1 could directly combine Cox2 *in vivo* and *in vitro*, providing supporting evidence for the role of mtHSC70-1 in COX assembly.

ARTICLE HISTORY
Received 7 November 2019
Revised 4 January 2020
Accepted 7 January 2020

KEYWORDS
Arabidopsis; mtHSC70-1; Cox2; cytochrome *c* oxidase

Eukaryotic complex IV or cytochrome *c* oxidase (COX), one of the respiratory complexes, catalyzes the transfer of electrons from reduced cytochrome *c* to the final electron acceptor O_2. COX is a multimeric enzyme formed by subunits of dual genetic origin whose assembly is intricate and highly regulated.[1] A large number of accessory factors are required to build a functional COX. The functions of these factors are required in all stages of the assembly process.[1,2] COX biogenesis has been widely studied in mammals and yeast. Current evidence suggests that COX is assembled from different modules, which are preassembled around the catalytic-core subunits.[3–5] In plants, COX biogenesis has not been extensively studied, and most proteins associated with its structure and assembly were annotated by sequence homology. Mansilla et al. performed a comparative analysis of COX composition and biogenesis factors in yeast, mammals and plants.[2] As a result, a total of 73 proteins from yeast, 62 proteins from mammals and 72 candidates from Arabidopsis were listed.[2,6–12] Arabidopsis putative COX-related proteins include 22 catalytic and structural subunits and 50 assembly factors.[2,3,13] It is worth noting that Arabidopsis mtHSC70-1 and mtHSC70-2 were also included in the list. These were identified as homologues of yeast Ssc1/Hsp70, which is involved in the regulation of Cox1 expression and assembly.[2]

In our first paper on mtHSC70-1, we demonstrated that Arabidopsis mtHSC70-1 plays important roles in the establishment of COX-dependent respiration and redox homeostasis. Using blue native-polyacrylamide gel electrophoresis and a polyclonal anti-Cox2 antibody, we found that the abundance of complex IV/COX was markedly lower in the *mtHSC70-1* mutants compared to WT plants,[14] indicating that the Cox2 level in COX was reduced by the *mtHSC70-1* knockout. Using real-time PCR and immunoblotting, we found that the levels of *Cox2* mRNA and total Cox2 protein were not obviously downregulated by the *mtHSC70-1* knockout,[14] suggesting that the stability of COX or the availability of Cox2 was impaired in the *mtHSC70-1* mutants. To address the role of mtHSC70-1 in the assembly of Cox2 into COX, we tested the interaction between mtHSC70-1 and Cox2 using the following three techniques. First, we carried out co-immunoprecipitation (co-IP) experiments using transgenic Arabidopsis seedlings containing GFP (GFP line) or mtHSC70-1-GFP (mtHSC70-1-GFP-1 and mtHSC70-1-GFP-2 lines). Co-IP was performed by adding GFP-Trap (ChromoTek, Planegg, Germany) to total protein extracts, and the immunocomplexes were analyzed through immunoblotting using an anti-Cox2 antibody or an anti-GFP antibody. We detected a distinct Cox2 band using mtHSC70-1-GFP-1 or mtHSC70-1-GFP-2 line after co-IP; however, no Cox2 band was detected using GFP line (Figure 1), indicating that mtHSC70-1 is likely to combine Cox2 *in vivo*. Second, we carried out a pull-down assay *in vitro*. The glutathione S-transferase (GST)–mtHSC70-1 fusion proteins and maltose-binding

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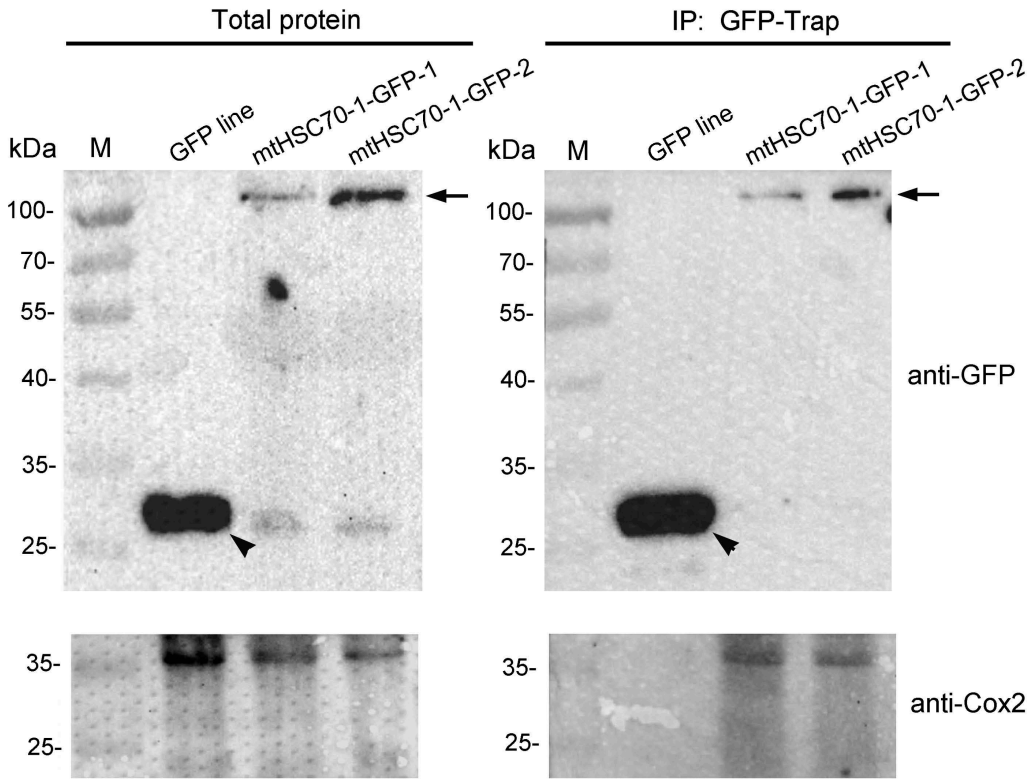

Figure 1. Interaction between mtHSC70-1 and Cox2 by co-IP assay. Total protein extracts from transgenic arabidopsis seedlings containing GFP (as control) or mtHSC70-1-GFP and the immunocomplexes were analyzed by immunoblotting using an anti-Cox2 antibody and an anti-GFP antibody. GFP line, a transgenic line containing GFP; mtHSC70-1-GFP-1 and mtHSC70-1-GFP-2, two transgenic lines containing mtHSC70-1-GFP; M, protein molecular weight marker; Arrows indicate the positions of mtHSC70-1-GFP; arrowheads indicate the positions of GFP. The experiment was repeated three times with reproducible results.

protein (MBP)–Cox2 fusion proteins were expressed in *Escherichia coli* BL21, and purified using glutathione agarose (Sigma-Aldrich, St Louis, MO, USA) or amylose resin (NEB, Ipswich, MA, England). Pull-down experiment was performed according to the protocol of Niu, et al.[15] The pull-down product was analyzed by immunoblotting using a mouse monoclonal anti-GST antibody (Sigma-Aldrich) or a mouse monoclonal anti-MBP antibody (Sigma-Aldrich). The result showed that GST–mtHSC70-1 were pulled-down by amylose resin with MBP–Cox2 but were not pulled-down by amylose resin with MBP alone (Figure 2(a)), indicating that mtHSC70-1 is able to directly bind to Cox2. Meanwhile, the interaction between mtHSC70-2 and Cox2 (Figure 2(b)) and the interaction between mtHSC70-1 and Cox1 (Figure 2(c)) were also tested; however, no positive reactivity was found. In addition, the binding of mtHSC70-1 or mtHSC70-2 to Cox1 or Cox2 was also examined through firefly luciferase complementation imaging (LCI) assay in tobacco leaves according to the protocol of Niu, et al.[15] The *mtHSC70-1/2* and *Cox1/2* coding sequence were cloned into the *pCAMBIA-NLuc* and *pCAMBIA-CLuc* vectors. The resulting constructs were separately introduced into the *Agrobacterium tumefaciens* strain GV3101 and then infiltrated into tobacco (*N. benthamiana*) leaves. Luciferin at 2 mM was sprayed onto the leaves, and the materials were kept in the dark for 6 min. A low-light cooled CCD imaging apparatus (VILBER Fusion FX5; VILBER, France) was used to capture luciferase (LUC) image. The result showed that none of the negative controls showed LUC complementation (Figure 3). The co-expression of *mtHSC70-1-NLuc* and *CLuc-Cox2* (Figure 3(a)), the co-expression of *CLuc-mtHSC70-2* (or *mtHSC70-2-NLuc*) and *Cox2-NLuc* (or *CLuc-Cox2*) (Figure 3(b)), and the co-expression of *CLuc-mtHSC70-1* (or *mtHSC70-1-NLuc*) and *Cox1-NLuc* (or *CLuc-Cox1*) (Figure 3(c)) also did not show LUC complementation. In contrast, the co-expression of *CLuc-mtHSC70-1* and *Cox2-NLuc* resulted in weak LUC complementation (Figure 3(a)), further confirming that mtHSC70-1, not mtHSC70-2, directly interacts with Cox2, not Cox1. Taken together, the data presented here and our previous data suggest that mtHSC70-1 plays an important role in the assembly of Cox2 into COX.

It is known that mature COX consists of three mitochondrial catalytic-core subunits (Cox1, Cox2 and Cox3) and more than 10 structural nuclear subunits.[2,16,17] At present, we only detected the interaction between mtHSC70-1 and two COX subunits; thus, the roles of

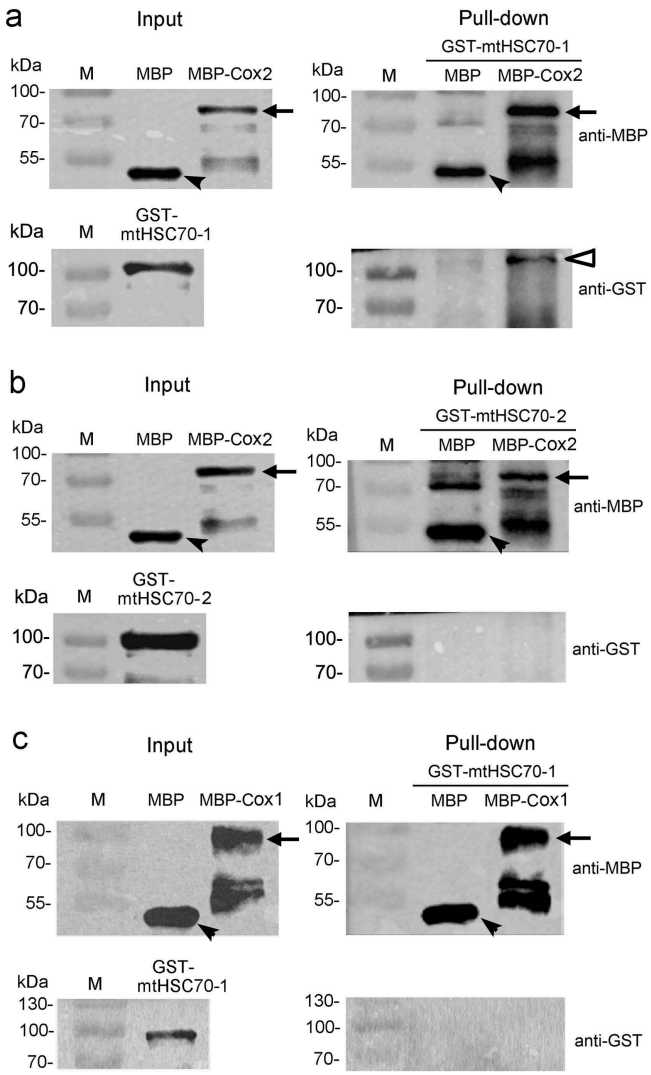

Figure 2. Interaction between mtHSC70-1 or mtHSC70-2 and Cox1 or Cox2 by pull-down assay. (a) interaction between mtHSC70-1 and Cox2; (b) interaction between mtHSC70-2 and Cox2; (c) interaction between mtHSC70-1 and Cox1. The purified MBP, MBP–Cox1, MBP-Cox2, GST-mtHSC70-1 and GST-mtHSC70-2 were detected by immunoblotting using a mouse anti-MBP monoclonal antibody or a mouse anti-GST monoclonal antibody (input). MBP-Cox1, MBP-Cox2 or MBP was first incubated with GST-mtHSC70-1 or GST-mtHSC70-2. The resulting complex was subsequently mixed with amylose resin. The binding of MBP-Cox1, MBP-Cox2 or MBP to amylose resin was detected by immunoblotting using a mouse anti-MBP monoclonal antibody. The Cox1- or Cox2-mediated binding of mtHSC70-1 or mtHSC70-2 to amylose resin was demonstrated by immunoblotting using a mouse anti-GST monoclonal antibody. M, protein molecular weight marker; Arrows indicate the positions of MBP-Cox1 or MBP-Cox2; solid arrowheads indicate the positions of MBP; hollow arrowhead indicates the target band. The experiment was repeated three times with reproducible results.

mtHSC70-1 in other COX subunits cannot be ruled out. Leaden et al. reported that mtHSC70-2 plays an important role in regulating the Fe-S assembly pathway in mitochondria.[18] Although the two Arabidopsis mtHSC70 isoforms are highly similar to each other (78% identical to each other), their functions are different. It has been reported that the expression patterns of these two genes are markedly different from each other.[14] We asked what causes functional divergence of these two mtHSC70 proteins – the differences in amino acid sequences or the differences in gene expression patterns. This is a question that we are very interested in, and it is one of the questions we will study in the future. Another question to be clarified is how mtHSC70-1 helps Cox2. To establish a functional respiratory complex, several steps must be followed in order, including the transcription of genes in the nucleus or mitochondria, the editing and processing of transcripts synthesized in the organelle, the synthesis and membrane translocation of proteins, the assembly of the subunits, and the insertion of the prosthetic groups.[2] In view of the proposed roles of HSP70s in protein *de novo* synthesis, protein translocation across membranes, native folding, oligomer assembly, and removal of toxic protein aggregates,[19–21] we speculate that mtHSC70-1 may act on the nascent Cox2 protein in the mitochondrial matrix to help them fold, transfer or insert into complex IV in the mitochondrial inner membrane; this conjecture has yet to be confirmed.

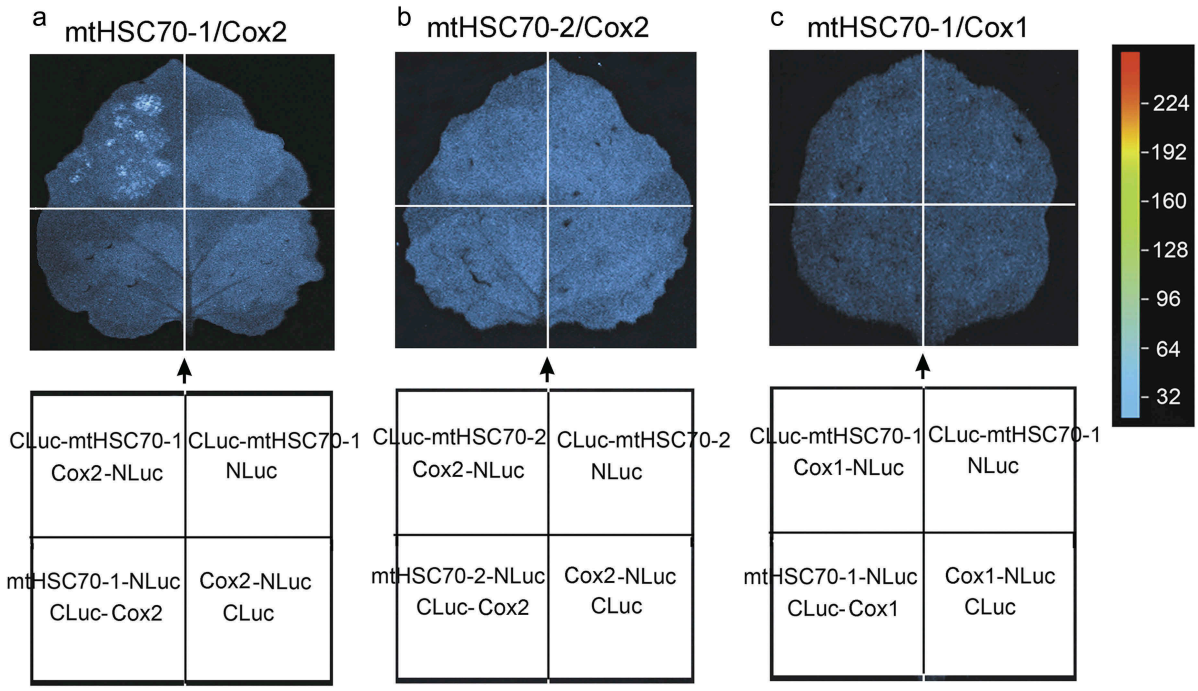

Figure 3. Interaction between mtHSC70-1 or mtHSC70-2 and Cox1 or Cox2 by LCI assay. (a) interaction between mtHSC70-1 and Cox2; (b) interaction between mtHSC70-2 and Cox2; (c) interaction between mtHSC70-1 and Cox1. NLuc, the N-terminal half of the firefly luciferase (LUC) protein; CLuc, the C-terminal half of the LUC protein. Upper panel, LUC images of tobacco leaves infiltrated with *Agrobacterium tumefaciens*; bottom panel, the plasmids contained in *A. tumefaciens* that were infiltrated into leaves; upper right corner, pseudocolour bar showing the range of luminescence intensity for each LUC image. The experiments were repeated four times with similar results, and representative images are shown.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the National Natural Science Foundation of China [grant no. 31870222], the Natural Science Foundation of Hebei Province, China [grant no. C2019205055] and Start-up funding from Hebei Normal University, China [grant no. L042018B01].

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