Final report

Project Title: Proteomic study of brassinosteroid responses in Arabidopsis

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Research Progress and Achievement

SUMMARY

The steroid hormone brassinosteroid (BR) is a major growth-promoting phytohormone. The specific aim of the current project is to identify BR-regulated proteins and characterize their functions in various aspects of plant growth, development, and adaptation. Our research has significantly advanced our understanding of how BR signal is transduced from the receptor at the cell surface to changes of nuclear gene expression and other cellular responses such as vesicle trafficking, as well as developmental transitions such as seed germination and flowering. We have also developed effective proteomic methods for quantitative analysis of protein phosphorylation and for identification of glycosylated proteins. Through this DOE funding, we have performed several proteomic experiments and made the following major discoveries.

1. We elucidated the phosphorylation events that mediate signal transduction from the cell surface receptor kinase BRI1 to the cytoplasmic kinase GSK3/BIN2 (Kim et al., 2009).
2. We identified PP2A as a component of the BR signaling pathway that mediates the dephosphorylation of the BZR1 family transcription factors (Tang et al. 2011).
3. We discovered that the GSK3-like kinase BIN2 of the BR pathway phosphorylates the MAP kinase kinase kinase YODA of the ERECTA receptor kinase pathway, to modulate stomata development (Kim et al., 2012),
4. We have identified about 90 proteins whose phosphorylation level is regulated by BR treatment (Xu et al., unpublished)
5. Among the BR-regulated phospho-proteins, we have characterized the NBR1 protein as a BR-regulated RNA splicing regulator that controls major developmental transitions such as seed germination and flowering. We have identified abour 80 proteins associated with NBR1, most of them are RNA-binding and splicing factors (Bi et al., manuscript in preparation).
6. We have characterized a BR-regulated phosphoprotein, TRS33, that plays a key role in cellular membrane/vesicle trafficking (Garcia et al., unpublished).
7. This project also supported a collaboration with Dr. Peter Quail on phytochrome signaling mechanisms, leading to the identification of ubiquitin ligase and kinases that mediate phytochrome signal transduction (Ni et al., 2013, 2014, 2017).
8. Further, our proteomic studies have identified the first large set of 262 O-GlcNAcylated plant proteins (previously only 3 are known), most of them playing regulatory roles in the nucleus (Xu et al., 2017). This opens a new field of glyco-signaling in plants.

In summary, this DOE-funded project has significantly advanced our understanding of not only BR signaling mechanisms but also functions of RNA splicing and protein O-GlcNAc modifications in plant growth and developmental regulation.

UNPUBLISHED RESEARCH ACHIEVEMENTS

Phosphoproteomic analysis of BR-regulated protein phosphorylation:

We have performed several BR treatment experiments followed by LC-MS/MS analysis, using ^{15}N-labeling. We purified phospho-peptides from total protein samples of BR treated and

untreated seedlings, and LC-MS/MS analysis identified and quantified 11,047 phosphopeptides on 3,135 proteins. We found phosphorylation decreases at 80 sites and increases at 25 sites on 91 proteins upon BR treatment. These include 7 known proteins of the BR pathway, and the rest unknown previously for a function in BR response. These new BR-responsive phosphoproteins represent new links from BR signaling to various cellular processes, including signaling by other receptor kinases, RNA splicing, translation, and crosstalk with other signaling pathways, as well as cellulose synthesis, cation exchangers, microtubule-associated proteins, and vacuolar protein sorting factor. Our proteomic analyses have identified a large number of proteins with potential functions in steroid regulated cellular growth.

BR regulation of vesicle trafficking.

We found that BR induces dephosphorylation (0.30x and 0.46x fold changes in repeat experiments) of the trafficking protein particle (TRAPP) complex subunit 6B (TPC6B, also named TRS33). The Arabidopsis AtTRS33 share about 49% sequence identity with the mouse TPC6B. Mammalian TRAPP complex plays a key role in the targeting and/or fusion of ER-to-Golgi transport vesicles with their acceptor compartment. TRAPP is a large multimeric protein complex that contains at least 10 subunits. The *trs33* mutant shows dwarfism and de-etiolation in the dark, similar to BR-insensitive mutants. The BR-regulated phosphorylation is at a Threonine residue that is part of seven Ser/Thr residues arranged in a pattern matching putative GSK3 phosphorylation sites. Our in vitro assays have shown that the BIN2 GSK3-like kinase phosphorylates wild type TRS33, but not a mutant TRS33 containing substitution of the seven Ser/Thr residues with alanine, confirming that BIN2 phosphorylates these residues. Together these results support our hypothesis that BR inactivation of BIN2/GSK3 leads to dephosphorylation of AtTRS33. We are currently testing the function of the BR-dependent TRS33 phosphorylation in BR-regulation of vesicle trafficking and plant growth.

BR regulation of cellular calcium levels.

We also found BR-induced phosphorylation of a conserved residue in the autoregulatory domain of two homologous cation exchangers (CAX1, and CAX3) at a conserved residue, implicating BR regulation of calcium homeostasis (the CAX1 phosphopeptide increased 3.2x and 9.2x, CAX3 phosphopeptide increased 2.2x and 2.0x). Recent studies have shown that BR induces cytosolic Ca^{2+} level increase. We have mutagenized the phosphorylation site of CAX1, and have transformed the mutant CAX1 gene into the *cax1,cax3* double mutant. If the phosphorylation mediates BR regulation of CAX1 activity and cytosolic Ca^{2+} elevation, we expect the transgenic plants to be insensitive to BR in Ca^{2+} responses.

A BR-regulated phosphoprotein controls alternative splicing of RNAs encoding key developmental regulators.

We have identified a BR-regulated nuclear protein (NBR1) in our proteomic analysis of BR-responsive proteins, using both two-dimensional difference gel electrophoresis (2D-DIGE). We have confirmed BR-induced NBR1 dephosphorylation using immunoprecipitation combined with 2D-DIGE and Stable Isotope-labeling in Arabidopsis followed by mass spectrometry (SILIA-MS) methods (Figure 1 and 3).

We have identified two T-DNA insertion lines of *nbr1* mutant, both of which displayed late-flowering phenotype (Fig. 2), with elevated expression of *FLC*, a known repressor of flowering.

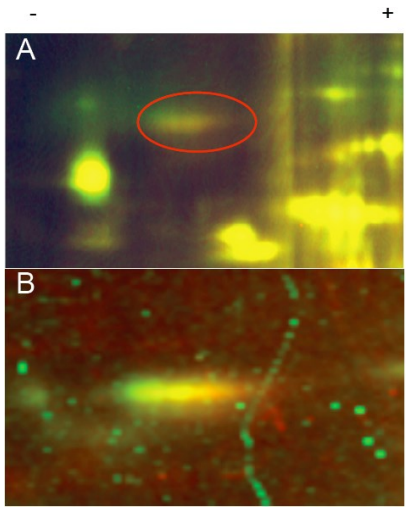

Fig. 1. A. 2-D DIGE analysis of nuclear proteins from the *det2*-1 mutant treated with BR (Red, Cy5-labelled) or with mock solution (green, Cy3-labelled). Red circle marks the spots shifted to the basic side by BR treatment, which contains the NBR1 protein. B. IP-DIGE analysis of BR-induced NBR1 modification. NBR1 was immunoprecipitated from BR-treated or mock-treated plants, labeled with Cy5 and Cy3, respectively.

NBR1 contains 38 putative BIN2 phosphorylation sites (Ser/Thr-X-X-X-Ser/Thr). We tested whether BIN2 phosphorylate NBR1. *In vitro* kinase assays showed that BIN2, but not a kinase dead version, phosphorylates NBR1 (Fig. 3A). We identified 22 *in vivo* phosphorylation sites by immunoprecipitation of NBR1 proteins followed by mass spectrometry analysis. Nine of these sites were also detected by BIN2 phosphorylation *in vitro* (Fig. 3B). We further used label free mass spectrometry quantification to quantify the relative phosphorylation level in mock-, bikinin- and BR-treated samples, and found that both bikinin and BR treatments reduced the level of phosphorylation on multiples sites (Fig. 3C-D), indicating BIN2, or BIN2 homologous GSK3 kinases, phosphorylates NBR1.

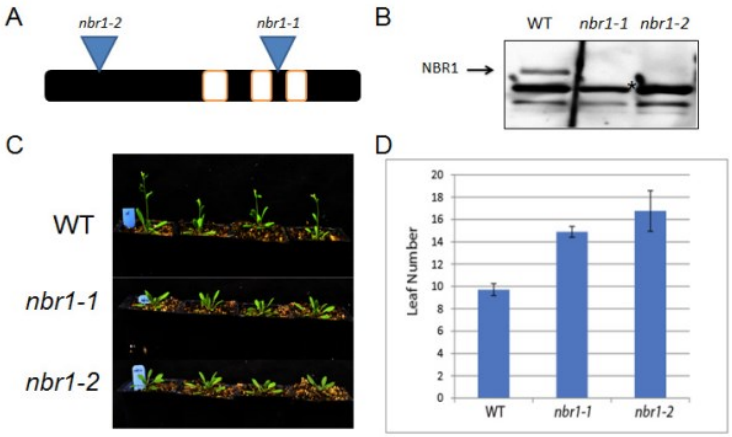

Fig.2. The *nbr1* mutants display a late flowering phenotype.
A.Schematic diagram shows the T-DNA insertion in *nbr1-1* and *nbr1-2*. Black box: Exons. White box: Introns.
B.Immunoblot of wild-type and *nbr1* mutants using anti-NBR1 antibody.
C.The *nbr1* mutants flower late compared to the wild-type plants.
D. Numbers of rosette leaves.

Recently, the human homolog of NBR1 was shown to be a subunit of the apoptosis and splicing-associated protein (ASAP) complex, which interacts with the exon-junction complex (EJC) to control post-transcriptional regulation. The study provides strong evidence that ASAP complex is a hub in the protein interaction network that regulates gene expression.

To characterize the molecular function of NBR1, we immunopurified NBR1 complex, using either anti-NBR1 antibody or an anti-GFP antibody and NBR1-GFP transgenic Arabidopsis, with the *nbr1-2* mutant or 35S:GFP lines as control, respectively. The proteins were analyzed by SDS-PAGE followed by mass spectrometry. About 80 proteins were specifically identified as NBR1 interacting proteins (not detected in the controls), of which about 70% are predicted to be involved in RNA processing or chromatin modification.

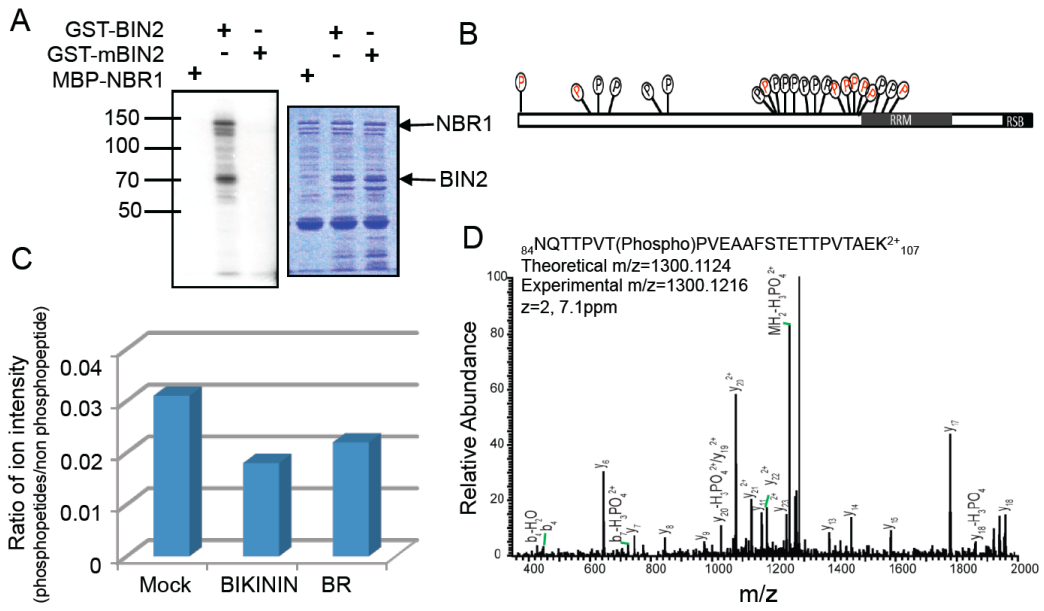

Fig. 3. Analysis of NBR1 phosphorylation.
A. I*n vitro* kinase assay shows BIN2, but not mutant (kinase dead) version, phosphorylates NBR1.
B. Diagram of domains and phosphorylation sites on NBR1. A total of 22 *in vivo* phosphorylated sites were identified on immunoprecipitated NBR1 protein by mass spectrometry using an Orbitrap mass spectrometer. Nine of these sites (highlighted in red color) were also identified on NBR1 phosphorylated by BIN2 in vitro.
C. Bikinin and BR treatments both reduce the level of phosphorylation of Thr90. Relative phosphorylation level was quantified as ratio between peak intensities of the phosphopeptide and its non-phosphorylated counterpart (aa84-107).
D. A mass spectrum of peptide from NBR1, identifying phosphorylation on Thr90.

Homologs of other components of the ASAP complex, Serine/arginine-rich 45 (the Arabidopsis homolog of RNPS1), and SAP18 were consistently detected in the NBR1 complex. Other NBR1-interacting proteins include the snRNPs proteins of splicesomes. Mutations of genes encoding some of these NBR1-associated proteins also display a late flowering phenotypes. Interestingly, EARLY FLOWERING 8 (ELF8) was also detected as an NBR1-associated protein. CTR9/ELF8 and PAF1/ELF7 are integral components of the PAF1 complex, which is involved in regulation of histone 3 trimethylation at the *FLC* locus {He, 2004 #2608}. We have confirmed the NBR1 interaction with ELF7 by co-immunoprecipitations (Fig. 4).

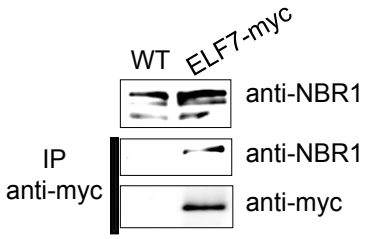

Fig. 4. Wild-type and ELF7-myc transgenic plants were immunoprecipitated with anti-myc antibody and the immunoblot was probed with anti-NBR1 and then with anti-myc antibody.

To unambiguously identify and quantify the NBR1 interactions, we have performed a metabolic ^{15}N-labeling experiment (Fig. 5). Reciprocal labeling of wild type and *nbr1-2* plants with ^{15}N and ^{14}N, followed by immunoprecipitation and mass spectrometry analysis, provided high confidence data (Fig. 5). Analysis of the spectra yielded high-confidence data that clearly distinguished real interacting proteins from false positives (Fig. 5).

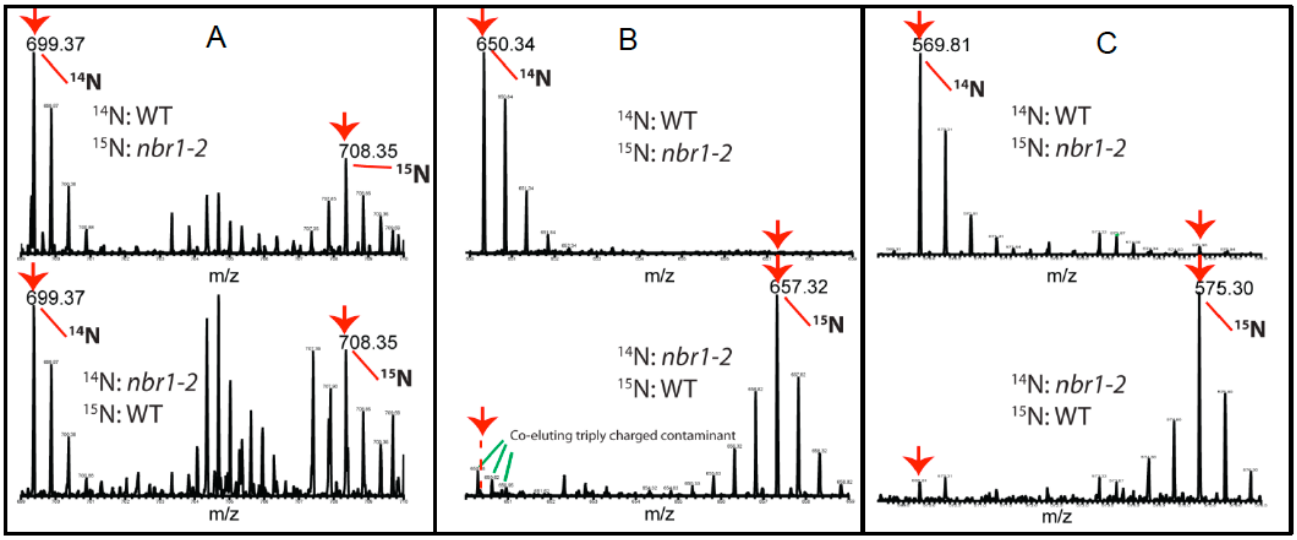

Figure 5: Quantitative analysis of NBR1-interacting proteins using reciprocal isotope labeling followed by immunoprecipitation and tandem mass spectrometry. Top panel: WT labeled with ^{14}N and *nbr1-2* with ^{15}N. Bottom panel: *nbr1-2* labeled with ^{14}N and WT with ^{15}N. Immunoprecipitations were performed using anti-NBR1 antibodies. Precipitated proteins for each pair of samples were mixed and digested by tripsin. The peptides were analyzed on a Velos-Orbitrap mass spectrometer. For all quantified peptides, at least one isoform peptide version was identified from MS/MS data by database searching. Arrows: monoisotopic peaks of ^{14}N- and ^{15}N-labeled peptides.
A: Extracted ion chromatogram of peptide VLENLGADPSNIR from non-specific interacting protein Chaperone protein ClpC1. Unlabeled, m/z=699.37, z=2; labeled, m/z=708.35, z=2.
B: Extracted ion chromatogram of peptide LSFAFVYPNNK from specific interacting protein histone deacetylase complex subunit SAP18. Unlabeled, m/z=650.34, z=2; labeled, m/z=657.32, z=2.
C: Extracted ion chromatogram of peptide DIWVTYLTK from specific interacting protein pre-mRNA-splicing factor SYF1. Unlabeled, m/z=569.81, z=2; labeled, m/z=575.30, z=2.

The mild phenotypes of *nbr1* mutant was surprising, given it's evolutionarily conserved in plants and animals. Studies in mammalian system have identified a distant homolog PNN, and a homolog of PNN is also present in Arabidopsis (AtPNN), although the similarity between NBR1 and PNN is limited to only a 15-aa region. The *pnn* single mutant showed no obvious phenotype, but the *nbr1,pnn* double mutant showed several growth defects and delayed flowering, suggesting that NBR1 and PNN play overlapping or redundant roles despite their limited sequence similarity.

We have performed RNA-seq analysis of changes in RNA level and RNA splicing in the *nbr1,pnn* double mutant, and identified over 258 intron retention events in 225 genes. Many of these genes are involved in stress responses, including signal transduction components of the stress hormone abscisic acid (ABA), which controls seed germination and abiotic stress responses. Indeed, we found that the *nbr1,pnn* double mutant is hypersensitive to ABA and show delayed seed germination. Our results support a model that BR-regulated NBR1 phosphorylation modulate alternative splicing of a number of specific genes, which in turn alter cellular response to ABA and developmental transition such as seed germination. For flowering regulation, our data show that BBR1 directly associates with the promoter of the *FLC* gene. Together, our results demonstrate that NBR1 (and likely PNN too) controls major developmental transition such as seed germination and flowering through alternative RNA splicing and transcriptional control of key regulators.

PUPLICATONS reporting works supported by this award:

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