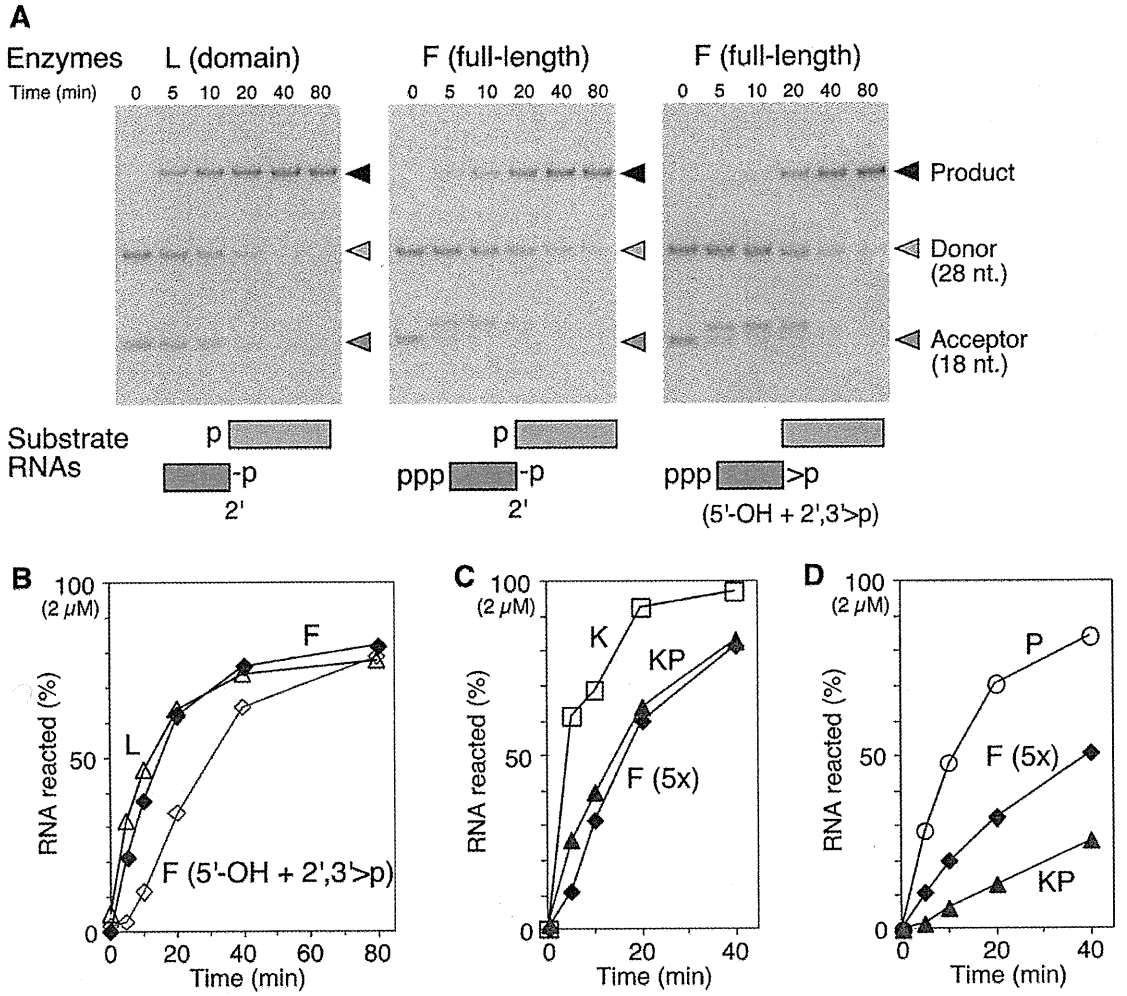

Fig. 3. Activities of the full-length and domain enzymes. (A) Denaturing polyacrylamide gels showing the time courses of the intermolecular ligation reactions catalyzed by the ligase domain and the full-length enzymes. The terminal structures of the substrate RNAs are schematically drawn at the bottom. The substrate RNAs used were 18NTGCIPCPD and GP1PNK (left panel), 18NTGCPD and GP1PNK (center panel), and 18NTG and GP1 (right panel). The concentrations of the enzymes and each RNA substrate were 0.57 μM (F), 0.70 μM (L), and 2 μM (RNA), respectively. The gels were stained with methylene blue. (B–D) The time courses of the reactions catalyzed by the full-length and each domain enzymes. The relative amounts of the substrate and product RNAs for the RNA ligase (B), 5′-kinase (C), and 2′,3′-cyclic phosphate-3′-phosphodiesterase (D) reactions are plotted. The initial concentration of the substrate RNAs was 2 μM. The enzyme concentrations were 0.57 μM for F and 0.70 μM for L in B, 0.43 μM for F, 0.18 μM for K, and 0.14 μM for KP in (C), and 0.14 μM for F, 0.076 μM for P, and 0.045 μM for KP in (D). Specific activities in mol (mol of enzyme)$^{-1}$ min^{-1} calculated from the time courses are 0.11 for L and 0.13 for F (5′-p, 2′-p-3′-OH ligase) in (B), 0.80 for K, 0.56 for KP, and 0.15 for F in (C), and 1.3 for P, 0.28 for KP, and 0.28 for F in (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enabled us to realize that the domain assembly into the multidomain RNA ligase caused a suppressive effect on the kinase and CPD domain activities. The embedded kinase and CPD domains may be well-regulated to solely meet the necessity for the following RNA ligation reaction. Our results shed new light on the way multidomain enzymes have been selected.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.030.

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METHODOLOGY ARTICLE Open Access

Biotinylated-sortase self-cleavage purification (BISOP) method for cell-free produced proteins

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Abstract

Background: Technology used for the purification of recombinant proteins is a key issue for the biochemical and structural analyses of proteins. In general, affinity tags, such as glutathione-S-transferase or six-histidines, are used to purify recombinant proteins. Since such affinity tags often interfere negatively with the structural and functional analyses of proteins, they are usually removed by treatment with proteases. Previously, Dr. H. Mao reported self-cleavage purification of a target protein by fusing the sortase protein to its N-terminal end, and subsequently obtained tag-free recombinant protein following expression in *Escherichia coli*. This method, however, is yet to be applied to the cell-free based protein production.

Results: The histidine tag-based self-cleavage method for purifying proteins produced by the wheat cell-free protein synthesis system showed high background, low recovery, and unexpected cleavage between the N-terminally fused sortase and target protein during the protein synthesis. Addition of calcium chelator BAPTA to the cell-free reaction inhibited the cleavage. In order to adapt the sortase-based purification method to the cell-free system, we next used biotin as the affinity tag. The biotinylated sortase self-cleavage purification (BISOP) method provided tag-free, highly purified proteins due to improved recovery of proteins from the resin. The N-terminal sequence analysis of the GFP produced by the BISOP method revealed that the cleavage indeed occurred at the right cleavage site. Using this method, we also successfully purified the E2 heterocomplex of USE2N and USE2v1. The c-terminal src kinase (CSK) obtained by the BISOP method showed high activity in phosphorylating the Src protein. Furthermore, we demonstrated that this method is suitable for automatically synthesizing and purifying proteins using robots.

Conclusion: We demonstrated that the newly developed BISOP method is very useful for obtaining high quality, tag-free recombinant proteins, produced using the cell-free system, for biochemical and structural analyses.

Background

Technology used for purifying a recombinant protein has a significant impact on its biochemical function, structural properties, and other aspects, such as generating an antibody against the protein. Currently available, established purification methods generally attach an affinity tag to the N-terminus or C-terminus end of the target protein, and then recover the target protein by affinity chromatography [1]. Purification tags used today are classified into peptide-tags and protein-tags based on their nature and form. His-tag, a typical and globally the most used peptide-tag, is highly effective in purifying the tagged protein by using immobilized metal affinity chromatography, such as nickel sepharose [2]. The glutathione-S-transferase (GST) tag, a typical protein-tag, has a high specific binding capacity for glutathione, and is, generally, known to have little or no effect on the activity of the fused recombinant protein [3]. In both cases, however, production of tag-free recombinant protein requires treatment with a protease, such as PreScission or TEV protease. Therefore, in order to purify a tag-free recombinant protein multiple chromatography steps are necessary.

Currently, there are only a few recombinant protein purification methods that combine affinity purification, cleavage, and separation of the fusion partner in one-step. One such one-step purification method was reported by Mao [4], in which the catalytic core of the transpeptidase

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sortase A (srtA, amino acid residues from 60-206), found in the cell envelope of *Staphylococcus aureus* [5,6], was used for fusion with the target protein. The SrtA enzyme is known to catalytically cleave the Thr-Gly bond of its recognition motif LPXTG (X is any amino acid) in the presence of calcium and triglycine [6-9]. Thus, the purified target protein eluted off the affinity column has only an extra Gly residue on the N-terminus end. This excellent approach was designed for the purification of recombinant proteins expressed in *Escherichia coli* cells. However, as discussed in that report [4], the fusion protein was partially self-cleaved during the expression, probably because of the difficulty in controlling the concentration of calcium in the living cells. Inability to suppress the srtA activity during the expression of the fused recombinant protein in *E. coli* was, therefore, a major limiting factor for using this otherwise excellent approach as a general tool for the production and purification of recombinant proteins.

At present, several types of cell-free protein production systems have been reported as alternative methods for obtaining recombinant proteins [see 10 and 11 for reviews]. In this regard, it is noteworthy that we are also developing wheat embryo based cell-free system for in vitro protein production [12-14]. Cell-free protein production is very flexible because it utilizes only the translational machinery of the cell without other factors, such as DNA replication and metabolic pathways, of the living system. The cell-free system, thus, could simply be modified by the addition or subtraction of reagents. In this study, we have adopted the self-cleavage activity of srtA in the wheat cell-free system for the production of tag-free recombinant proteins, and demonstrated an improved self-cleavage purification method by incorporating biotinylation reagents and calcium chelates in the cell-free synthesis system.

Results and Discussion

Self-cleavage activity of srtA during the cell-free protein synthesis

First, based on the previous report, we constructed the expression vector pEU-His-srtA-GW by inserting the DNA fragment required for the Gateway (GW) recombination technology into the previously described wheat germ cell-free expression vector pEU-E01 [13]. The Gateway system allows easy recombination of the targeted genes. The srtA cleavage site, LPETG, was introduced in the forward PCR primer according to the instructions provided with the Gateway system (Figure 1A). To test the self-cleavage purification system, we selected the human protein kinases and malaria vaccine candidate Pfs25 (*Plasmodium falciparum* 25 kDa ookinete surface antigen precursor) as candidate proteins because they are very important proteins for practical use. PCR products of the coding regions of Pfs25 and six protein kinases were amplified, and each PCR amplified fragment was individually inserted into the pDONR221 vector by BP recombination reaction. Subsequently, the inserted LPETG-gene fragment was cloned into the pEU-His-srtA-GW vector by LR recombination reaction, resulting in a pEU-His-srtA-LPETG-gene plasmid. These pEU-His-srtA-LPETG-gene plasmids were then used for ^{14}C-Leu-labeled protein synthesis using the wheat cell-free system. Unfortunately, in all cases, 20 to 40% of the synthesized proteins were cleaved during the cell-free synthesis (Figure 1B). The cleavage rate was dependent on the type of the gene used in creating the plasmid construct. For example, cleavage of Pfs25 (S25 lane in Figure 1B) and GFP (Figure 1C) during the protein synthesis process were very low, whereas almost 40% of the synthesized SGK495 protein was cleaved during the cell-free synthesis. Since calcium supplementation is known to induce the srtA activity [4,9], the cell-free system was treated with the calcium chelator, BAPTA (Figure 1C). Treatment with more than 4 mM BAPTA dramatically blocked the inexpedient cleavage of SGK495. However, protein synthesis was inhibited as the BAPTA concentration was increased (Figure 1D). For example, levels of SGK495 and GFP proteins produced in the presence of 5 mM BAPTA were approximately 20% and 30%, respectively, of their respective levels in the absence of BAPTA. In other proteins, the synthesis conditions in the presence of 1 and 3 mM BAPTA were investigated (Figure 1E and 1F). By comparing the results of the BAPTA-concentration dependent blocking of the srtA activity and inhibition of the protein synthesis, we concluded that 1 mM BAPTA is optimal for the cell-free synthesis of srtA-fusion proteins, because at this concentration of BAPTA the recovery of all full-length srtA fusion proteins improved by approximately 10-20% without any major inhibition of the protein synthesis.

Self-cleavage purification of His-srtA-fusion proteins

A total of eight plasmid constructs, each containing a different gene (generalized here as pEU-His-srtA-LPETG-gene), were used for the cell-free protein production and self-cleavage purification studies, and the results are shown in Figure 1G. Out of eight proteins, three proteins, GFP, p38g and Pfs25, clearly eluted from a nickel-nitrilotriacetic acid (Ni-NTA) sepharose column, whereas other five proteins could not be recovered in the eluted fraction (Figure 1G, left panel). To confirm protein synthesis and self-cleavage, column resins of all samples were analyzed by SDS-PAGE after boiling with SDS-sample buffer (Figure 1G, right panel). Surprisingly, even though AMPKa1, CSK, SGK and SGK495 were synthesized and self-cleaved on the column resin, their cleaved forms were not eluted off the resin. We could not find the RIPK3 protein in the

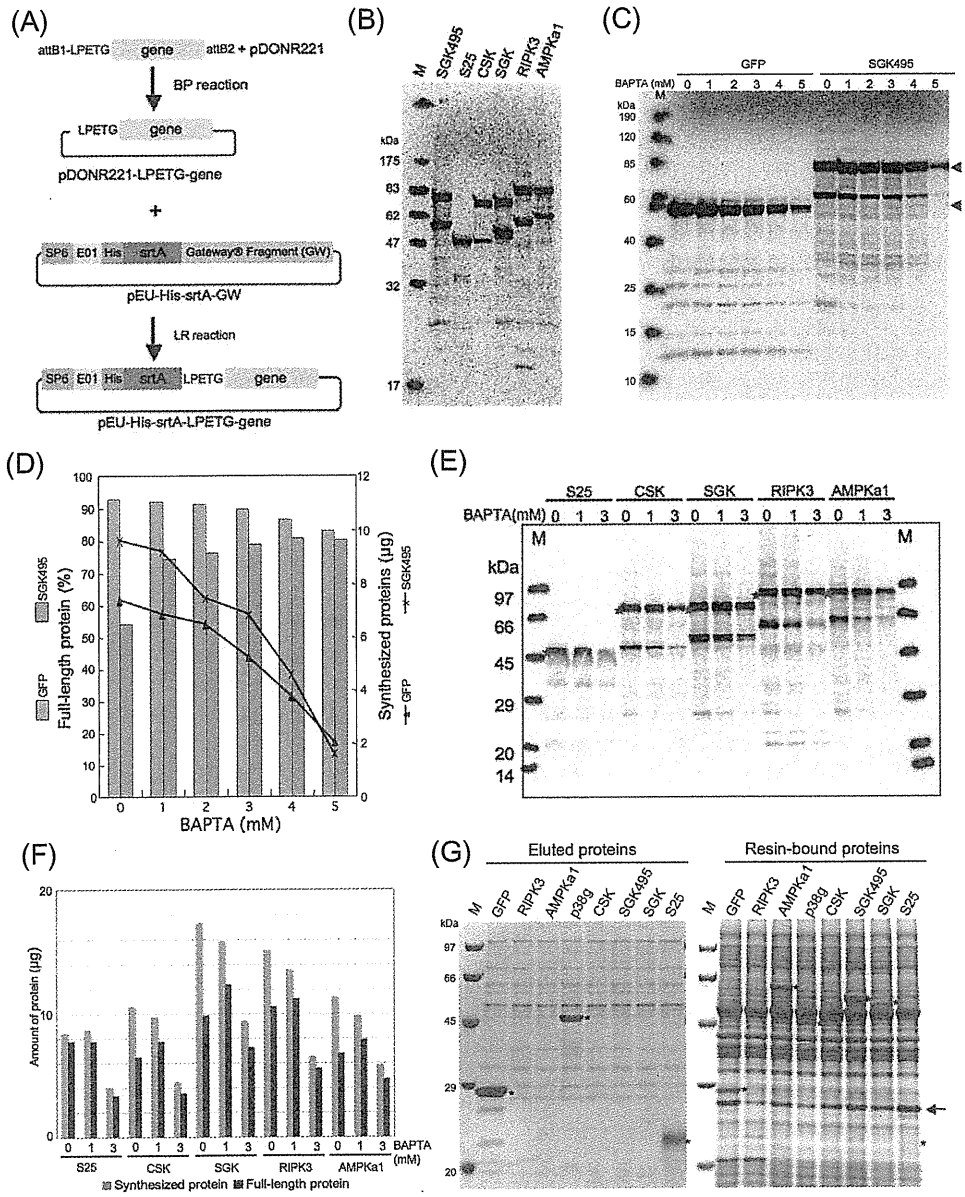

Figure 1 Synthesis of srtA-fusion proteins using the wheat germ cell-free system. A. Schematic representation of the pEU-His-srtA-LPETG-Gene plasmids created using the Gateway system. **B**. Autoradiogram of SDS-PAGE of proteins synthesized using the cell-free system in the presence of [^{14}C] Leu. Lane M, Protein MW standards labeled by using [^{14}C]-containing felt pen. **C**. Autoradiogram of [^{14}C] Leu incorporated GFP and SGK495 proteins synthesized by the wheat cell-free system in the presence of the Ca^{2}+ chelating reagent BAPTA. The number represents concentration (mM) of BAPTA used in the protein synthesis reaction. Arrowheads denote the sizes of the full-length proteins. **D**. Rate of synthesis of the full-length protein and productivity of GFP (pink-colored bar and red-colored line) and SGK495 (purple-colored bar and blue-colored line) in the presence of different concentrations of BAPTA. **E**. Autoradiogram of [^{14}C]-Leu incorporated proteins synthesized by the cell-free system in the presence of BAPTA. Asterisk denotes the sizes of the full-length proteins. **F**. Rate of synthesis of the full-length protein and productivity of proteins in the presence of different concentrations of BAPTA. Productivities of total synthesized and full-length proteins indicated as blue and red bars respectively. **G**. Purifications of proteins by the cell-free synthesis using the pEU-His-srtA-LPETG-Gene plasmid constructs. CBB-stained protein bands on the SDS-PAGE gel of the eluted (left panel) and resin-bound (right) target proteins are indicated using asterisk. Arrow represents the cleaved His-tagged srtA. Lane M (both panels): Protein MW standards.

eluted or in the resin-bound fraction, suggesting that this protein was expressed at a very low level. In addition, purity of the protein in the eluted fraction was not high, as there were several similar protein contaminants in every lane. Furthermore, many proteins bound to the Ni-NTA resin (right panel in Figure 1G). These results suggested that further technical improvements were necessary to achieve high quality purified proteins with high-efficiency from the cell-free based His-srtA system.

Biotinylated sortase self-cleavage purification (BISOP)

Recently we successfully adapted the biotin-labeling system of *E. coli* biotin ligase and biotin to the wheat cell-free protein synthesis system [15]. The biotinylation reaction modifies a specific lysine residue at the biotin ligation site (bls: GLNDIFEAQKIEWHE, the underline is the ligation site). The biotinylated proteins could be directly used for an assay without further purification because of very low biotin concentration. Since the His-tag based approach showed many contaminated proteins in the eluted fraction of the Ni-NTA column, we next used the biotin-labeled tag for protein purification. For this purpose, we constructed the pEU-BISOP-LPETG-GFP plasmid based on the pEU-His-srtA-LPETG-GFP vector as shown in Figure 1A and 2A. Next, we compared whether the His-tagged or the biotin-tagged protein could be better purified by the sortase self-cleavage method, processed either manually or using automated robots to eliminate any human error. Clearly, on the CBB-stained SDS-PAGE, a single major protein band (with low background) was found in the eluted fraction following the sortase self-cleavage of the biotin-tagged srtA fusion protein; in contrast, contaminating proteins were found along with the major protein band in the eluted fraction of the self-cleaved His-tagged srtA fusion protein (Figure 2B). Reaction performance of robot for His-srtA-LPETG-GFP or BISOP-LPETG-GFP was 63 or 58% of recovery, 52 or 88% of purity, and 68 or 62 μg of yield respectively. These results suggest that the BISOP method is better suited for producing tag-free purified proteins by the cell-free system.

Next, to examine whether the number of bls has any effect on the purification, two vectors, one having a single bls and the other having double bls, were constructed (Figure 2A). Coding regions of GFP, Pfs25 and CSK were individually cloned into each one of these two vectors, and then the resultant recombinant plasmids were used for the protein synthesis using the cell-free system. Staining with Alexa488-labeled streptavidin revealed slight mobility shifts for proteins containing double bls, as compared to those containing single bls (Figure 2C). We did not observe any difference between the single and double bls containing proteins with respect to biotinylation, elution and resin binding characteristics. Similar to the self-cleavage purification of GFP (shown in Figure 2B), all three eluted proteins were highly purified (left panel in Figure 2D). The higher purity of proteins obtained using the BISOP method might be due to the presence of very few contaminating proteins on the streptavidin-conjugated resin (compare right panel in Figure 2D with Figure 1G). Interestingly, the CSK protein, prepared by the BISOP method was eluted of the resin, whereas with the His-tag based method it was not found in the eluted fraction (Figure 1G). Total amounts of purified GFP, S25 and CSK by the BISOP method were 44, 37, and 55 μg per reaction respectively. Also full-length GFP, S25 and CSK proteins remaining on the column were approximately 6, 3 and 3 μg respectively, and cleaved S25 and CSK proteins on the column were 3 and 3 μg respectively. These data means that rate of target proteins remaining on the column was approximately 15%. Next, the BISOP method was used for purifying several other proteins. For this purpose, four protein kinase genes were individually inserted into the Gateway system vector pEU-BISOP-GW following the procedures described above, and the results are shown in Figure 2E. Both SGK495 and AMPKa1, which were not recovered earlier from the resin when the His-tag based method was used (Fgiure 1G), were also purified by the BISOP method (Figure 2E).

Total amounts of purified CSK, SGK495, AMPKa1 and p38g by the BISOP method were 55, 42, 24 and 57 μg per reaction respectively. In addition, analysis of the N-terminal sequence of the GFP protein purified by the BISOP revealed the expected cleavage of the Thr-Gly bond of the inserted LPETG sortase-recognition site (Figure 2F). These results suggested that the BISOP method would be suitable for the purification of the cell-free produced proteins with high efficiency and purity.

Purification of E2 heterocomplex by BISOP

Analysis of protein complex is one of important targets for their structural and biochemical analysis. Thus, next we examined whether a protein heterocomplex, co-expressed using the wheat cell-free system, could be purified by the BISOP method. To test this notion, we next co-expressed UBE2N and UBE2v1, two proteins forming the heterodimer complex of the ubiquitin-conjugate (E2) enzyme [16], using the BISOP method. Specific complex formation between these two proteins produced by the cell-free system has already been reported [15]. At first, we confirmed co-expression of the biotinylated srtA-LPETG-UBE2N and UBE2v1 (tag-free form) by incorporating ^{14}C-Leu during the cell-free synthesis (Figure 3A). Notably, we recovered the E2 heterocomplex consisting of UBE2N and UBE2v1 when both UBE2v1 and srtA-LPETG-UBE2N were co-expressed using the cell-free system and then purified by the BISOP method (Figures 3B and 3C). The protein band corresponding to UBE2v1

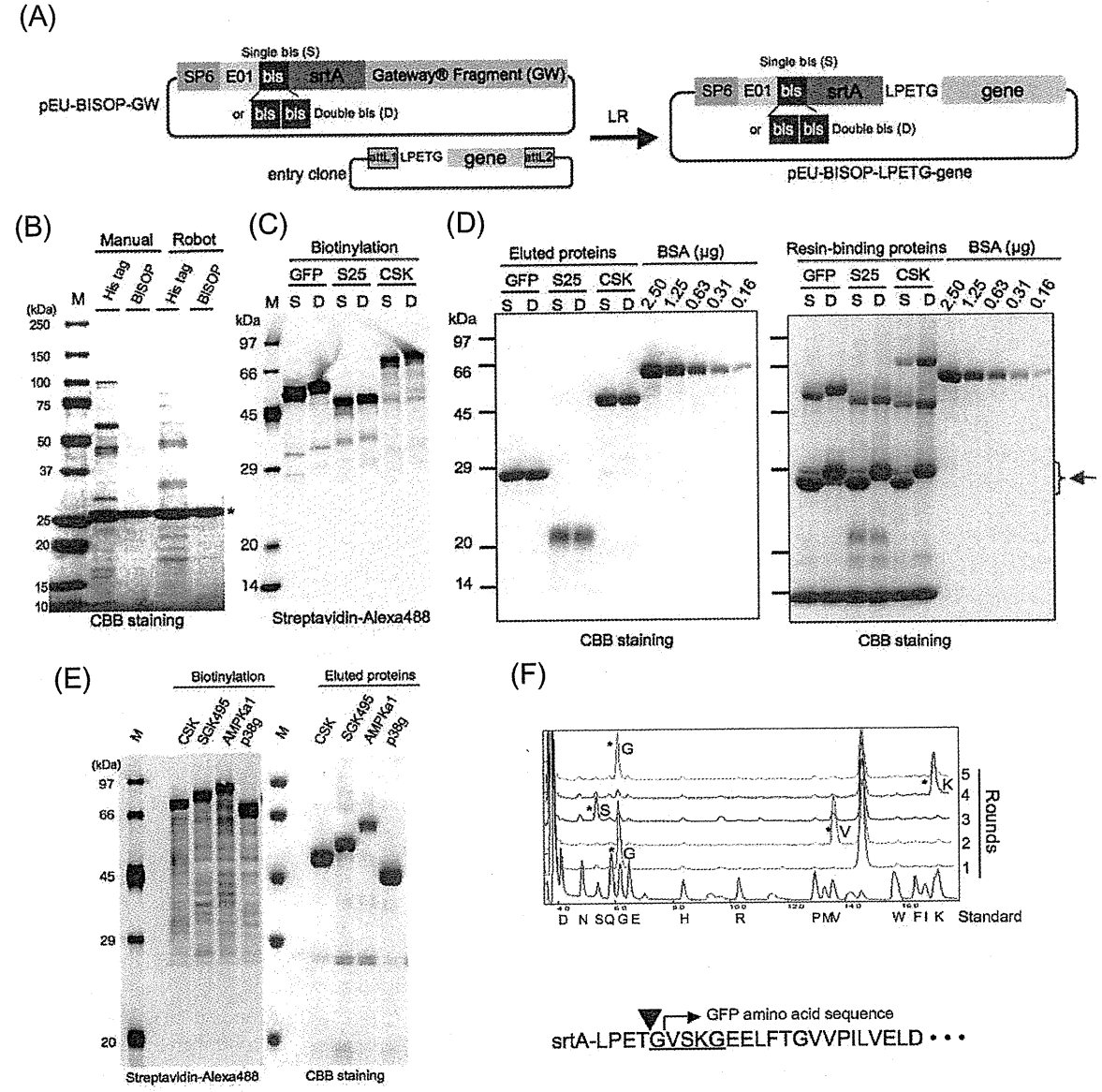

Figure 2 Preparation of proteins by the biotinylated-sortase self-cleavage purification (BISOP) method. A. Scheme for creating the pEU-BISOP-LPETG-gene plasmids using the Gateway recombination method. The LPETG-fused target gene containing entry clone plasmid was recombined with the pEU-BISOP-GW vector by using the Gateway LR reaction. **B.** CBB-stained SDS-PAGE comparing the His- or biotin-tagged GFP protein (indicated using an asterisk) purified manually or by using an automated robot. Lane M: Protein MW standards. **C.** Biotinylation of GFP, Pfs25 (S25) and CSK proteins synthesized by the cell-free system with vectors having a single (S) or a double (D) biotin ligation site. Biotinylated proteins were detected by labeling the separated protein bands with streaptavidin-Alexa488 as described in the text, followed by scanning using the Typhoon Imager. **D.** CBB-stained eluted (left panel) and resin-bound (right panel) proteins. Arrow indicates the cleaved biotinylated srtA protease left on the resin. Samples (5 or 10 μL in left or right panel respectively) were loaded on the gel. **E.** Left panel: Biotinylated proteins were detected by streaptavidin-Alexa488; Right panel: CBB-stained proteins purified by the BISOP method. Samples (5 μL) were loaded on the gel. **F.** Amino acid sequences from the N-terminal end of GFP purified by the BISOP method were determined by using an amino acid sequencer (asterisks, upper panel). Rounds indicate the number of Edman degradation cycle. Underlined amino acid sequence in the lower panel shows the determined sequence. Arrowhead indicates the site cleaved by the sortase enzyme.

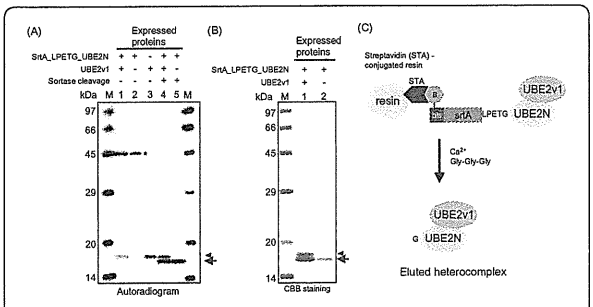

Figure 3 Application of BISOP method to purify the UBE2N/UBE2v1 heterocomplex co-expressed by the cell-free system. A. The proteins were synthesized from the plasmid constructs using the cell-free system in the presence of ^{14}C-Leu and the synthesized proteins were detected by autoradiography after electrophoresis on a SDS-PAGE (5-20% gradient gel). Lane 1: Proteins synthesized from a mixture of pEU-BISOP-LPETG-UBE2N and pEU-UBE2v1 plasmids; Lane 2: Protein synthesized using the pEU-BISOP-LPETG-UBE2N plasmid; Lane 3: Protein synthesized using the pEU-UBE2v1 plasmid; Lane 4: Sortase cleavage of lane 1 by the BISOP method; Lane 5: Sortase cleavage of lane 2 by the method; and Lane M, Protein MW standards. Asterisk, arrow and arrowhead indicate srtA-LPETG-UBE2N, G-UBE2N and UBE2v1 (tag-free), respectively. **B.** BISOP was performed after expression of biotinylated srtA-LPETG-UBE2N with (lane 1) or without UBE2v1 (lane 2). The eluted proteins were separated on the SDS-PAGE gel and then stained with CBB. Arrow and arrowhead indicate the purified G-UBE2N and UBE2v1, respectively. **C.** Scheme for the preparation of UBE2N/UBE2v1 heterocomplex by BISOP.

was however not found when the biotinylated srtA-LPETG-UBE2N was expressed alone. Therefore, this result suggests that the BISOP method would be useful for purification of protein complexes produced by the cell-free system.

Activity of CSK purified by the BISOP method

It is very important that the purification method provide functionally active protein. We, therefore, investigated whether the CSK protein purified using the BISOP method could specifically phosphorylate the Tyr-530 residue of human Src protein (Swiss-Prot no. P12931) [17]. For this purpose, biotinylated Src was synthesized by the cell-free system and the synthesized protein was partially purified using the magnetic streptavidin-conjugated beads. The bead-bound Src was then treated with [^{32}P]-labeled or unlabeled ATP and the CSK protein that was purified by the BISOP method. Both autoradiogram of the SDS-PAGE separated proteins from the reaction mix containing [^{32}P]-labeled ATP and immunoblot analysis of the SDS-PAGE separated proteins from the reaction mix containing the unlabeled ATP using the specific anti-phospho-Src antibody showed specific phosphorylation of the Y530 residue of Src by CSK (Figure 4). These results suggest that BISOP would be suitable for the in vitro synthesis of active proteins.

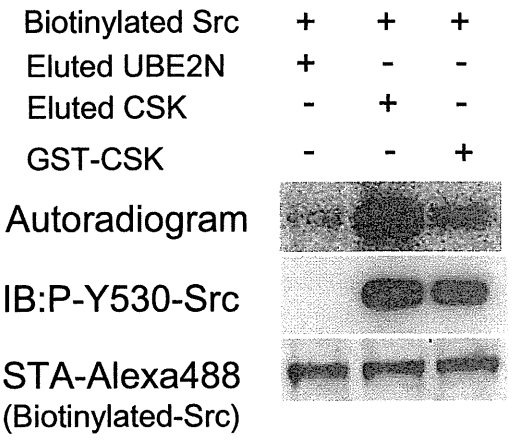

Figure 4 Activity of CSK prepared using the BISOP method. Biotinylated Src protein was incubated with UBE2N, CSK purified using the BISOP method (eluted CSK and UBE2N), or commercially available GST-CSK fusion protein in the presence of cold (for immunoblot) or radioisotope-labeled (for autoradiogram) ATP. Src phosphorylation was assayed by immunoblot analysis using the anti-Phospho-Src (Y530) antibody. Autoradiogram shows phosphorylation of the Src protein by CSK.

Conclusion

The cell-free system is an easy to use method for synthesizing recombinant proteins. In this study, we have developed a new biotinylated-sortase self-cleavage purification (BISOP) method to achieve high quality purification of several proteins, including a protein heterocomplex, without any affinity tag. In addition, we showed that this method could be automated using robots. Results of this study indicate that the combination of the cell-free protein synthesis system and BISOP could provide a simple and easy method for the preparation of high quality recombinant proteins without any affinity tag. Since the cell-free system is suitable for high-throughput protein production, this combined method could also be utilized at the genome-wide level.

Methods

General

The following procedures have been either described in detail or cited [13,15,18,19]: isolation of the wheat germs and preparation of the extract, generation of the DNA template by polymerase chain reaction (PCR) using split-primers, parallel synthesis of mRNA and protein, estimation of the amount of protein synthesized by densitometric scanning of the Coomassie brilliant blue (CBB)-stained band and autoradiogram of radiolabeled-proteins, and detection of biotinylated proteins using Alexa488-conjugated streptavidin (Invitrogen) and the

Typhoon Imager (GE Healthcare) fitted with 532 nm laser and 526 emission filter.

Template genes

The cDNA clone of the malarial parasite *Plasmodium falciparum* 25 kDa ookinete surface antigen precursor (Pfs25) was kindly provided by Dr. Tsuboi (Cell-free Research and Technology Center, Ehime University). cDNAs of GFP, UBE2N (GenBank accession no. BC003365), and UBE2v1 (GenBank accession no. BC000468) were described in our previous reports [15,18]. Mammalian gene collection (MGC) cDNA clones of CSK (BC104875), SGK (BC001263), SGK495 (BC007835), AMPKa1 (BC048980), p38g (BC015741), RIPK3 (BC062584) and Src (BC011566) were also used in this study.

Construction of the srtA-based self-cleavage vector

DNA fragment encoding the mature-form of srtA (corresponding to amino acids 60-206, GenBank accession no. AF162687) was artificially synthesized and inserted into the pUC57 to create the plasmid pUC57-srtA-EcoRV-SpeI by the GenScript Corporation (Boston). The pUC57-srtA-EcoRV-SpeI plasmid as used as a template to amplify the mature srtA fragment (DraI-His or bls-srtA-EcoRV-SpeI) by PCR using the following pair of primers: M13(M3) (5'-GTAAAACGACGGCCAGT) and DraI-A-His-srtA (5'-GAGATTTAAATGGCCAGCAGC CATCACCATCACCATCATAGCAGCGGCCTGGTGC CGC) or M13(M3) and Dra1-A-bls-srtA (5'-GAGAT TTAAATGGCCAGCAGCGGCCTGAACGACATCTTC GAGGCCCAGAAGATCGAGTGGCACGAAAGCAGC G GCCTGGTGCCGC). The DraI-A-His-srtA primer included 6 × His-tag codons and a DraI restriction enzyme site, and the DraI-A-bls-srtA primer included a bls (biotin ligase site) recognition sequence and a DraI restriction enzyme site. After digestion with DraI and SpeI enzymes each fragment was inserted into the EcoRV and SpeI sites in pEU-E01-MCS provided by CellFree Sciences, Ltd http://www.cfsciences.com/eg/index.html to create pEU-His-srtA-MCS and pEU-BISOP-MCS plasmids, respectively. To create the Gateway®-based plasmids pEU-His-srtA-GW and pEU-BISOP-GW, DNA fragment needed for Gateway® recombination cloning technology (Invitorogen) was inserted into the EcoRV site of the pEU-His-srtA-MCS and pEU-BISOP-MCS plasmids. PCR reaction was performed by PrimeStar enzyme according to instruction (Takara Bio, Otsu, Japan). Nucleotide sequences of the DNA inserts in all plasmid constructs were subsequently confirmed by using the ABI PRISM 310 Genetic Analyzer using the BigDye terminator v1.1 Cycle sequence kit (Applied Biosystems, Foster City, CA).

Plasmid construction for the cell-free protein production

We introduced the srtA self-cleavage site (DNA encoding for the amino acid sequence LPETG) into the recombinant plasmid construct for the cell-free production of proteins. The DNA fragments coding the respective protein were amplified by PCR using two gene specific primers: forward primer attB1-LPETG-Gene (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC-**CTGCCCGAGACCGGC**atg(n)$_{19}$; uppercase, lowercased and bold sequences indicated common, gene specific and LPETG sequences, respectively; n represent gene specific sequence) and reverse primer attB2-Gene (5'-GGGGACCACTTTGTACAAGAAAGCTG-GGTCxxxnnnnnnnnnnnnnnnn; xxx is the complementary sequence of the stop codon; n represent gene specific sequence). PCR reaction was performed using the PrimeStar enzyme (Takara Bio, Otsu, Japan) and following the supplier's instructions. The amplified attB1-LPETG-Gene-attB2 fragments were inserted into the donor vector pDONR221 by BP reaction to generate the entry plasmids. The LPETG-fusion Gene in the entry plasmid was transferred to the pEU-His-srtA-GW or pEU-BISOP-GW by LR reaction to generate the pEU based-plasmid clones. BP and LR reactions were carried out according to the instructions provided by the supplier of the reagents (Invitrogen, Carlsbad, CA). Nucleotide sequences of the DNA inserts in all the plasmid constructs were subsequently confirmed by using the ABI PRISM 310 Genetic Analyzer described method above.

Cell-free protein production

For the cell-free protein production, we employed the wheat germ cell-free protein expression system using the bilayer translation method described previously [18,19]. Cell-free protein production was carried out using the ENDEXT® Wheat Germ Expression S Kit and according to the instructions provided by the supplier (CellFree Sciences Co., Ltd., Matsuyama, Japan). Briefly, 250 μL of transcriptional mixture [80 mM HEPES-KOH, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM DTT, 2.5 mM NTP mix, 1 U/μL SP6 RNA polymerase (Promega, Madison, WI), 1 U/μL RNase inhibitor, RNasin (Promega), and 100 ng/μL undigested plasmid DNA] was incubated at 37°C for 6 h, and then mixed with 250 μL of wheat embryo extract (120 A 260/mL, CellFree Sciences Co., Ltd.) and 1 μL of 20 mg/mL creatine kinase (Roche Applied Science, Indianapolis, IN). This mixture, called the translational mixture, was then carefully transferred to the bottom of the well of a 6-well tissue culture plate (Whatman Inc., Clifton, NJ) that already contained 5.5 mL of TSB (30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM DTT, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP and 16 mM creatine phosphate) by

inserting the pipette tip down to the bottom of the well, thereby creating two distinct layers. The plate was then covered with the sealing film, and was incubated at 17°C for 18 hr without shaking. For the calcium chelating experiment, 1 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) (Sigma-Aldrich, St Louis, MO) was added to both the translational mixture and TSB. For biotin labeling of proteins [15,20], 2 μg of biotin protein ligase (BirA, GenBank accession no. NP_0312927) produced by the wheat cell-free system and 6 μM D-biotin (Nacalai Tesque, Kyoto, Japan) were added to the bottom translational mixture.

For co-expression of the biotinylated srtA-LPETG-UBE2N and UBE2v1, the cell-free production method was slightly modified. Each translation mixture was prepared independently and pre-incubated at 26°C for 30 min, following which they were mixed, and were subsequently used in the bilayer translation reaction as described above.

Self-cleavage purification of proteins produced by the cell-free system

Reaction mixture (6 mL) from the cell-free expression system described above was mixed with 100 μL of Ni-NTA sepharose (GE Healthcare) or 100 μL of streptavidin-sepharose (GE helthcase). The sepharose beads were pre-equilibrated with phosphate buffered saline (PBS) for 4 to 6 hours (His tag) or 30 min (BISOP) at 4°C. Sepharose bead-captured srtA-fusion proteins were collected by centrifugation (3,000 × g) and the beads were washed three times with PBS buffer. Self-cleavage purification of the target protein was performed by incubation of the beads with 100 μL of Elution buffer [20 mM Tris-HCl (pH 7.5), 5 mM Tri-Gly (Sigma-Aldrich), 5 mM CaCb, 150 mM NaCl, 1 mM DTT and 2% glycerol] for 4 hours at 16°C. The buffer-bead mixture was then transferred into a micro spin-column (GE Healthcare) and the eluted fraction was recovered by flash centrifuge (3,000 × g) at 4°C. The N-terminal end of the eluted GFP was determined by amino acid sequence analysis using the Applied Biosystems ABI 473A protein sequencer and according to the instructions provided by Applied Biosystems.

The cell-free protein production and self-cleavage purification were also carried out using an automatic robot, Protemist DTII (CellFree Sciences Co., Ltd.), basically according to manufacturer's instructions. Addition of biotinylation reagents and BAPTA were also carried out as mentioned above.

Phosphorylation assay

The phosphorylation assay was mainly performed according to the previous published methods [13,21]. To assay for phosphorylation of the biotinylated Src by CSK, 40 μl of the reaction mixture was mixed with 15 μl of biotin magnetic beads (Promega, MI), and was washed twice with PBS buffer and once with protein kinase (PK) buffer [50 mM Tris-HCl (pH 7.6), 500 mM potassium acetate, 50 mM $MgCl_2$, 0.1 mM DTT]. Beads were suspended in 10 μl PK buffer supplemented with cold ATP (for immunoblot) or [γ-^{32}P]-ATP (for autoradiogram), and CSK purified using the BISOP method, UBE2N or commercially available GST-CSK fusion protein (purchased from Carna Biosciences Inc., Kobe) was added in the reaction mixture. The mixtures were incubated at 37°C for 30 min, following which they were boiled in the SDS-denaturing buffer and the proteins were separated on 12.5% SDS-polyacrylamide gel. Autoradiogram of Src phosphorylation was analyzed by BAS-2500 (FUJIFILM, Tokyo, Japan). For immunoblot analysis, proteins were transferred from the SDS-PAGE gel to PVDF membrane (Millipore Bedford, MA, USA) following standard procedures. The blots were then processed using the Immobilon Western detection reagents (Millipore) and antibody against phosphorylated Src (Y527) or Src (Cell Signaling Technology, Beverly, MA) according to the manufacturer's procedure. The anti-phospho-Src (Y527) antibody recognizes the phosphorylated Y530 residue in human Src.

Abbreviations

srtA: sortase SrtA; GFP: green fluorescent protein; SGK: serum/glucocorticoid regulated kinase 1; CSK: c-src tyrosine kinase; UBE2N: ubiquitin-conjugating enzyme E2N; UBE2v1: ubiquitin-conjugating enzyme E2 variant 1; AMPKa1: 5'-AMP-activated protein kinase alpha 1 catalytic subunit; PRKAA1: protein kinase, AMP-activated: alpha 1 catalytic subunit; MAPK12: mitogen-activated protein kinase 12; p38g: p38 gamma; Pfs25: *Plasmodium falciparum* 25 kDa ookinete surface antigen precursor; BAPTA 1: 2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; PBS: phosphate buffered saline.

Authors' contributions

SM conceived the study and performed some of the experiments; KM and KS performed also participated in performing the experiments; YE conceived the study and supervised the work; TS conceived and designed the study, supervised the work and contributed to writing the manuscript. All authors read and approved the final manuscript.

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Regulation of Arabidopsis defense responses against *Spodoptera littoralis* by CPK-mediated calcium signaling

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Abstract

Background: Plant Ca^{2+} signals are involved in a wide array of intracellular signalling pathways after pest invasion. Ca^{2+}-binding sensory proteins such as Ca^{2+}-dependent protein kinases (CPKs) have been predicted to mediate the signaling following Ca^{2+} influx after insect herbivory. However, until now this prediction was not testable.

Results: To investigate the roles CPKs play in a herbivore response-signaling pathway, we screened the characteristics of Arabidopsis CPK mutants damaged by a feeding generalist herbivore, *Spodoptera littoralis*. Following insect attack, the *cpk3* and *cpk13* mutants showed lower transcript levels of plant defensin gene *PDF1.2* compared to wild-type plants. The CPK cascade was not directly linked to the herbivory-induced signaling pathways that were mediated by defense-related phytohormones such as jasmonic acid and ethylene. CPK3 was also suggested to be involved in a negative feedback regulation of the cytosolic Ca^{2+} levels after herbivory and wounding damage. *In vitro* kinase assays of CPK3 protein with a suite of substrates demonstrated that the protein phosphorylates transcription factors (including ERF1, HsfB2a and CZF1/ZFAR1) in the presence of Ca^{2+}. CPK13 strongly phosphorylated only HsfB2a, irrespective of the presence of Ca^{2+}. Furthermore, *in vivo* agroinfiltration assays showed that CPK3-or CPK13-derived phosphorylation of a heat shock factor (HsfB2a) promotes *PDF1.2* transcriptional activation in the defense response.

Conclusions: These results reveal the involvement of two Arabidopsis CPKs (CPK3 and CPK13) in the herbivory-induced signaling network via HsfB2a-mediated regulation of the defense-related transcriptional machinery. This cascade is not involved in the phytohormone-related signaling pathways, but rather directly impacts transcription factors for defense responses.

Background

One of the significant factors determining successful plant growth and reproduction is an efficient defense against insect attacks. After herbivore feeding there is a dramatic Ca^{2+} influx limited to a few cell layers lining the damage zone [1,2]. Signals induced rapidly by herbivore attack have been found to spread over the leaf, leading to a strong Ca^{2+}-dependent transmembrane potential (*Vm*) depolarization in the damage zone followed by a transient *Vm* hyperpolarization in the close vicinity and a constant depolarization at distances greater than 6-7 mm [1]. These initial cues are transmitted within the plant by signal transduction pathways that include phosphorylation cascades, such as mitogen-activated protein (MAP) kinases, and the jasmonic acid (JA) pathway, which play a central and conserved role in promoting resistance to a broad spectrum of insects [3]. However, there is a missing link to downstream signaling and gene regulation for defense responses. In this network, Ca^{2+}-binding sensory proteins are of particular interest, since following Ca^{2+} influx the sensory proteins may secondarily decode information contained in the temporal and spatial patterns of

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the signal trafficking to control metabolism and gene expression [4].

Plants possess several classes of Ca^{2+}-binding sensory proteins, including calmodulins, calmodulin-like proteins, calcineurin B-like proteins, and Ca^{2+}-dependent protein kinases (CPKs) [4]. The CPKs are of special interest, since they represent a novel class of Ca^{2+} sensors, having both a protein kinase domain and a calmodulin-like domain (including an EF-hand calcium-binding site) in a single polypeptide [5,6]. CPKs constitute a large family of serine/threonine protein kinases that are broadly distributed in the plant kingdom. For example, the Arabidopsis genome is predicted to have 34 different CPKs [7]. Arabidopsis CPK1 was the first CPK to be characterized, and is known to be activated by phospholipids and 14-3-3 proteins, which are small, highly conserved eukaryotic proteins that regulate multiple cellular enzymes, including protein kinases [8]. AtCPKs 3, 4, 6, 11 and 32 act as abscisic acid (ABA) signaling components, and are involved in ABA-responsive gene expression, seed germination, seedling growth, and stomatal movement [9-11]. Especially, AtCPKs 4, 11 and 32 are likely to interact with ABA-related leucine zipper class transcription factors [10,11], indicating the proximate involvement of CPKs in transcriptional regulation.

Curiously, little attention has been given to the role of CPKs in defense responses. Only one case has been reported: in tobacco, NtCDPK2 modulates the activation of stress-induced MAP kinases, and this interaction requires the synthesis and perception of wound hormones [12]. The role of CPKs involved in the defense response against insect herbivory has never been reported. In this report, we show the involvement of two Arabidopsis CPKs (CPK 3 and CPK 13) in the herbivory-induced signaling network via post-translational regulation of the defense-related transcriptional machinery. Implications for possible signal trafficking via CPKs are discussed.

Results

cpk3 and *cpk13* mutants showed decreased transcript levels of defense genes in *S. littoralis*-damaged leaves

We obtained 19 T-DNA insertion mutant lines that were putative AtCPK-deficient mutants from the European Arabidopsis Stock Centre. Homozygous T-DNA insertion lines corresponding to each CPK gene were confirmed using the PCR method (data not shown) and challenged with herbivore damage. Transcript levels of plant defensin gene *PDF1.2*, which is induced in wild-type (WT) leaves exposed to *Spodoptera littoralis* larvae for 24 h, were investigated in the mutant leaves (Figure 1). Compared to *S. littoralis*-damaged WT leaves, T-DNA insertion line *cpk3* (Salk_022862) and two different *cpk13* lines (*cpk13-1* (Salk_057893) and *cpk13-2* (Salk_135795)) showed significantly lower transcript levels in *S. littoralis*-damaged leaves. The molecular analysis of CPK T-DNA insertion mutants and gene expression profiling of the mutants are shown in Additional file 1. In addition to our study of *PDF1.2*, the time-courses of the transcript levels of two other defense-related genes (*Thi2.1* and *VSP2*) after herbivore attack were examined in *cpk3*, *cpk13-1*, and *cpk13-2* leaves and compared to those in WT leaves (Figure 2). The transcript level of *Thi2.1* in WT leaves was increased about 22 fold after 6 h but declined after 24 h. The *cpk3* mutant showed a comparable level of the *Thi2.1* expression during the time course, whereas the transcript levels in *cpk13-1* and *cpk13-2* leaves remained unchanged at 6 h, but increased after 24 h. Thus, WT and *cpk13* probably have different temporal regulation. Throughout the time-course, the induced transcript levels of *VSP2* were comparable between the WT and all the mutants (Figure 2).

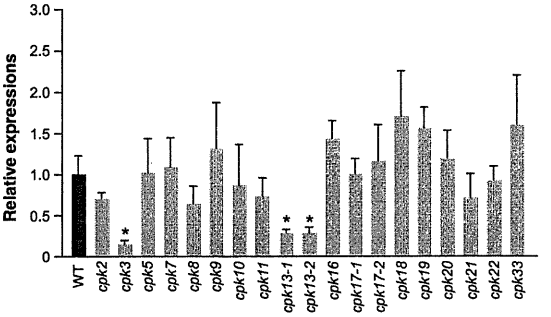

Figure 1 Comparison of the transcript levels of plant defensin gene *PDF1.2* between *S. littoralis*-infested leaves of WT and CPK T-DNA insertion mutants. Transcript levels of *PDF1.2* in mutant leaves (gray columns) were normalized by those of *ACT1* measured in the same samples and expressed relative to the normalized transcript levels in leaves of infested WT plants (filled columns). An asterisk (*) indicates that mutants were significantly different from WT leaves ($P < 0.05$). WT, wild-type; *cpk2*, Salk_131765; *cpk3*, Salk_022862; *cpk5*, Salk_138808; *cpk7*, Salk_035601; *cpk8*, Salk_036581; *cpk9*, Salk_034324; *cpk10*, Salk_105108; *cpk11*, Salk_023086; *cpk13-1*, Salk_057893; *cpk13-2*, Salk_135795; *cpk16*, Salk_020716; *cpk17-1*, Salk_140527; *cpk17-2*, Salk_057146; *cpk18*, Salk_061352; *cpk19*, Salk_057587; *cpk20*, Salk_073448; *cpk21*, Salk_029412; *cpk22*, Salk_125850; *cpk33*, Salk_059467

Possible involvement of phytohormone signaling in the herbivory-related CPK cascades

To assess whether loss of CPK function affects the signal transduction involved in the defense response, we explored the biosynthesis of JA, jasmonyl-L-isoleucine (JA-Ile, an active form of JA [13]), ethylene, and abscisic acid (ABA, known to be involved in protective wound-healing processes [14]). As shown in Figure 3, all the phytohormones examined in this study were formed and accumulated at a similar rate in the infested WT, com-

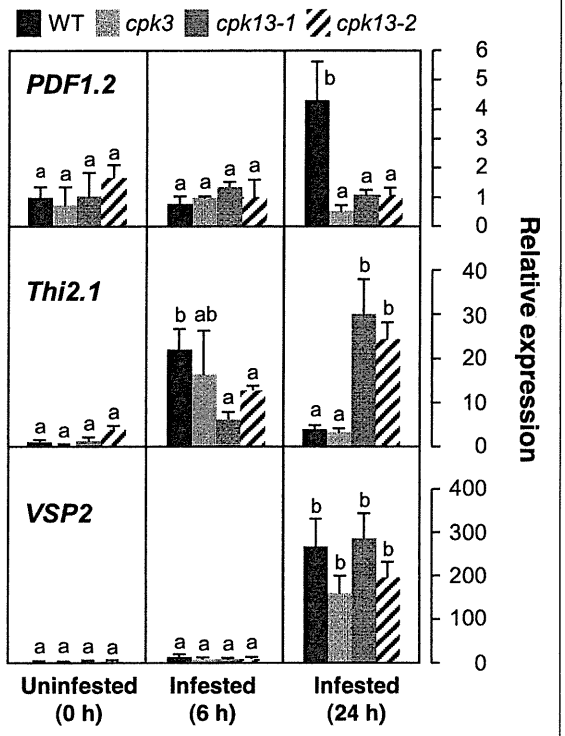

Figure 2 Relative mRNA levels of defense-related genes *PDF1.2*, *Thi2.1* and *VSP2* in *S. littoralis*-infested leaves. Transcript levels of genes in WT, *cpk3*, *cpk13-1* and *cpk13-2* leaves were normalized by those of *ACT1* measured in the samples and expressed relative to the normalized transcript levels in the leaves of uninfested WT plants. Means followed by different small letters are significantly different ($P < 0.05$)

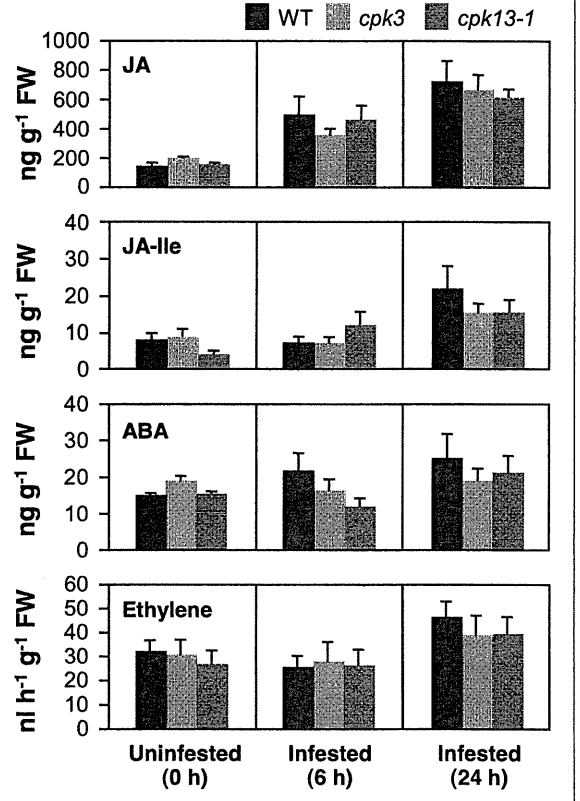

Figure 3 Levels of JA, JA-Ile, ABA and ethylene in WT, *cpk3*, and *cpk13-1* leaves fed on by *S. littoralis* larvae for 6 and 24 h.

pared to the *cpk* mutant leaves (Figure 3), indicating that CPK3 and CPK13 are not upstream signal kinases for the biosynthesis of JA, ABA and ethylene. We also investigated the *PDF1.2* expression levels in WT and in *cpk3* and *cpk13* mutants by applying an exogenous solution of either JA, ethephon (a chemical which releases ethylene), or ABA (Figure 4). Compared to the control, after 6 h the transcript levels of *PDF1.2* were very slightly induced by JA or ABA to levels comparable to those in WT and mutant leaves. Ethephon treatment resulted in drastic but comparable increases of the transcript in both WT and mutant leaves. We therefore conclude that the CPK3 and CPK13 cascades are not closely linked to the above phytohormone signaling and biosynthesis cascades.

Increased intracellular Ca^{2+} levels in *cpk3* leaf cells after herbivore and mechanical damage

It is interesting to note that the *cpk3* mutant showed abnormal changes of the cytosolic Ca^{2+} level after insect damage. As shown in Figure 5, when the membrane-permeable Fluo-3 AM [Ca^{2+}-sensitive fluorescent probe] was applied to WT leaf tissues, it showed a cytoplasmic subcellular localization at sites damaged by *S. littoralis* (Fig-

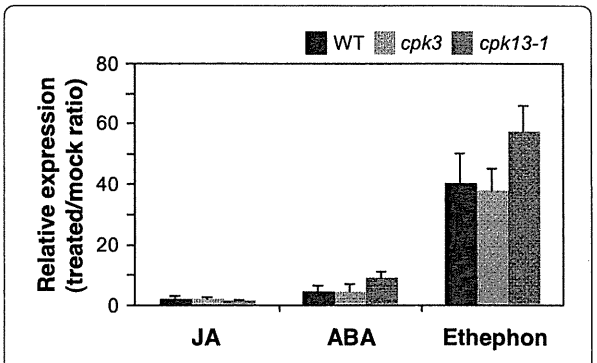

Figure 4 Effects of loss of CPK function on signaling pathways for defense response. Effect of exogenous application of JA, ABA or ethephon (an ethylene releasing chemical) on *PDF1.2* expression levels after 6 h was investigated. Transcript levels of *PDF1.2* in WT, *cpk3*, and *cpk13-1* leaves were expressed relative to the normalized transcript levels in leaves of mock-treated (water-treated or buffer-treated) wild-type plants. Significant differences between treatments were not observed

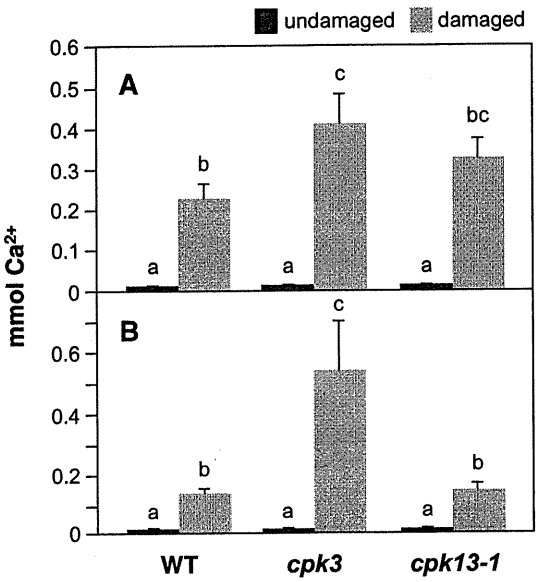

Figure 5 Intracellular Ca^{2+} levels in WT and *cpk* leaf cells. Undamaged WT leaves served as a control. Leaves were treated with Fluo-3 AM for 1 h and damaged with a *S. littoralis* larva (A) or mechanical wounding (B). Thirty minutes after damage, the cytosolic Ca^{2+} concentrations in leaf cells were determined and taken as the saturation value. Undamaged WT leaves served as a control. Means followed by different small letters are significantly different ($P < 0.05$).

ure 5A) or after mechanical wounding (Figure 5B). *cpk3* but not *cpk13-1* plants showed a more marked increase of the cytosolic Ca^{2+} level after the damage, compared to WT.

Substrate targeting of CPK3 and CPK13

In previous studies, CPK3 was found to be localized in the nucleus and the cytosol [15]. In this study, we additionally tested the subcellular localization of CPK13-GFP fusion proteins in transiently expressing onion peels, which revealed nuclear, cytosolic and plasma membrane localizations of the fusion proteins (Figure 6). Accordingly, in order to screen the protein target of CPK3 and CPK13, protein kinase assays with 100 nuclear and cytosolic protein substrates synthesized using the wheat germ cell-free system (see Additional file 2) were carried out. CPK3 or CPK13 proteins purified from a cell-free extract were incubated with radiolabeled ATP, $CaCl_2$ and the 100 distinct substrates. Both CPK3 and CPK13 showed autophosphorylation in the presence of radiolabeled ATP and $CaCl_2$ (Figure 7A). Notably, CPK3 was auto-phosphorylated along with increased concentration of Ca^{2+}, whereas CPK13 was not, indicating that CPK3 was strictly Ca^{2+}-dependent. Auto-phosphorylation of CPK stringently reflects the intensity of the phosphorylation of substrate targets [16]. As shown in Figure 7B and Additional file 3, CPK3 phosphorylated three transcription factors (TFs) [JA/ethylene-inducible APE/ERF domain transcription factor 1 (ERF1) [17], heat shock factor HsfB2a (Hsf22), and the wound-inducible CZF1/ZFAR1 transcription factor [18]]. ATL2, a member of a multigene family of highly related RING-H2 zinc finger proteins that function as E3 ubiquitin ligases [19,20] and a potent regulator of *PDF1.2* transcription [21], was also phosphorylated by CPK3 (discussed in Additional file 4). Addition of BAPTA, a cal-

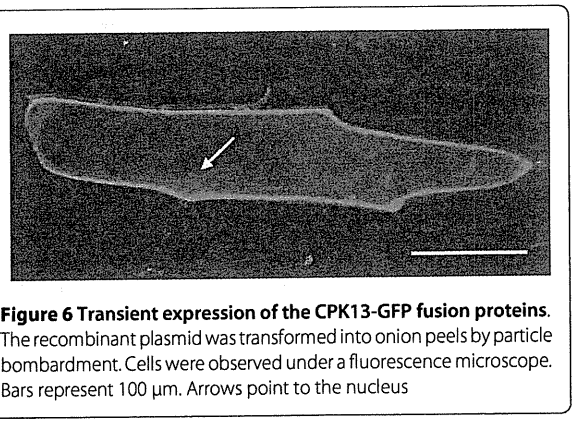

Figure 6 Transient expression of the CPK13-GFP fusion proteins. The recombinant plasmid was transformed into onion peels by particle bombardment. Cells were observed under a fluorescence microscope. Bars represent 100 μm. Arrows point to the nucleus

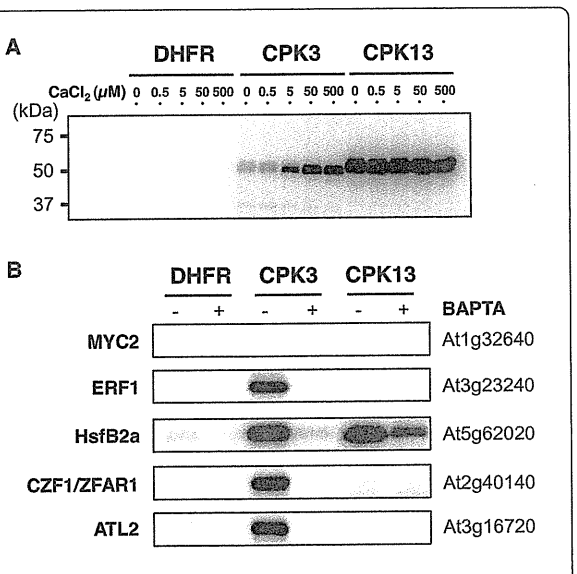

Figure 7 Substrate targeting of CPK3 and CPK13. A. Auto-phosphorylation signals from each CPK protein. Recombinant CPK was pretreated with BAPTA (2.5 mM; a calcium chelator) and then subjected to auto-phosphorylation assays with several concentrations of $CaCl_2$. B. *In vitro* kinase assays of the CPK3 and CPK13 proteins with a suite of TFs and ATL2 (following cell-free protein synthesis and purification) revealed their substrate targets. In the presence (+) or absence (-) of BAPTA (500 μM), kinase assays were performed in the presence of $CaCl_2$ (100 μM). The DHFR protein served as a control. Each experiment was performed two or three times, with similar results each time. See the quantitative values in Additional file 3.

cium chelator, to the reaction mixture abolished the phosphorylation by CPK3, suggesting that these phosphorylations were strictly Ca^{2+}-dependent. This result was in line with the Ca^{2+}-dependent auto-phosphorylation of CPK3, described above. In contrast, the CPK13-derived protein labeling (auto-phosphorylation) was scarcely activated by the addition of Ca^{2+} ion (Figure 7A), suggesting that CPK13 has very high sensitivity for auto-phosphorylation, like soybean CDPKα [22]. CPK13 strongly phosphorylated HsfB2a, irrespective of the presence of BAPTA.

Although it has been claimed that MYC2 (At1g32640) is also involved in *PDF1.2* regulation [23], this transcription factor was not phosphorylated by either CPK3 or CPK13 (Figure 7B).

HsfB2a takes part in the regulation of the herbivore-induced transcription of *PDF1.2*

We investigated the *PDF1.2* expression levels in *S. littoralis*-damaged leaves of Arabidopsis WT and HsfB2a T-DNA insertion mutants (Salk_027578) (Figure 8A). Compared to *S. littoralis*-damaged WT leaves, *hsfB2a* plants showed significantly lower transcript levels in *S. littoralis*-damaged leaves. Thus, HsfB2a appeared to be a positive regulator of herbivore-induced *PDF1.2* expression. In addition, to investigate the *in vivo* function of CPKs, a constitutively active form of CPK and HsfB2a were co-expressed as cofactors for the transient expression of a reporter (GUS) gene under the control of the *PDF1.2* promoter in *Nicotiana benthamiana* leaves, in *Agrobacterium tumefaciens*-mediated transient expression (agroinfiltration) assays. For these assays, we prepared a constitutively active form of CPK proteins which lacks junction and calmodulin-like domains and thus no longer shows Ca^{2+} dependency. As shown in Figure 8B, the reporter gene activity was drastically increased when co-expressed with HsfB2a as effector. The activation by HsfB2a was further enhanced (6.6- and 1.9-fold) when CPK3 and CPK13 were co-expressed, respectively. However, when CPK3 or CPK13 was co-expressed in the absence of HsfB2a, scarcely any increase of the expression of the reporter gene was observed. Similarly, other CPK substrate transcription factors (ERF1 or CZF1/ZFAR1) were co-expressed as effectors, but neither of them resulted in significant transactivation of the GUS marker gene (data not shown).

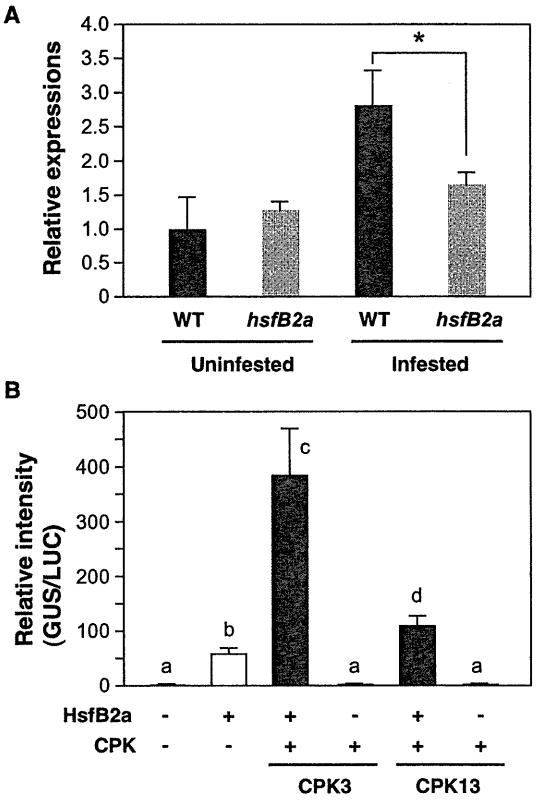

Figure 8 Involvement of CPK3, CPK13 and HsfB2a in herbivore-induced *PDF1.2* regulation. A. Transcript levels of *PDF1.2* in WT and *hsfB2a* mutant Arabidopsis leaves fed on by *S. littoralis* larvae for 24 h. An asterisk (*) indicates that mutants were significantly different from WT ($P < 0.05$). B. *PDF1.2* promoter activity in *N. benthamiana* leaves co-expressing CPK and HsfB2a. The 1-kb *PDF1.2* promoter region was fused to a GUS reporter gene including the intron. Transient activation of the reporter gene according to co-expressed effector(s), HsfB2a, or truncated variant of CPK3 or CPK13, in *N. benthamiana* leaves was assessed. *Agrobacterium*, carrying 35S promoter::LUC including intron, was used to normalize for the efficiency of agroinfiltration. Means followed by different small letters are significantly different ($P < 0.05$).

Discussion

Regulation of Ca^{2+} homeostasis is important, particularly when Ca^{2+} is involved as a signaling ion. In plant cells, Ca^{2+}-binding proteins also serve as regulators of internal free Ca^{2+} levels. Protein phosphorylation is the most common type of post-translational modification, and functions through phosphorylation-induced conformational changes [24]. Since CPKs may be involved in the specificity and cross-talk of signal transduction for a variety of biotic and abiotic stresses, their possible involvement in active signaling cascades in herbivore responses needs to be investigated [6]. The present study provides a new view of a signaling network for plant-insect interactions. This cascade is not involved in the phytohormone (JA, ethylene and ABA)-related signaling pathways, but rather is able to directly impact transcription factors for defense responses. In fact, we did not observe striking effects of loss of CPK function on the biomass of *S. littoralis* larvae by feeding *cpk3* or *cpk13* mutants compared to WT plants for up to 3 days (Additional file 5). This suggests that more genes than just *PDF1.2* contribute in a

complex manner to the onset of acquired resistance to the generalist herbivore *S. littoralis*. For instance, compared to the levels in WT leaves infested by *S. littoralis*, the leaf transcript levels of *Thi2.1* were higher in *cpk13* after 24 h but lower after 6 h (Figure 2).

Herbivory responses in Arabidopsis may not be mediated strikingly by the ABA signaling network (Figures 3 and 4). In guard cells, CPK3 appears to act in the phosphorylation of plasma membrane S-type anion channels for the Ca^{2+}-reactive stomatal closure response [9]. Our data show that a loss of CPK3 function may consequently lead to an increase of cytosolic Ca^{2+} concentration in the infested leaf cells (Figure 5). If we consider that ABA activation of plasma membrane Ca^{2+}-permeable channels is, in contrast, impaired in *cpk3* or double *cpk3cpk6* mutant guard cells [9], we can argue that mesophyll cells, which are the most responsive to herbivore attack, respond in a different way compared to guard cells. In summary, the present findings are consistent with a model in which additional signaling branches function in the herbivory signal transduction network in parallel to CPK3-imposed feedback regulation of Ca^{2+} channels, and these additional branches are different from those of the stomatal closure response controlled via ABA signaling. In contrast to CPK3, CPK13 does not act upstream of herbivore-stimulated Ca^{2+} transients (Figure 5).

Screening of the databases for *cis*-acting regulatory DNA elements revealed the presence of a GCC box ((A)GCCGCC [25]) in the *PDF1.2* promoter that is potentially recognized by ERF1, which is one of the CPK3 substrates (Figure 7) and a potent regulator of *PDF1.2* [26]. However, our agroinfiltration assays showed that ERF1 as a cofactor failed to transactivate the reporter GUS gene under the control of the GCC consensus sequence (fused to a minimum TATA box) and the *PDF1.2* promoter in *N. benthamiana* (data not shown). We therefore infer that ERF1 may interact only indirectly with the *PDF1.2* promoter, similarly to MYC2 [27]. Otherwise, additional *cis*/*trans*-factors or protein modifications might be required to fully facilitate this hetero *planta* system.

According to our agroinfiltration assays, CPK3-or CPK13-derived phosphorylation(s) of a heat shock factor (HsfB2a) appeared to be directly and positively involved in *PDF1.2* transcriptional regulation (Figure 8B). Heat shock factors (Hsfs) are well known to function in the regulation of stress-inducible genes (e.g., *Hsp*) by recognizing a conserved binding motif (heat shock element [HSE]: three inverted repeats of nGAAn units [28,29]). However, the *PDF1.2* promoter lacks intact HSE motifs. It is therefore considered that HsfB2a can act as part of the transcriptional machinery of *PDF1.2* transcription by directly binding to non-HSE sequence(s) present in the promoter, and then CPK3 and CPK13 phosphorylate HsfB2a to modulate its activity. Similarly, Kumar et al. [30] reported that double knockout *hsfb1*/*hsfb2b* plants showed up-regulation of the basal mRNA-levels of *PDF1.2* in mutant plants. Therefore, it will be of great interest to identify a novel non-HSE DNA recognition site for the class B-Hsf transcription factors.

HsfB2a belongs to the Hsf class B transcription factors (B-Hsfs). However, the function of class B-Hsfs differs from that of class A-Hsfs due to a structural variation within the oligomerization domain and the lack of an AHA-motif, which is required for the transcriptional activation function of class A-Hsfs [31]. Since B-Hsfs have the capacity to bind to similar or the same HSE sites in the heat shock gene promoters as class A-Hsfs, most of them may act as repressors of target gene expression [32,33]. If class B-Hsfs generally antagonistically interact with A-Hsfs by binding (or competing for binding) to the HSE consensus sequence, their regulatory mechanisms would in most cases be different from the positive regulation of the herbivore-induced *PDF1.2* promoter lacking intact HSE motifs. A-Hsfs should be investigated as possible additional cofactors in further studies.

Conclusions

These results reveal the involvement of two Arabidopsis CPKs (CPK3 and CPK13) in the herbivory-induced signaling network via HsfB2a-mediated regulation of the defense-related transcriptional machinery. To reveal whether protein phosphorylation has significant effects on the transcript levels in response to feeding by caterpillars, future studies such as genetic analyses investigating double mutants defective for both CPKs and substrates or plants overexpressing those genes will be needed.

Methods

Plants, caterpillars, and treatments

Arabidopsis plants (Col-0) were grown in soil. Individual plants were grown in plastic pots in a growth chamber at 22°C (160 μE m^{-2} s^{-1} during a 12-h photoperiod) for 5 weeks. Larvae of *S. littoralis* Boisd. (Lepidoptera, Noctuidae) were reared on artificial diet [34] in a plastic box (25 ± 1°C; 14 h light: 10 h dark). For the herbivory treatments, three second-and third-instar larvae were placed on leaves of an *Arabidopsis* plant (non bolting). For chemical treatment, (±)-jasmonic acid or abscisic acid (Sigma-Aldrich) at a concentration of 0.3 mM or 50 μM, respectively, in aqueous solution, or ethephon (0.3 mM, Sigma-Aldrich) at a concentration of 3 mM in sodium phosphate buffer (50 mM, pH7), was evenly sprayed (about 3 ml) onto intact plants.

Genetic analysis

The homozygous T-DNA lines were screened using two PCR tests http://signal.salk.edu/tdnaprimers.html using

either a pair of primers consisting of a T-DNA left border primer (LBa1) and a specific primer for the corresponding T-DNA insertion region or a pair of gene-specific primers which straddle the outer T-DNA flanking regions. Genomic DNAs were isolated from the leaves following the CTAB method [35], and were used as the template for polymerase chain reaction (PCR) following the method described in: http://signal.salk.edu/tdnaprimers.2.html. These PCR analyses were performed at least twice through the two generations along T3 or T4 to confirm whether the lines are certainly homozygous.

Reverse transcription (RT)-PCR and real-time PCR

Total RNA was isolated from leaf tissues using a Qiagen RNeasy Plant RNA kit and an RNase-Free DNase Set (Qiagen) following the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript II RT, oligo(dT)$_{12-18}$ primer, and 1 μg of total RNA at 42°C for 50 min. The real-time PCR was done on an M×3000 P Real-Time PCR System (Stratagene) [36]. PCR conditions were chosen by comparing threshold values in a dilution series of the RT product, followed by non-RT template control and non-template control for each primer pair. Relative RNA levels were calibrated and normalized with the level of ACT1 (At2G37620) mRNA.

Quantification of JA, JA-Ile, ABA and ethylene

Leaves (200 mg) were harvested in FastPrep tubes containing 0.9 g of FastPrep matrix (BIO 101, Vista, CA), flash-frozen in liquid nitrogen, and stored at -80°C until use. Ethyl acetate (1 ml), spiked with 200 ng each of internal standards $^{2}H_{2}$-JA, $^{13}C_{6}$-JA-Ile and $^{2}H_{6}$-ABA, was added to each sample and then the mixture was homogenized using a FastPrep homogenizer (Savant Instruments, Holbrook, NY). After centrifugation at 12,000 *g* for 20 min at 4°C, supernatants were transferred to Eppendorf tubes. Each pellet was re-extracted with 1 ml of ethyl acetate and centrifuged; supernatants were combined and then evaporated to dryness under vacuum. The residue was resuspended in 0.5 ml of 70% methanol/water (v/v) and centrifuged to clarify phases, and the supernatants were analyzed using a 1200 L LC/MS system (Varian, Palo Alto, CA) as described in [37].

Ethylene production was measured in real-time with a photoacoustic laser spectrometer (ETH-PAC1-TR, http://www.invivo-gmbh.de, Germany) in combination with a gas multiplexer (4 channels) [38], in which an Arabidopsis plant in a pot was infested with larvae for up to 24 h.

Intracellular calcium concentration measurement

A solution of Fluo-3 AM (acetoxy-methyl ester of Fluo-3, 5 μM, Fluka, Buchs, Switzerland), 0.5 mM calcium sulphate, and 2.5 μM DCMU [3-(3',4'-dichlorphenyl)-1,1-dimethylurea] in 50 mM MES buffer, pH 6.0, was used for initial treatment of leaves of an intact Arabidopsis plant as previously described [39]. A leaf was cut once with a razor blade in order to allow the dye to enter the tissues. One hour after treatment with Fluo-3 AM, the leaf was fixed on an Olympus FLUOview confocal laser scanning microscope (CLSM) stage without detaching it from the plant. The microscope was operated with a krypton/argon laser at 488 nm and 568 nm wavelengths: the first wavelength excited the Fluo-3 dye emitting green light, while the second excited mostly chloroplasts emitting red fluorescence. Images generated using FluoView software were analyzed with NIH Image J software. Earlier microscopic analysis showed the false-color subcellular localization of the dyes, which indicated that the dyes are loaded mainly into the cytosol [40].

Vector construction and transient expression of GFP fusion proteins

Gateway Technology (Invitrogen) was used for the generation of p2GWF7 transformation constructs, which consisted of a target gene (CPK13 ORF cDNA) bearing an N-terminal fusion to eGFP under the control of the dual Cauliflower Mosaic Virus 35S promoter for plant transformations [41]. The attB adaptor-bearing PCR primers (see Additional file 6) were designed for the generation of attB PCR products for recombination with the donor vector pDONRzeo via BP Clonase reactions (Invitrogen). Fully sequenced entry clones were recombined in LR Clonase reactions with the p2GWF7 vector [41]. One microgram of the plasmid was precipitated onto 1.0-μm spherical gold particles (Bio-Rad). Onion peels were bombarded using a particle gun PDS-1000/He (Bio-Rad) according to the manufacturer's instructions. After 24 h, GFP fluorescence of the onion peel was observed under a BX51 fluorescent microscope (OLYMPUS).

Transcription and cell-free protein synthesis

In order to prepare recombinant proteins fused with GST or a biotin ligase recognition site at their N-terminus, Riken Arabidopsis full-length cDNA clones (RAFL) were used. The DNA constructs were made, according to [42], by two rounds of "Split-Primer" PCR, with the first PCR performed with a target protein-specific primer (5'-CCACCCACCACCACCAatgnnnnnnnnnnnnnnnn-3'; lowercase indicates the 5'-coding region of the target gene) and the AODA2306 primer. The second PCR was performed with an SPu primer, AODA2303 primer, and a deSP6E02bls-S1 primer, which contains a biotin ligase recognition site sequence (for the CPK substrates). For the CPK-GST fusion proteins, a full-length ORF was reinserted into pEU-E01-GST-TEV-MCS vector (Cell-free Sciences, Yokohama, Japan) and used for *in vitro* transcription. *In vitro* transcription, cell-free protein syn-

thesis, and protein purification were performed as described [42,43].

Auto-phosphorylation reaction of recombinant CPK

Crude GST-tagged recombinant CPK protein (20-40 μg) produced by the dialysis method [44] was precipitated with glutathione Sepharose™ 4B (GE Healthcare). The protein on the Sepharose was washed twice with PBS buffer and then treated with PBS buffer containing 2.5 mM 1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N',N'*-tetra acetic acid (BAPTA, Sigma-Aldrich) at 4°C for 10 min, to remove free Ca^2. After washing with PBS buffer, the recombinant CPK was eluted with 45 μl of PBS buffer containing 0.1 U of AcTEV protease (Invitrogen), which cleaved the CPK from the GST-tag. Autophosphorylation reactions were carried out in 10 μl of total reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 μl of partially purified CPK, 0 to 500 μM $CaCl_2$, 10 mM potassium acetate, 50 mM $MgCl_2$, 0.5 mM DTT and 37 kBq of [γ-^{32}P] ATP at 30°C for 30 min. To stop the reaction, 5 μl of 3x-sample buffer [150 mM Tris-HCl (pH 6.8), 6% SDS, 3% 2-mercaptoethanol, and 0.012% bromophenol blue] was added to the reaction mixture. After boiling the reaction mixture, recombinant CPK was separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE). The labeled signals were detected with BAS-2500 (FUJIFILM, Japan).

In vitro phosphorylation of target protein

In vitro phosphorylation of target was carried out according to a previously described method with a minor modification [44], using partially purified recombinant CPK and substrate. Partially purified GST-tagged recombinant CPK was prepared as described above, but excluding BAPTA from the PBS buffer. Forty microliters of each crude biotinylated substrate protein produced by the bilayer method [43] were attached to Streptavidin Magnesphere Paramagnetics particles (Promega). After washing, the substrate protein on the particles (1-2 μg) was incubated in 15 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 μl of purified CPK, 100 μM $CaCl_2$, 500 mM potassium acetate, 50 mM $MgCl_2$, 0.5 mM DTT and 37 kBq of [γ-^{32}P] ATP, in the presence or absence of 500 μM BAPTA at 30°C for 30 min. Following the reaction, the beads were washed twice with PBS, then boiled in sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 1% 2-mercaptoethanol, and 0.004% bromophenol blue]. For the detection method, see above.

Agrobacterium tumefaciens-mediated transient expression (agroinfiltration) in *Nicotiana benthamiana*

A cDNA fragment of a truncated variant lacking junction and calmodulin-like domains was cloned into the pER8 (XVE) binary vector [45]. The full-length coding region of *HsfB2a* was inserted into the GUS reporter gene site of the binary vector pGreen 0229 (35S promoter::GUS including intron) [46]. The 1-kb *PDF1.2* promoter region upstream of the transcription start site was inserted into the 35S promoter region of the above pGreen-GUS vector.

Agroinfiltration was carried out according to a modified protocol from Kobayashi et al. (2007) [47]. Binary plasmids were transformed into *Agrobacterium* strain GV3101, which contains the transformation helper plasmid pSoup [46], and the bacteria were cultured overnight. The culture was diluted 10-fold in Luria-Bertani medium/rifampicin with kanamycin or spectinomycin, and then was cultured until OD_{600} 0.6. Cells were harvested by centrifugation and resuspended in 10 mM MES-NaOH, pH 5.6 and 10 mM $MgCl_2$. The bacterial suspensions were adjusted to OD_{600} 0.5, and then acetosyringone was added to a final concentration of 150 μM. The suspensions were incubated for 2-4 h at 22°C, and a mixture of those carrying CPK, HsfB2a, luciferase (LUC, see below) and *PDF1.2* promoter::GUS vectors at an approximate ratio (1:1:1:3) was infiltrated into leaves of 4- to 5-week-old *N. benthamiana* plants by using a needleless syringe. One day after bacterial infiltration, β-estradiol (10 μM) was infiltrated into the same position of the leaf to induce the expression of a chimeric transcription activator XVE gene [45], and the plant was kept for 1 day. We then prepared an enzyme extract by homogenization of a leaf with a grinding buffer containing 100 mM potassium phosphate (pH 7.8), 1 mM EDTA, 7 mM 2-mercaptoethanol, 1% Triton X-100 and 10% glycerol, followed by centrifugation. GUS activity was measured by monitoring cleavage of the β-glucuronidase substrate 4-methylumbelliferyl β-D-glucuronide (MUG) [48]. *Agrobacterium* carrying pGreen 0229 (35S promoter::LUC including intron) was used to normalize for the efficiency of agroinfiltration. The luciferase activity in protein extracts was measured using a PicaGene luciferase kit (Toyo Ink, Japan) according to the manufacturer's protocol.

Data and statistical analysis

At least five repetitions with individual biological sample sets were used for the statistical treatment of the data. The data are expressed as mean values; error bars indicate the standard error. To evaluate the significance of differences of data, ANOVA followed by Fisher's PLSD test was performed.

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At least five repetitions with individual biological sample sets were used for the statistical treatment of the data. The data are expressed as mean values; error bars indicate the standard error. To evaluate the significance of differences of data, ANOVA followed by Fisher's PLSD test was performed.

Additional material

Additional file 1 Molecular analysis of CPK T-DNA insertion mutants and gene expression profiling in the mutants. A, Disruption of CPK mRNA expression in leaves of the respective *cpk* mutants and the Col-0 wild-type (WT). None of the mutant CPK genes was expressed in the corresponding mutant leaves under the growth conditions, whereas all of them were expressed in WT leaves. B, T-DNA insertion site in *cpk3* (Salk_022862), *cpk13-1* (Salk_057893) and *cpk13-2* (Salk_135795). PCR was performed with a primer pair consisting of a left border primer of the T-DNA and a gene-specific primer, and PCR products were sequenced to determine the T-DNA insertion positions (solid lines). ATG and TGA/TAG indicate start and stop codons. White boxes indicate exons. The T-DNA inserts in *cpk3* and *cpk13-1* are located in the first exon, while the insertion in *cpk13-2* is located in the promoter region upstream of the *CPK13* gene. Note that Southern blot analyses of homozygous plants showed only a single T-DNA insertion in all four mutants (data not shown).

Additional file 2 Substrate targeting for CPKs. A table listing protein substrates synthesized using the wheat germ cell-free system.

Additional file 3 Substrate targeting of CPK3 and CPK13. The quantitative values for data in Figure 7B are shown.

Additional file 4 Supplemental discussion. Implications for possible involvement of ubiquitination in the CPK signaling pathway are discussed.

Additional file 5 Growth of *S. littoralis* larvae on a WT, *cpk3* and *cpk13-1* plant. Freshly hatched *S. littoralis* larvae were grown on artificial diet. The second instar of the larva was subjected to growth on a WT, *cpk3* and *cpk13-1* plant in a pot at 25°C. The larva was allowed to feed for up to 3 days, and its biomass was recorded every 24 h.

Additional file 6 Primers used for this study. A table listing primers used for this study.

Authors' contributions

CNK carried out all the biological and genetic analyses. HT and AM carried out cell-free protein synthesis and *in vitro* phosphorylation assays and participated in writing the methods section. SQ, CB and SAZ performed the RT-PCR and real-time PCR experiments. MEM and SB carried out calcium measurement. NI and HY helped with agroinfiltration assays and participated in writing the methods section. WB, JT and YE participated in the coordination of the work. TS and GA conceived the intellectual design of the project and wrote the manuscript. All authors read and approved the final manuscript.

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