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Knock-down of protein phosphatase 2A subunit B'γ promotes phosphorylation of CALRETICULIN 1 in *Arabidopsis thaliana*

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Controlled protein dephosphorylation by protein phosphatase 2A (PP2A) regulates diverse signaling events in plants. Recently, we showed that a specific B'γ regulatory subunit of PP2A mediates basal repression of immune reactions in *Arabidopsis thaliana*. Knock-down *pp2a-b'*γ mutants display constitutive defense reactions and premature yellowing conditionally under moderate light intensity. Here we show that knock-down of *PP2A-B'*γ renders CALRETICULIN 1 (CRT1) highly phosphorylated. Calreticulins are ER-resident chaperonins that operate in the unfolded protein response to prevent ER-stress, components of which are differentially regulated at mRNA level in *pp2a-b'*γ leaves. We speculate that in dephosphorylated state, CRT1 promotes the degradation of unfolded proteins in ER. Our findings suggest that in wild type plants, dephosphorylation of CRT1 is mediated by PP2A-B'γ dependent signaling effects. In *pp2a-b'*γ, strong phosphorylation of CRT1 may partially imbalance the quality control of protein folding, thereby eliciting ER-stress and premature yellowing in leaves.

Different types of plant pathogens may cause enormous losses in agriculture and also have an ecological impact in the nature. On the molecular level, disease resistance is acquired through the action of tightly interconnected signaling pathways that may induce highly specific immune reactions in plant cells. Controlled protein dephosphorylation through protein phosphatase 2A activity is emerging as a crucial mechanism that regulates diverse signaling events in plants. PP2A is predominantly trimeric, and consists of a catalytic subunit, a scaffold subunit A, and a variable regulatory subunit B, which determines the target specificity of the PP2A holoenzyme.[1] Recently, we uncovered a specific role for a regulatory subunit B'γ of PP2A as a negative regulator of immune reactions in *Arabidopsis thaliana* (hereafter Arabidopsis).[2] Knock-down *pp2a-b'*γ mutants show constitutive activation of defense related genes, imbalanced antioxidant metabolism and premature disintegration of chloroplasts upon aging. Proteomic analysis of soluble leaf extracts further revealed that the constitutive defense response in *pp2a-b'*γ leaves associates with increased levels of Cu/Zn superoxide dismutase, aconitase as well as components of the methionine-salvage pathway, suggesting PP2A-B'γ modulates methionine metabolism in leaves.

Constitutive Defense Response of *pp2a-b'*γ Involves Strong Phosphorylation of CALRETICULIN 1

Here, we have utilized ProQ-staining (ProQ Diamond, Invitrogen) to further explore the phosphoproteome of *pp2a-b'*γ leaves. Analysis of photosynthetic thylakoid membranes and total leaf soluble extracts by 1D SDS-PAGE and subsequent ProQ-staining of phosphoproteins revealed a number of differentially labeled proteins in the soluble fractions from *pp2a-b'*γ leaves as compared with the wild type (**Fig. 1A**). In thylakoid membranes, no differentially phosphorylated proteins could be detected (**Fig. 1A**). To further explore the differentially phospho-labeled soluble proteins, oligomeric protein complexes of soluble fractions were separated by clear native electrophoresis (CN-PAGE), followed by SDS-PAGE and subsequent ProQ-staining in the second dimension. Designing the electrophoretic conditions in CN-PAGE to specifically separate small protein complexes and free-running proteins allowed identification of two highly phosphorylated protein spots (**Fig. 1B**), which were barely detected upon staining with Sypro Ruby in *pp2a-b'*γ leaves (**Fig. 1C**). These presumably low abundance proteins did not co-migrate with any detectable protein complex. Mass spectrometric analysis identified these protein spots as CALRETICULIN 1 (CRT1; At1g56340) and tubulin α-chain 6 (TUA; At4g14960), which on Sypro-stained gels were found to be present in similar levels in wt and *pp2a-b'*γ leaves (**Fig. 1C**). Densitometric quantification and normalization of spot intensities on ProQ-stained gel against spot intensities on Sypro-stained gel for these individual proteins revealed ratios of 25.9 and 12.4 for phospho-CRT1/CRT1 in *pp2a-b'*γ and wt, respectively, and ratios of 13.3 and 8.8 for phospho-TUA/TUA in *pp2a-b'*γ and wt, respectively.

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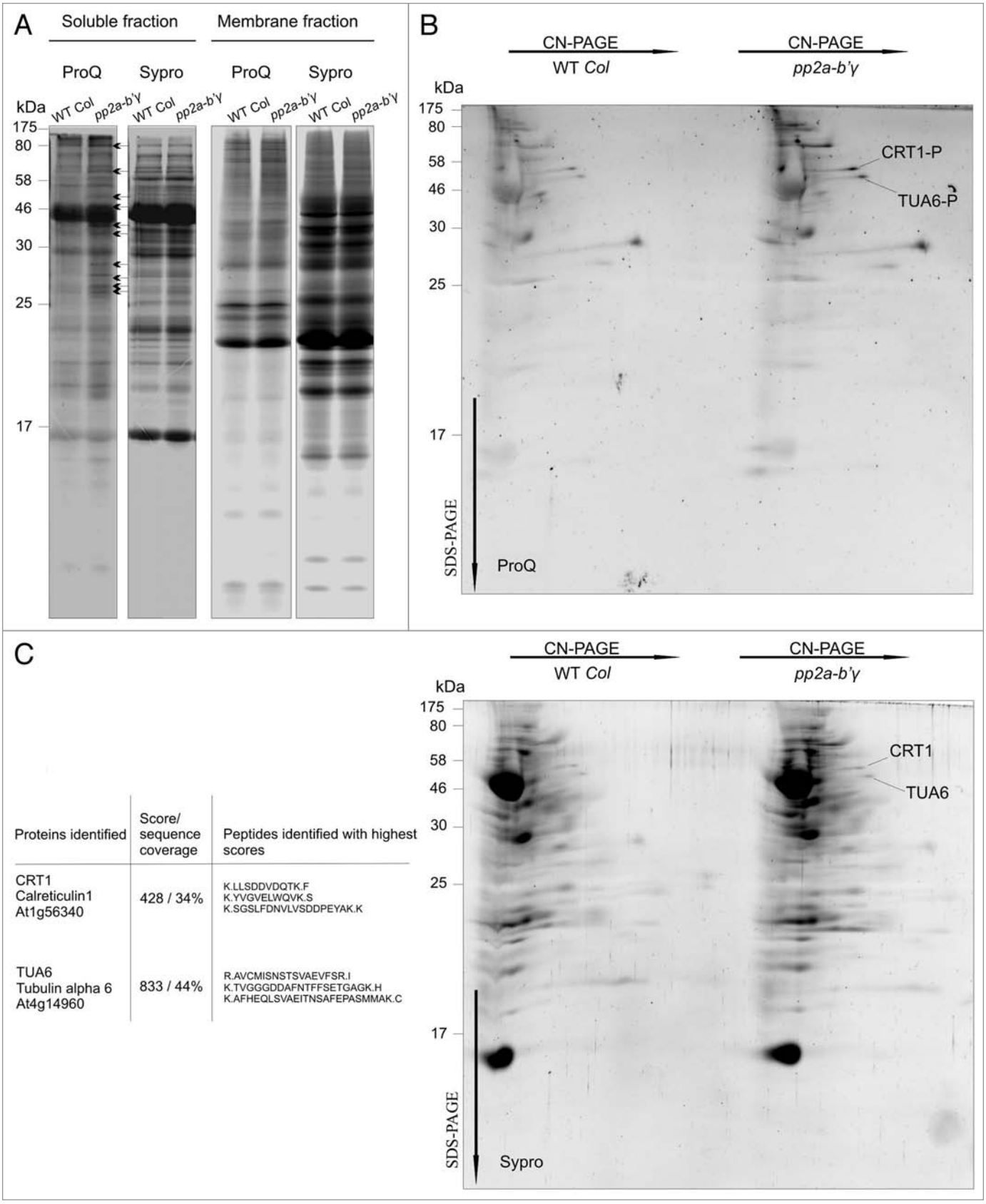

Proteins identified	Score/ sequence coverage	Peptides identified with highest scores
CRT1 Calreticulin1 At1g56340	428 / 34%	K.LLSDDVDQTK.F K.YVGVELWQVK.S K.SGSLFDNVLVSDDPEYAK.K
TUA6 Tubulin alpha 6 At4g14960	833 / 44%	R.AVCMISNSTSVAEVFSR.I K.TVGGGDDAFNTFFSETGAGK.H K.AFHEQLSVAEITNSAFEPASMMAK.C

Figure 1. For figure legend, see page 1667.

Figure 1 (See opposite page). Proteomic approach to identify differentially phosphorylated proteins in *pp2a-b'γ* leaves. (A) One-dimensional SDS-PAGE of soluble and membrane fractions of 5-week-old wild type (WT Col) and *pp2a-b'γ* leaves. Phosphoproteins were detected with ProQ (ProQ Diamond, Invitrogen), and total proteins were subsequently stained with Sypro Ruby (Invitrogen). Proteins strongly phosphorylated in soluble fraction of *pp2a-b'γ* are indicated by arrows. Samples corresponding to 80 μg of protein were loaded in each well. (B) ProQ and and (C) Sypro staining of soluble oligomers separated by CN-PAGE in first dimension, followed by SDS-PAGE is the second dimension. A gradient of 5–12% acrylamide was used to obtain separation of free-running proteins. The identified peptides are presented on the left hand side of the Sypro-stained gel.

Does PP2A-B'γ Modulate the Extent of ER-Stress through Dephosphorylation of CALRETICULIN 1?

Calreticulins are endoplasmic reticulum (ER)-resident chaperonins involved in the unfolded protein response (UPR), which becomes upregulated to activate protein folding and degradation machineries upon high rate of protein synthesis and/or accumulation of improperly folded proteins in ER.[3,4] CRT1 has been found to undergo dephosphorylation in response to oligogalacturonide treatment of tobacco cells, and this dephosphorylation event is sensitive to the PP2A inhibitor okadaic acid, suggesting that PP2A activity is involved in the dephosphorylation of CRT1.[5] Oligogalacturonides are released from the cell wall upon certain plant-pathogen interactions, and their perception by wall associated kinases (WAKs) elicit a subset of immune reactions to combat the invading attacker.[6] Even though the physiological significance of CRT1 phosphorylation in plants has not been revealed,[7,8] it appears to form a connection between PP2A-B'γ, ER stress and oligogalacturonan signaling, all of which have been assigned important roles in plant immunity.

CRTs together with membrane-bound calnexins (CNXs) operate in ER quality control (ERQC), which ensures successful folding of glycosylated proteins in the secretory pathway.[4,9,10] Other main components of the ERQC system include HSP70 family binding proteins (BiPs), which bind nascent polypeptides upon N-glycosylation to monitor their folding and protein disulphide isomerases (PDIs), which in concert with CNXs/CRTs prevent misfolding and aggregation of the client proteins.

ER quality control is based on successive removal of three glucose residues from the N-glycosylated client proteins. The first two glucose residues are removed by glucosidases I and II, which is followed by release of the protein from BiP and subsequent recognition by the CNX/CRT/PDI protein folding machinery. Removal of the third and innermost glucose residue by glucosidase II releases properly folded proteins for export from ER. Misfolded proteins, in contrast, are recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT), which re-directs the unfolded protein to the CNX/CRT chaperonin complex for a further round of folding. Upon irreversible misfolding or prolonged shuttling between UGGT and CNX/CRT, an additional mannose residue is removed from the glycan to target the protein to ER-associated degradation (ERAD) machinery. In this way, misfolded proteins are directed into the cytosplasm, where they become ubiquitinylated and finally degraded by proteasome activity.[4,9,10]

Accumulation of unfolded proteins is sensed by ER-resident membrane-spanning transcription factors bZIP17, bZIP28 and bZIP60, which become released to promote the transcription of UPR-related genes. In *pp2a-b'γ* leaves, only bZIP60, known to be responsible for activation of a sub-set of UPR-related genes, is induced.[11-13] Of the three CRTs encoded by the Arabidopsis genome,[9,10] *CRT2* and *CRT3* are strongly upregulated at mRNA level in *pp2a-b'γ* leaves, similarly to the UPR genes encoding BiP2, BiP3 and a number of PDIs.[2] *CRT1*, however, is not transcriptionally differentially regulated, and genes encoding UGGT and a mannosidase (At1g27520) that operate in the last steps of ERQC are downregulated in *pp2a-b'γ*.[2] These findings do not follow the general consensus that activation of both the folding and degradation machineries of the ER quality control is required for ER stress to become relieved.

The unfolded protein response is tightly connected with formation of systemic acquired resistance (SAR) in plants,[14] and is commonly induced already at early phases of pathogen attack in order to prepare the ER for massive translation of defense-related proteins.[15] Indeed, pathogen related proteins and plasma membrane-targeted pattern recognition receptors are processed in ER before they become released to the extracellular space, thereby over-loading the ER machinery. CRT3, in particular, has been shown to be specifically required for the quality control of the EF-Tu receptor EFR, which is a pattern recognition receptor (PRR) involved in immunity induced by microbe-associated molecular patterns.[16,17] Strong prevalence of ER-stress may also elicit cell death upon biotic and abiotic challenges in plant cells.[18]

We speculate that dephosphorylated form of CRT1 promotes the degradation of unfolded proteins in ER. Our results suggest that in basal state, the dephosphorylation of CRT1 is mediated by PP2A-B'γ-dependent signaling effects in wild type plants. Since PP2A-B'γ is predominantly cytosolic, CRT1 is unlikely to represent a cognate target for B'γ-dependent dephosphorylation by PP2A.[2] The constitutive immune response of *pp2a-b'γ*, however, involves strong phosphorylation of CRT1 and misregulation of genes related to the UPR. Together, these regulatory imbalances may elicit ER-stress and promote premature senescence in *pp2a-b'γ* leaves.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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