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).

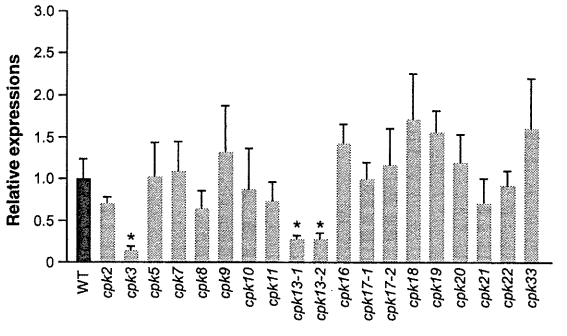

Figure 1 Comparison of the transcript levels of plant defensin gene *PDF1.2* between *S. littoralis*-infested leaves of WT and CPK T-DNA insertion mutants. Transcript levels of *PDF1.2* in mutant leaves (gray columns) were normalized by those of *ACT1* measured in the same samples and expressed relative 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-

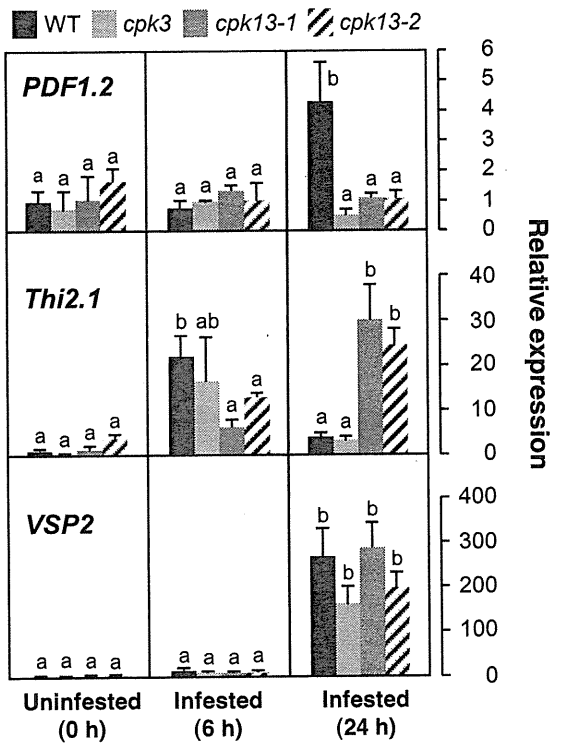

Figure 2 Relative mRNA levels of defense-related genes *PDF1.2*, *Thi2.1* and *VSP2* in *S. littoralis*-infested leaves. Transcript levels of genes in WT, *cpk3*, *cpk13-1* and *cpk13-2* leaves were normalized by those of *ACT1* measured in the samples and expressed relative to the normalized transcript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

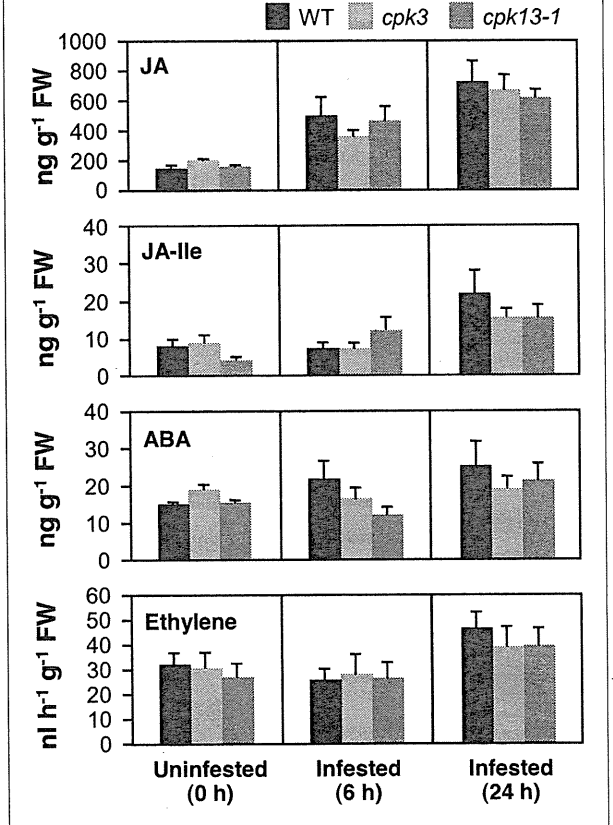

Figure 3 Levels of JA, JA-Ile, ABA and ethylene in WT, *cpk3*, and *cpk13-1* leaves fed on by *S. littoralis* larvae for 6 and 24 h.

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-

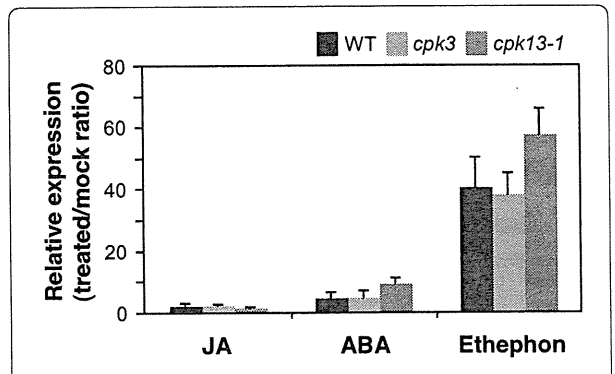

Figure 4 Effects of loss of CPK function on signaling pathways for defense response. Effect of exogenous application of JA, ABA or ethephon (an ethylene releasing chemical) on *PDF1.2* expression levels after 6 h was investigated. Transcript levels of *PDF1.2* in WT, *cpk3*, and *cpk13-1* leaves were 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

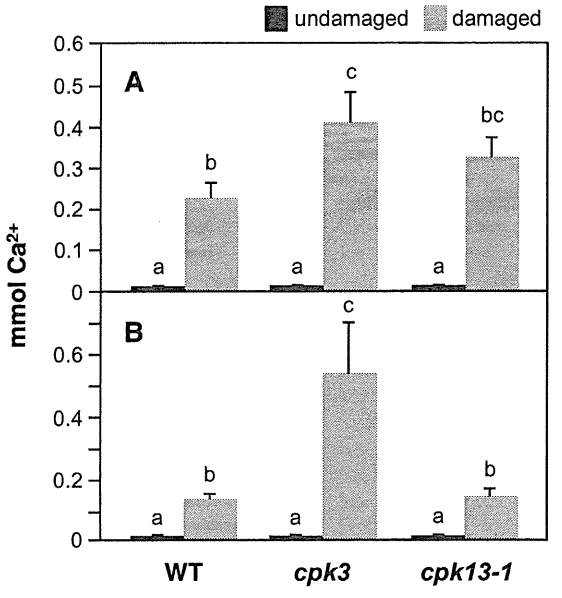

Figure 5 Intracellular Ca^{2+} levels in WT and *cpk* leaf cells. Undamaged WT leaves served as a control. Leaves were treated with Fluo-3 AM for 1 h and damaged with a *S. littoralis* larva (A) or mechanical wounding (B). Thirty minutes after damage, the cytosolic Ca^{2+} concentrations in leaf cells 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 auto-phosphorylation 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-

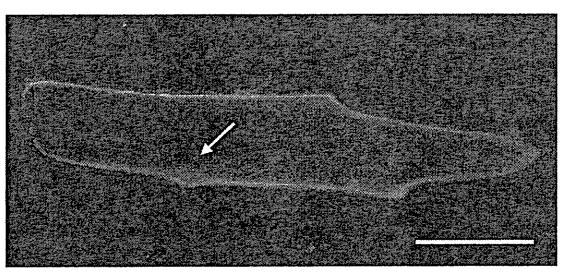

Figure 6 Transient expression of the CPK13-GFP fusion proteins. The recombinant plasmid was transformed into onion peels by particle bombardment. Cells were observed under a fluorescence microscope. Bars represent 100 μm. Arrows point to the nucleus

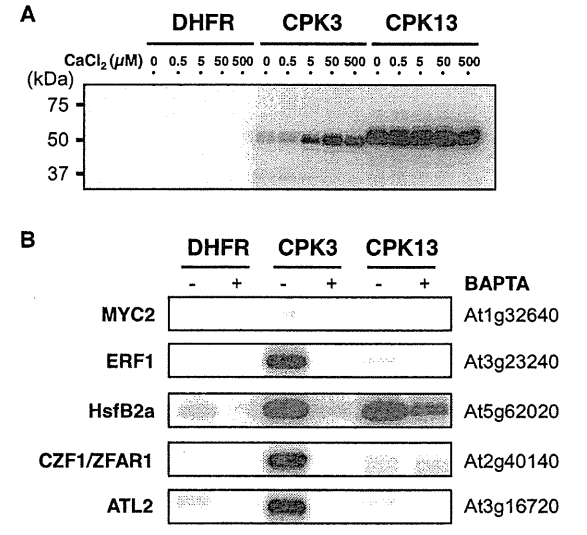

Figure 7 Substrate targeting of CPK3 and CPK13. A. Auto-phosphorylation signals from each CPK protein. Recombinant CPK was pre-treated with BAPTA (2.5 mM; a calcium chelator) and then subjected to auto-phosphorylation assays with several concentrations of $CaCl_2$. B. *In vitro* kinase assays of the 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).

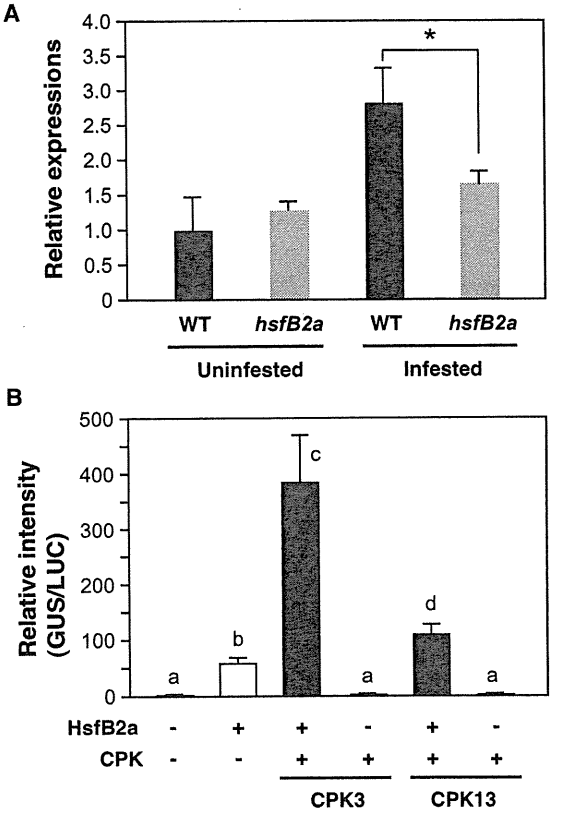

Figure 8 Involvement of CPK3, CPK13 and HsfB2a in herbivore-induced *PDF1.2* regulation. A. Transcript levels of *PDF1.2* in WT and *hsfB2a* mutant Arabidopsis leaves fed on by *S. littoralis* larvae for 24 h. An asterisk (*) indicates that mutants were significantly different from WT ($P < 0.05$). B. *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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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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The Wheat-Germ Cell-Free Expression System

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Abstract: We have made a dramatic improvement of the wheat cell-free protein synthesis system. The first key improvement is the method for preparation of the cell-free extract that is free of inhibitory factors of translation reaction. Additional improvements include a method for preparation of transcription-ready templates by PCR, an expression vector for the cell-free system, and the "bilayer" mode reaction method that is much more efficient than the batch mode method and at the same time easy to be performed by human hands and by liquid handling machines. We review here the history of the development and describe the protocols for the most handy "bilayer" method and a more efficient but complicated methods. Information on many examples and variations of the wheat cell-free protein synthesis methods already published elsewhere is then provided so that the readers can understand the power and potential applications of the methods.

Keywords: Cell-free protein synthesis, wheat, split-primer PCR, automation, pEU.

INTRODUCTION

Cell-free protein synthesis was first established with mammalian cell extracts in 1950's [1]. It was soon found that extracts from *E. coli* catalyze efficient translation, which led to the discovery of poly(Phe) synthesis directed by poly(U) in 1961 [2]. The first cell-free translation system from wheat embryo, which could catalyze poly(Phe) synthesis, was described in 1964 [3]. It was later shown that an extract from commercial wheat germ can efficiently translate tobacco mosaic virus (TMV) RNA and rabbit globin mRNA [4,5], demonstrating that the extract contains all the ingredients required for protein synthesis. The amounts of proteins synthesized by these cell-free methods were generally small, however. In an attempt to increase the protein production, Spirin's group showed that cell-free protein synthesis with wheat germ extracts can last for very long time when performed in an ultrafiltration chamber with continuous supplementation of the substrates and removal of the products, which was termed the continuous-flow cell-free (CFCF) method [6]. This seemed to mean that the translation apparatus is stable under a steady state-like condition that mimics intracellular environments. As the CFCF method is complicated, it was substituted by the less-complicated continuous-exchange cell-free (CECF) method, in which the reaction proceeds within a semi-permiable membrane bag being dialyzed against the substrate solution (dialysis method). We made a dramatic improvement in yield and achieved automation of the wheat cell-free protein production technology, as summarized in the following sections.

TECHNOLOGY DEVELOPMENTS

1. Extract Preparation

We speculated that the conventional wheat cell-free translation system contains factor(s) that catalytically inactivate(s) the ribosomes during the translation reaction. One such factor is tritin, present in commercial wheat germ, which attacks and modifies the sarcin-ricin loop of the ribosomes [7-9]. We have tested many different methods for inhibiting or removing the intrinsic ribosome inhibitors and found that extensive water wash of embryo particles largely removes white matter originating from endosperm and thereby dramatically improves the efficiency of translation catalyzed by the extract [10]. Such water wash has additional effects: most of other potential catalysts, such as proteases, DNases, and RNases, are also removed, and translation may be activated during the wash, for a substantial amount of water is absorbed into the embryo particles.

The method for preparation of the wheat embryo particles, which will be subsequently subjected to water wash, includes crushing wheat seeds, mesh sieving, flotation, and eye selection. This procedure was problematic because the flotation step needed use of carbon tetrachloride which is tightly regulated in Japan, and because eye selection was too laborious. These problems have been minimized in CellFree Sciences, Co., Ltd. (CFS), Matsuyama, Japan. The conventional method of crushing the washed embryo particles was to grind the liquid nitrogen-frozen particles with mortar and pestle in a cold room, after which oil was removed and the supernatant of the 30,000×g centrifugation (S30) was recovered. We could prepare several milliliter of the S30 extract by this conventional method at a time. This process is now replaced by a quality-controlled method utilizing a blender with rotating blades, and the extract can be obtained from CFS. We have recently described a laboratory-level version of this blender method elsewhere [11].

2. mRNA Preparation

As the cap analog and uncompleted transcripts inhibit cell-free translation, it would be necessary, if a capped mRNA is used for translation, to purify the complete transcript for reproducible results. Instead, an Ω sequence from TMV can be used for activation of downstream translation when placed in the 5'-untranslated region (5'-UTR) of un-

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capped mRNAs [12]. With the Ω sequence, the transcript can be directly introduced into the cell-free translation reaction without purification. The pEU vector that contains an SP6 promoter, the Ω sequence, and a multiple cloning site in this order was then constructed [12]. The wheat cell-free system thus developed produced up to 10 mg of proteins per 1-ml reaction mixture by the dialysis CECF method [12]. We have also developed several artificial sequences that are different from the Ω sequence but have almost the same functionality as Ω. The E01 sequence is one of such artificial sequences, and is regularly used in our group [13]. Furthermore, a method was developed for preparation of transcription-ready templates by PCR reactions without cloning of the target ORF into pEU as below. This facilitated parallel production of many different proteins encoded in a cDNA library [12].

3. Reaction Format

The dialysis method is not simple enough for translation of many different samples in parallel. Thus, a more handy method for CECF translation was developed [14]. The extract is slightly denser than the substrate solution. Thus, the reaction mixture can be put under the substrate solution to form a bilayer without the need for the separating membrane (Fig. (**1**)). Both solutions (*i.e.,* extract and substrate solution) mix together gradually with time, accompanied by supply of fresh substrates to and dilution of the byproducts from the extract layer. While the productivity of this "bilayer" method is lower than that of the dialysis method, it is much higher than that of the simple batch mode method. We have learned at the same time that the dilution of the byproducts, such as pyrophosphate and inorganic phosphate, has a larger effect on the productivity of translation than the supplementation of the substrates. The most important feature of the bilayer method was that it fits automation due to the absence of the semi-permeable membrane.

4. Automation

The bilayer method made automation possible. A robotic machine named "GenDecoder" performs a parallel small-scale bilayer protein synthesis, and a robot named "Protemist" performs a large-scale synthesis of small number of proteins (some Protemist machines also perform tag purification after the synthesis). One of the latest machines can perform gram-scale synthesis based on the "intermittent exchange" or "discontinuous batch" method [15,16]. This can synthesize nearly 1 gram of a model protein by an overnight operation.

In the following section, we describe basic protocols for synthesizing proteins encoded in a cDNA subcloned into a pEU plasmid. The protocols of the bilayer methods are essentially the same as those provided by CFS: basic protocols and typical results are available from the CFS website (www.cfsciences.com). As a lot of variations of these protocols are possible and have already performed by many people, we review such variations in the last section, which we expect will be useful for the readers to determine whether they will try the wheat cell-free system for their purposes.

PROTOCOLS AND EXAMPLES

Transcription of pEU Plasmids

The wheat embryo protein synthesis system is primarily a translation system, although both transcription and translation can be done in one tube. mRNA solutions are prepared by *in vitro* transcription with a DNA template, ribonucleoside 5'-triphosphates (NTPs), and SP6 RNA polymerase. The DNA template to be transcribed can be either a PCR product or a plasmid DNA, if it contains an SP6 promoter with a GAA trinucleotide at the transcription start site, an Ω sequence or an E01 or E02 enhancer sequence, the ORF sequence to be translated, and a long 3'-UTR of >500 nucleotides of any sequence. Such template DNA can be prepared by the split-primer PCR method as below or by subcloning the ORF sequence into the pEU vector. The protocol presented here is for an ORF subcloned in the pEU vector. We have observed that a G between the 5' enhancer sequence and the initiation codon sometimes causes alternative translation initiation that may result in no band or a doublet band in the SDS gel of the final products. Closed circular DNA is directly used for transcription.

For transcription in the small-scale bilayer method (see next section), mix Milli-Q water, 10 μl of 5× TB (400 mM HEPES-KOH, pH 7.8, 80 mM $Mg(OAc)_2$, 10 mM spermidine, and 50 mM DTT), 5 μl of 25 mM NTP, 50 units of RNase Inhibitor from human placenta (Promega), 50 units of SP6 RNA polymerase (Promega), and 5 μg of the plasmid DNA in a 50-μl solution. Incubate the solution at 37°C for 6 h. White pellet will appear during the incubation. For the large-scale method, perform the same reaction in a 250-μl solution. The transcription solution is directly used for translation (without purification).

For checking the transcription product, incubate 1-3 μl of the sample in a standard formamide dye at 65°C for 3 min and load the sample on a standard 1% agarose gel run in 1× TAE (40 mM Tris-acetate, pH 7.5-7.8, 1 mM EDTA). The ethidium bromide stained gel will show a few bands with smear signals in the upper (slowly migrating) region. The major band may be from the RNA molecule stopped near the plasmid replication origin: there is a transcription terminator around there. If there are several bands or a smear signal below the major band, it is possible that the transcription mixture was contaminated with some nuclease that cannot be inhibited by the RNase inhibitor. In such cases, we recommend to further purify the plasmid and to prepare all the solutions freshly.

Bilayer Method

In the bilayer method, the starting translation reaction mixture containing all the components required for translation (*i.e.*, creatine kinase, the wheat extract components, and mRNA) is first layered below a substrate solution. The substrate solution contains the standard 20 amino acids, nucleotides required for translation, an energy source substrate (creatine phosphate), a reducing agent dithiothreitol (DTT), salts, and a buffer. The substrate solution had been called Dialysis Buffer (DB) because it is used for the external solution for the dialysis method below. In this paper, however, we call it Buffered Substrate Solution (BSS). The wheat extract is prepared in BSS.

The efficiency of the bilayer translation may depend on the shape of the container in which the reaction is performed. The ratio of the volume to the contacting surface area can affect the rate of the exchange of the substrates and byprod-

ucts. While the following protocol using a flat-bottomed 96-well microtiter plate will give reproducible results, standard 0.2 ml PCR tubes can also give good results.

For protein synthesis, mix 10 μl of the mRNA solution (the transcription product above) with 0.8 μl of 1 mg/ml creatine kinase and 10 μl of 240 OD/ml wheat embryo extract. Then, pour 206 μl of BSS (30 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 2.7 mM $Mg(OAc)_2$, 0.4 mM spermidine, 2.5 mM DTT, 0.3 mM each amino acid, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate) into a well of a flat-bottomed 96-well microtiter plate. Put the reaction mixture containing mRNA, creatine kinase, and the extract under the BSS at the bottom of the microtiter well carefully NOT to mix these two solutions together (Fig. **1**). Cover the well using a standard seal in order to minimize evaporation. Incubate the plate at 15°C for 20 h.

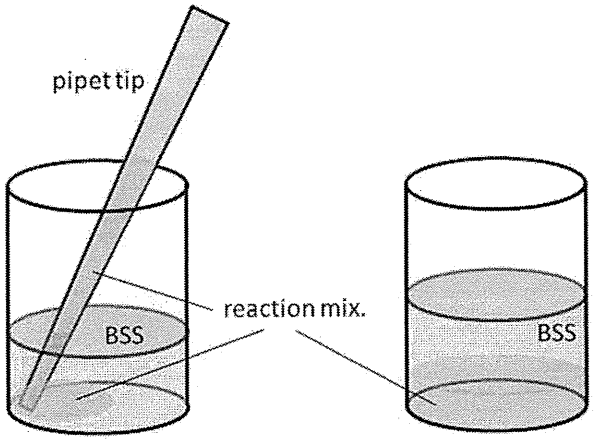

Fig. (1). The bilayer method.
Schematic representations showing how the reaction mixture should be layered under the substrate solution without perturbation (left) and the bilayer to be formed by this operation (right).

After the incubation, mix the sample and analyze 3 μl of it by a standard SDS-PAGE stained with Coomassie Brilliant Blue. The band for the product protein may be visible among many bands of the wheat proteins. If the band overlaps with that of a wheat protein, or the band is too faint to be detected, it is recommended to label the protein with [^{14}C]leucine during the translation reaction and to detect the band by autoradiography. A larger protein tends to give a fainter band.

The above system can be scaled-up in the large-scale bilayer mode useful for sub-milligram synthesis of selected proteins. The reaction is performed in a 6-well plate with 500 μl of the reaction mixture in each well. For translation, first mix 250 μl of the transcription product with 1 μl of 20 mg/ml creatine kinase and 250 μl of the 240 OD/ml extract. Then, pour 5.5 ml of BSS into a well of a 6-well plate (Cat. No. 92006, Techno Plastic Products). Layer the reaction mixture under the solution at the bottom of the well, and seal the well. Incubate the plate at 15°C for 20 h. Mix the solution after the incubation, and analyze the products on an SDS-polyacrylamide gel.

Dialysis Method

The dialysis method is useful for synthesizing a large amount of protein, though the method is rather complicated. The reaction mixture, which is essentially the same as that in the bilayer method, is placed in a dialysis membrane or a dialysis cartridge, and the substrate solution is placed in the outer container. The dialysis membrane containing the reaction mixture is dipped into the outer solution, and the container is sealed and incubated. When utilizing this method, we usually add the mRNA solution every 12 h to the inner solution and exchange the outer solution every 24 h to maximize the productivity per unit amount of the extract. The reaction could be prolonged for more than 2 weeks if maintained carefully, while 60 h may be the most convenient duration time: a longer incubation may cause growth of fungi. For a 50-μl reaction we usually use a dialysis cup (Biotech International, Japan) for the reaction mixture with a 9-ml polypropylene tube (Cat. No. 0204-03, Maruemu Corp., Japan) as the reservoir of BSS. For a 2-ml reaction, a 5-ml Spectra/Por Float-A-Lyzer™ G2 device (MWCO of 8-10 K, Cat. No. G235055, Spectrum Laboratories, Inc.) can be used with about 20 ml of BSS.

ADVANTAGES AND DISADVANTAGES

PCR-Based Template Preparation

The wheat embryo cell-free translation system can utilize not only the pEU-subcloned templates but also PCR-produced templates amplified from cDNA library clones of any format. The PCR-based method is useful for high-throughput parallel production of natural and tag-fused proteins [17-19]. It is required to introduce an SP6 promoter and an enhancer sequence upstream of the ORF and a suitable 3'-UTR sequence by the PCR. For this purpose the "split-primer" PCR method mentioned above is useful [12]. This method utilizes two primers, each of which has an incomplete SP6 promoter sequence, and combination of the two generates a complete promoter. This method seldom generates byproducts containing a complete promoter that could produce an unintended RNA molecule during the following transcription reaction. The sequence in the 3'-UTR can be the sequence in the vector in which the cDNA sequence is inserted. There is no need for the poly(A) sequence characteristic of the eukaryotic mRNA. In the case of the cDNA clones subcloned in pEU or other pUC-related plasmid vectors, reverse primers that define the 3' terminus of the transcript near the plasmid replication origin are useful for the split-primer PCR method.

Modification of the Extract

The extract is highly resistant to treatments with chromatography resins and ultrafiltration membranes. The buffer can be exchanged by passing the sample through a Sephadex G-25 column. The extract may be diluted after passage through a column, while it can be concentrated by the use of an ultrafiltration cartridge, such as Amicon Ultra-15. Taking advantage of this robust nature of the extract, a highly productive gram order synthesis method was developed and automated as mentioned above. In this method, the buffer and substrates surrounding the protein synthesis apparatus are intermittently exchanged by ultrafiltration during the

reaction. The extract can be freeze-dried without severe loss of activity and can be stored at –20°C for years.

Spectrum of Suitable Proteins

We have recently shown that 12,996 (97.2%) of randomly chosen 13,364 human cDNA clones produced detectable amounts of proteins using the wheat embryo cell-free translation system [20]. The results clearly show that in most cases the amino acid sequence of the cDNA-encoded polypeptide could successfully be synthesized in the wheat cell-free system. Of those 12,996 polypeptides, 12,682 were detected in the soluble fraction. It was apparent in Ref. [20] and as shown in Fig. (**2**) that the cell-free system is essential for highly parallel production of many different proteins, and the wheat cell-free system exhibits a very high success rate.

Number	Resource of ORF (FLJ ID)	Mw (kDa)	TM	Wheat germ (*in vitro*)		*E. coli* (*in vitro*)		*E. coli* (*in vivo*)	Cultured cell (DM2)	Cultured cell (CHO)
				5'GST	3'His	5'GST	3'His	3'His	3'His	3'TAP
1	FLJ20659	10.4	0	○	○	○	×	○	○	○
2	FLJ21903	14.7	0	○	○	×	×	×	○	×
3	FLJ20616	15.2	0	○	○	×	○	○	○	○
4	FLJ20852	17.1	0	○	○	×	N.D.	○	×	×
5	FLJ22873	17.8	0	○	○	×	×	○	○	○
6	FLJ21700	18.9	0	○	○	×	×	○	○	○
7	FLJ22881	20.0	0	○	○	○	×	○	○	○
8	FLJ20819	23.5	0	○	○	×	×	△	○	○
9	FLJ22273	25.3	0	○	○	○	×	○	○	○
10	FLJ22538	29.6	0	○	○	○	×	×	△	×
11	FLJ22880	33.3	0	○	○	○	×	○	○	○
12	FLJ20621	33.3	0	○	○	○	×	△	○	○
13	FLJ20855	33.5	0	○	○	○	×	○	○	○
14	FLJ21182	33.7	0	○	○	○	×	○	○	○
15	FLJ21486	34.9	0	○	○	○	×	○	○	○
16	FLJ22325	35.7	0	○	○	○	×	○	○	○
17	FLJ20586	37.0	0	○	○	○	○	×	○	○
18	FLJ22320	38.2	0	○	○	○	×	×	○	○
19	FLJ22491	39.0	0	○	○	○	×	○	○	N.D.
20	FLJ21861	50.1	0	○	○	○	○	△	○	×
21	FLJ21235	50.1	0	○	○	○	○	△	×	○
22	FLJ22470	52.7	0	○	○	○	×	△	△	N.D.
23	FLJ22779	57.3	0	○	○	○	○	×	○	N.D.
24	FLJ20768	66.9	0	○	○	○	×	△	○	○
25	FLJ22818	91.3	0	○	○	×	○	○	×	×
26	FLJ21907	101.3	0	○	○	△	×	△	×	×
27	FLJ21076	7.0	1	○	△	×	×	△	○	N.D.
28	FLJ21791	10.1	1	○	○	○	×	○	○	×
29	FLJ21558	15.3	2	○	○	×	×	○	○	×
30	FLJ20556	17.4	1	○	×	×	×	○	×	×
31	FLJ20489	25.7	1	○	△	×	×	×	×	○
32	FLJ20507	25.8	4	○	○	○	N.D.	N.D.	○	○
33	FLJ12627	26.0	1	○	×	○	×	△	×	×
34	FLJ10199	27.8	2	○	×	○	×	○	△	△
35	FLJ10191	28.6	4	○	○	×	×	×	×	N.D.
36	FLJ12791	32.3	4	○	○	○	×	×	×	○
37	FLJ10099	35.1	1	○	○	○	×	△	△	N.D.
38	FLJ12133	36.2	3	○	○	×	×	×	○	○
39	FLJ21879	37.7	7	△	△	○	×	×	×	○
40	FLJ11856	45.8	10	○	△	○	×	×	×	N.D.
41	FLJ10583	46.3	3	○	○	○	×	×	×	×
42	FLJ12397	48.5	1	○	○	×	×	×	△	△
43	FLJ12769	51.2	1	○	○	×	×	×	○	×
44	FLJ10494	53.9	9	○	○	△	×	△	×	N.D.
45	FLJ12646	63.1	1	○	○	×	×	×	△	N.D.
46	FLJ22958	64.7	8	○	○	○	×	×	×	N.D.
47	FLJ10375	68.3	9	○	○	×	×	△	×	N.D.
48	FLJ12756	76.1	14	○	○	×	×	×	×	×
49	FLJ10407	76.3	7	△	△	×	×	×	×	×
50	FLJ10572	80.1	1	○	×	×	×	△	○	○

Fig. (2). A comparison of different expression systems in Goshima *et al.* (2008).
Expression of fifty different ORFs was compared for the wheat cell-free, *E. coli* cell-free, *E. coli in vivo*, DM2 cultured cell, and CHO cultured cell systems. The numbers in the "TM" column show the numbers of predicted transmembrane domains. Tags shown below the names of the expression systems were fused to the ORFs by using the Gateway system. The expression levels were determined by visual inspection of the SDS gels stained with CBB. Circles and triangles, high and low expression, respectively; crosses, no expression; N. D., no data. Reproduced from Ref. [20].

While many expression systems suffer from low productivity due to biased codon preferences within the ORF for the target protein, the effects appear to be small for the wheat cell-free system. For example, while malaria proteins are generally difficult to produce partly because the codon preferences are biased extremely, many of them have been successfully produced in the wheat cell-free system [21]. A template with a very high A/T content may rather be troublesome at the level of DNA/RNA technologies. It has also been revealed that the wheat cell-free system is excellent in producing DNases, such as restriction enzymes [22]. Other examples of produced proteins are summarized elsewhere [23-30].

Modifications on Synthesized Proteins

The N-termini of the cell-free synthesized proteins are processed according to the normal eukaryotic "N-end rule", and are acetylated if the cell-free reactions are supplied with acetyl-coenzyme A [31]. N-teminal lipoylation, such as palmitoylation, may also occur if the substrate is supplied [32]. Glycosylation does not occur during wheat cell-free reactions. We have observed that some expressed proteins give SDS-PAGE bands of very high molecular weights, which may correspond to ubiquitination.

Quality of Synthesized Proteins — Protein Degradation and Co-Translational Folding

The proteins produced in the wheat cell-free protein synthesis system are only minimally susceptible to degradation and thus can be used without purification. In fact, the wheat extract does not contain detectable activity of 26S proteasome [33]. This may be why some proteins give bands for ubiquitinated forms that have not been degraded.

Another prominent feature in the wheat cell-free system is that more proteins are produced in soluble forms than in the bacterial expression system [34,35]. It is suggested that the wheat cell-free system has a mechanism to minimize inter-domain interactions, presumably due to both a lower elongation rate than in bacterial systems and the presence of eukaryote-specific chaperones that bind to growing polypeptides [35,36]. In fact, we have successfully produced an active multi-domain RNA ligase that had been difficult to produce in an active form in a bacterial system [37]. Protein kinases are also difficult to produce in bacterial systems, in general, but not in the wheat cell-free system [38]. Successful production of native homopolymer of polyhedrin [39] and heterodimer protein [40,41] was also possible in the wheat cell-free system. Both of the apo- and holo-forms of an enzyme were also successfully produced [42].

It is possible, in the wheat cell-free system, to perform the protein synthesis reaction at lower temperatures than that presented in the above protocol. We have observed that translation proceeds even at 4°C, which facilitates production of proteases (unpublished results). Therefore, it may also be possible to promote correct folding and subunit assembly by performing the translation reaction at a low temperature.

Applications to Structural Biology

The low protease activity within the extract makes the method useful for protein structural analyses. HSQC spectra can be obtained using the product of protein synthesis with ^{15}N amino acids just after a simple buffer exchange without purification [43]. The cell-free labeling is useful also because only smaller amounts of labeled amino acids are required, and because the labeled amino acids can be in part recovered from the reaction mixture, as seen in the structural genomics project at the Center for Eukaryotic Structural Genomics (CESG) in Wisconsin University [44,45].

The wheat cell-free method has been found to be quite useful for amino acid-specific stable-isotope labeling that should accelerate 3D-structure determination. The amino acid-specific labeling technology in general may suffer from "scrambling" of the isotope due to metabolic reactions that simultaneously occur with the protein synthesis reaction. In the case of the wheat cell-free translation system, only a few inhibitors were found to be sufficient for eliminating the effects of such metabolic reactions [46-48]. Therefore, the wheat cell-free method is expected to be also useful for labeling with "SAIL" amino acids that should increase the size limit of NMR protein structure determination from around 20 kDa to 40 kDa [45,49].

The wheat cell-free method is also useful for protein structure determination through X-ray crystallography [22]. Selenomethionine labeling for this purpose is quite easy in the cell-free method: it is achieved just by substituting Met with selenomethionine. The buffer in the extract can be exchanged by simply passing through a Sephadex G-25 column.

Production of membrane proteins may be one of the major technologies in structural biology, protein engineering and drug discovery. Conventionally canine microsomal fraction has been used for *in vitro* production of membrane proteins and glycosylation. However, the microsomal fraction contains many degrading enzymes, and can be used for only limited purposes. It has been demonstrated that by adding a detergent and liposomes prepared from soybean asolectin to the wheat cell-free translation system, a membrane transporter can be produced in an active form on the liposome membrane [50].

Disadvantages

Cell-free methods are not very good at producing proteins with disulfide bonds. This is because the reaction mixture should contain a sufficient amount of reducing agent, such as DTT. We have shown that a single-chain variable fragment with two disulfide bonds can be made using a minimal amount of DTT in the reaction mixture, though the productivity is not very high [51]. This technology needs much more improvement, which may include regulation of reducing potential and introduction of membrane technologies.

The major disadvantage of the wheat cell-free protein synthesis system is the cost of the extract [45]. This is because the extract is prepared from wheat embryo particles selected by human eyes. This process cannot be compromised, as it ensures the quality of the extract. Another possible point is due to the presence of unknown components and mRNA degrading enzymes in the extracts. Such problems are minimal in the PURE system from *E. coli* (see the next

paper in this volume; sold by Wako Pure Chemical Industries, Ltd., Osaka, and New England Biolabs).

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Evaluating the Role of Rheumatoid Factors for the Development of Rheumatoid Arthritis in a Mouse Model with a Newly Established ELISA System

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Enzyme-linked immunosorbent assays (ELISA) have been widely used to determine quantitatively autoantibodies. However, the processes for the purification and immobilization of antigens in conventional ELISA methods include multiple steps, which have hampered the application for screening of autoantibodies. Here, we have developed a novel ELISA system using the plates pre-coated with glutathione casein to capture recombinant proteins fused to N-terminal glutathione S-transferase (GST). The GST-fused proteins were synthesized with the wheat germ cell-free protein production system. Thus, the present system combined the GST-capture ELISA with the cell-free protein production system, which allowed immobilization of the recombinant proteins with one-step purification. Using this ELISA method, we determined whether rheumatoid factors (RF), which have been considered as one of the representative disease – specific autoantibodies for rheumatoid arthritis (RA), were genetically associated with severity of arthritis in a mouse model for RA, MRL/Mp-*lpr/lpr* (MRL/lpr). GST-fused human IgG1-Fc (GST-Fc), synthesized with the robotic protein synthesizer, were used as reactants for RF. Serum samples for RF were prepared from 11 lines of a recombinant inbred mouse strain, MXH/lpr, which was established from intercrosses between MRL/lpr and non-arthritic C3H/HeJ-*lpr/lpr* (C3H/lpr) strains, composed of a different genomic recombination derived from the parental strains in each line. A correlation of RF titers with the severity of the arthritis in these lines was not significant, indicating genetic dissociation of RF from arthritis and that RF is not necessarily required for the development of RA. The present method may provide high-throughput screening for determining the disease-specific autoantibodies in autoimmune diseases.

Keywords: MRL/lpr; recombinant inbred strain; MXH/lpr; GST; IgG-Fc
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Enzyme-linked immunosorbent assays (ELISA) have been widely used to determine quantitatively the reactant proteins for autoantibodies in various diseases involving autoimmunity and infection (Gawryl et al. 1986; Moore et al. 1986; Tan 1989; Valdés Veliz et al. 2003). However, conventional ELISA methods are not suitable for the wide screening of autoantibodies because various processes and conditions are necessary for the purification and immobilization of each protein.

The wheat germ cell-free protein production system was established in our laboratory, in which any proteins could be synthesized in the presence of the cDNA template by using wheat germ ribosome more effectively than in *E. coli*-based system (Madin et al. 2000; Sawasaki et al. 2002a), and made it possible to synthesize proteins in a high-throughput manner (Sawasaki et al. 2002b). In fact, almost 380 kinds of proteins could be automatically synthesized at once overnight based on cDNA templates by using the robotic protein synthesizer GenDecorder 1000A® (CellFree Sciences, Matsuyama, Japan). Among cell-free

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systems for protein synthesis, this wheat germ-based system is of special interest for its eukaryotic nature; it has the significant advantage of producing a large amount of eukaryotic multi-domain proteins in folded state (Endo and Sawasaki 2006; Goshima et al. 2008; Takai et al. 2010). Thus, this system may be suitable for high-throughput synthesis of any reactant proteins for autoantibodies. Moreover, when recombinant proteins fused to N-terminal glutathione S-transferase (GST) are prepared in this system and captured on glutathione-coated ELISA plates (Sehr et al. 2001), the multi-step processes for the purification and immobilization of each protein required in conventional ELISA methods may not be necessary.

This report describes the development of a capture ELISA system using synthesized proteins fused to GST in the wheat germ cell-free protein production system. This method allowed the simple immobilization and purification of the proteins in one-step on ELISA plates pre-coated with glutathione casein. Then, this system was used to measure the rheumatoid factor (RF) in a murine model for rheumatoid arthritis (RA), MRL/Mp-*lpr/lpr* (MRL/lpr) (Murphy and Roths 1978; Nose et al. 1989). This mouse strain spontaneously develops arthritis resembling RA associated with high titers of IgG-RF, supporting the concept that IgG RF is one of disease-specific autoantibodies in RA (Theofilopoulos and Dixon 1985). Our results may reveal that the RF activity does not genetically correlate with the severity of arthritis in this model.

Materials and Methods

IgG-Fc with GSTtag in cell free system

The Fc portion of human IgG1 (IgG-Fc) was prepared as RF reactant according to a cell-free protein synthesis system using wheat germ ribosomal RNA (Madin et al. 2000; Sawasaki et al. 2002a; Soga et al. 2009). In brief, the Fc portion of human IgG1 cDNA (Kitai et al. 1988) was inserted into a pEUE01-GST-N2 expression vector (CellFree Sciences, Matsuyama, Japan) containing a GST region and SP6 promotor. The GST-IgG-Fc proteins were automatically synthesized by the Robotic Protein Synthesizer Protemist® DT (CellFree Sciences) as previously described (Soga et al. 2009). Namely, 250 μl of transcription mixture containing 25 μg of the plasmid DNA, 80 mM HEPES-potassium hydroxide, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM dithiothreitol, 2.5 mM each of nucleoside triphosphates, 250 U of SP6 RNA polymerase (Promega, Madison, WI, USA) and 250 U of RNasin (Promega) was incubated for 6 hr at 37°C. After the incubation, the transcription solution containing transcribed mRNA was mixed with 250 μl of wheat germ extract WEPRO1240 (CellFree Sciences) supplemented with 2 ml of 20 mg/ml creatine kinase in a single well of a six-well plate. The 5.5 ml substrate mix (30 mM HEPES-potassium hydroxide, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM dithiothreitol, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP and 16 mM creatine phosphate; CellFree Sciences) was then added on top of the translation mix and then was incubated at 17°C for 20 hr. The reaction mixture was used without any purification procedures as IgG-Fc-GST to be captured on the ELISA plates. In some experiments, the IgG-Fc-GST in the mixture was purified by glutathione Sepharose 4B (GE Healthcare, Uppsala, Sweden) according to the instructions for use.

GST capture ELISA

GST capture ELISA was performed basically according to the previously described method (Sehr et al. 2001). In brief, to conjugate glutathione to casein, casein (Wako, Osaka, Japan) at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) was incubated at room temperature (RT) with 0.4 mM N-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO, USA) and then the single cysteine residue was blocked in casein. Next, sulfosuccinimidyl 4-[p-maleimidophenyl] butyrate (SSMBP; Pierce, Rockford, IL) was added at 4 mM as a cross-linker. Free SSMBP and NEM were separated from casein on PD10 columns (GE Healthcare). The protein fraction was then supplemented with 10 mM glutathione (Wako) and the coupling reaction was executed for 1 h at RT. The glutathione casein was separated from unbound glutathione by gel filtration with PD10, using PBS as buffer and stored at −20°C until use. Next, plastic plates, consisting of 96 wells (Thermo Labsystems, Franklin, MA), were coated overnight at 4°C with 100 μl/well of glutathione casein, 2 μg/ml in 50 mM carbonate buffer, pH 9.6. The optimal concentration of glutathione casein was determined in a titration study by using plates pre-coated with it in a various concentration (0.5 to 8 μg/ml). Thereafter, the wells were incubated for 1 h at 37°C with blocking buffer (0.2% casein in PBS, 0.05% Tween 20), followed by incubation for 1 h at 4°C with the reaction mixture (100 μl/well) of IgG-Fc-GST proteins synthesized in cell free system diluted in blocking buffer.

The coated ELISA plates were then incubated for 1 h with 100 μl/well of goat anti-human IgG-Fc antibodies conjugated with alkaline phosphatase (AP; Sigma-Aldrich) or diluted sample solution of mouse monoclonal antibodies and mouse sera. Bound antibodies of the later were detected by polyclonal goat anti-mouse immunoglobulin IgG (Sigma) or IgM (Zymed Laboratories Inc., San Francisco, CA), conjugated to AP, diluted 1/2000 in blocking buffer. In some experiments, the conventional ELISA using *E. coli.*-generated IgG-Fc with the same construct (Nose et al. 1990) which were purified by protein A column (GE Healthcare) was performed in the same procedure. Commercialized glutathione-coated ELISA plates for GST-proteins, which are pre-coupled with glutathione via polyethylene glycol as a spacer, were reacted overnight at 4°C with GST or IgG-Fc-GST proteins synthesized in cell free system according to the manufacturer instructions.

Mouse sera

Serum samples for RF were prepared from MRL/MpJ-*lpr/lpr* (MRL/lpr) and C3H/HeJ-*lpr/lpr* (C3H/lpr) mice and 11 lines of a recombinant inbred (RI) strain of mice, MXH/lpr, established by intercrosses between an MRL/lpr and a C3H/lpr strain of mice. These lines had a different genome recombination each other derived from the parental strains in homozygote. Sera from C3H/HeJ mice without the *lpr* gene were used as normal control for RF. The parental strains of mice were originally obtained from Jackson Laboratory (Bar Harbor, ME). All of the mice used in this study were bred and housed in an animal facility in a pathogen-free and climate-controlled environment with 12 hr light/dark cycles. All experiments were done according to the Guidelines for the Care and Use of Laboratory Animals at Ehime University.

RF monoclonal antibodies

Hybridomas producing IgM-RF monoclonal antibodies (clones; Zao2 and Zao7) were prepared by the cell fusion of NS-1 myeloma cells with unmanipulated spleen cells from a 20 wk-old male C57BL/6-*lpr/lpr* mouse, which were originally obtained from the Jackson Laboratory (Bar Harbor), according to a method described elsewhere (Takahashi et al. 1993). The selection of IgM-RF-producing hybridoma cells was performed in conventional ELISA using *E. coli.*-generated IgG-Fc (Nose et al. 1996). Ant-TNP monoclonal IgM antibodies (a mouse hybridoma clone; Sp6) (Nose and Wigzell 1983) were used as a murine IgM control.

Histopathology of arthritis

The mice were killed under ether anesthesia, and their hind limbs were processed for histopathology. Whole ankle joints were fixed in 10% formalin in 0.001 M phosphate buffer, pH 7.2, decalcified in 10% formic acid, and embedded in paraffin. Serial sections 2-3-μm thick were taken sagittally through the talus and stained with hematoxylin and eosin for examinations by light microscopy. The lesions, including the calcaneus bone and anterior and posterior synovial tissue at the ankle joints, were evaluated histopathologically. To evaluate the severity of synovitis, the following grading was used: normal, grade 0; thickening and proliferation of synovial lining, grade 1; and grade 1 with granulomatous and/or fibrous lesions in synovial sublining tissue, grade 2 (Kamogawa et al. 2002). Pannus formation was categorized as follows: normal, grade 0, extending to bone cortex, grade 1; and further to bone marrow, grade 2. Each score of synovitis and pannus formation in the individual mice was represented as the maximum score in the bilateral ankles.

Statistical analysis

Student's *t*-test was applied to analyze the statistical significance of the results. A p value of < 0.05 was considered to be significant. Correlation between any two parameters of the severity of arthritis and RF activity; synoviti vs. pannus, IgG-RF vs. IgM-RF, IgG-RF vs. synovitis/pannus, and IgM-RF vs. synovitis/pannus, was estimated by the Spearman rank-correlation coefficient as indicated r.

Results

One-step capture of IgG-Fc-GST fusion proteins on the ELISA plates

Synthetic IgG-Fc-GST in the reaction mixture was successfully captured on ELISA plates coated with glutathione-conjugated casein. The reaction mixtures of GST or IgG-Fc-GST prepared in cell free system themselves showed multiple bands on PAGE as shown in Fig. 1A. However, each fraction obtained after reacting on and then eluting from the ELISA plates coated with glutathione-conjugated casein, but not with casein only, clearly showed a single band with the expected molecular sizes corresponding GST or GST-IgG-Fc, except a few extra bands with 22-25kD in molecular sizes derived from wheat germ extract (Fig. 1B).

The dose dependency of IgG-Fc capture on the glutathione casein-coated plates was measured by using anti-human IgG-Fc antibodies (Fig. 2). Synthetic IgG-Fc-GST in the reaction mixture was reacted with the glutathione casein-coated plates at a series of concentrations generated by serial dilution. The results showed the capture of IgG-Fc-GST to be saturated at concentrations of less than 1.0 μg/ml under this condition. The background coating only with glutathione casein was quite low and GST itself did not react at any concentrations.

Reactivity of monoclonal RF on the capture ELISA

Next, to confirm the specificity of RF reactivity of the

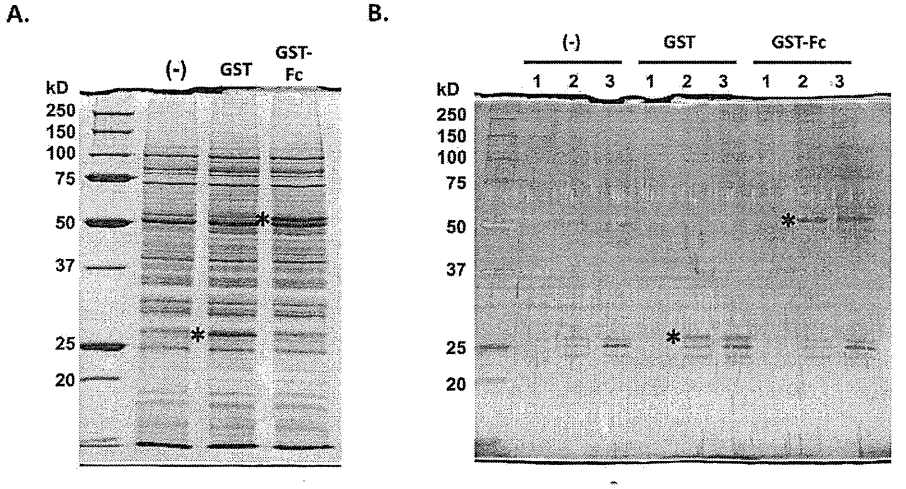

Fig. 1. Capture of the synthetic IgG-Fc-GST on glutathione casein-coated ELISA plates. A. The reaction mixture (0.5μl) of vector free (–), GST or IgG-Fc-GST was loaded on SDS-PAGE. B. The same mixture of samples (100μl) was loaded after reacting on and then eluting by using 10μl of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH8.0) from the ELISA plates coated with; casein only (lane 1) or glutathione-conjugated casein (10 mg/ml; lane 2, 1 mg/ml; lane 3). Each eluate (0.5μl) was loaded on SDS-PAGE. GST and IgG-Fc-GST seemed to be specifically bound to the glutathione casein-coated plates. Extra bands around 25 kDa seemed to be from wheat germ extract. Asterisk indicates the expected band on each lane.

capture ELISA plates, the reactivity of mouse monoclonal IgM RF, clones Zao2, Zao7 and Sp6 (see Materials and Methods) was compared with that of the conventional ELISA plates coated with *E. coli.*-generated IgG-Fc (Fig. 3). Both monoclonal IgM RF showed almost the same reactivity between the capture ELISA and the conventional ELISA systems. And, mouse IgM control showed no reactivity in both ELISA systems.

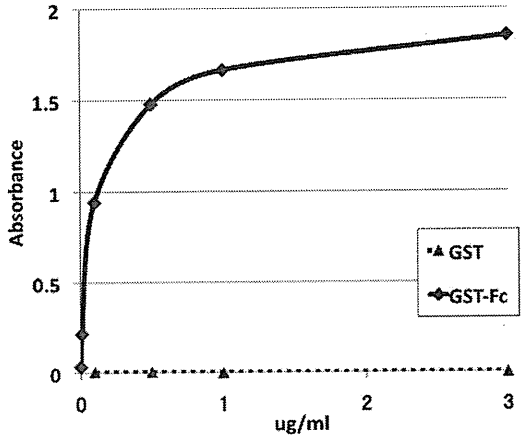

Fig. 2. Dose dependency of IgG-Fc to be captured on the glutathione casein-coated plates. IgG-Fc-GST in the reaction mixture was loaded on the glutathione casein coated plates. The binding of IgG-Fc-GST was measured by using AP-conjugated anti-human IgG-Fc antibodies. The capture of IgG-Fc-GST was in dose-dependent manner, and saturated at concentrations of less than 1.0 μg/ml. The coating background with glutathione casein was quite low and GST itself did not react at any concentration. Absorbance indicates the mean value of OD_{405}-OD_{510} in triplicate samples.

Measurement of RF activity of mouse sera

Finally, the IgG RF activity in mouse sera was compared between the arthritis-prone strain of mice MRL/lpr and the non-arthritic strain of mice C3H/lpr, both of which possess the Fas deletion mutant lpr (Nagata, 1994) (Fig. 4A). MRL/lpr mice showed a higher reactivity than C3H/lpr mice as previously reported (Theophilopoulus and Dixon 1985). And, sera from C3H/HeJ mice without the *lpr* gene showed no reactivity for IgG-Fc. At the same time, the results were compared with those obtained using commercialized GST-ELISA plates (Fig. 4B). Unexpectedly, GST-ELISA did not show any IgG-Fc specificity in both mouse sera.

Genetic dissociation of RF activity from the severity of arthritis in the MXH/lpr mice

The capture ELISA system was used to determine the correlations between the RF activity and the severity of arthritis in the recombinant inbred strains of mice, MXH/lpr. MXH/lpr mice were established by intercrosses between MRL/lpr and C3H/lpr strains of mice, among which genetic dissociation of arthritis might be generated due to genome recombination between the parental stains, based on a previous study of chromosomal mapping of the susceptibility loci to arthritis (Nose et al. 1989; Kamogawa et al. 2002). Expectedly, genetic dissociation of arthritis was observed among the MXH/lpr mice (Fig. 5); that is, several lines among the MXH/lpr mice showed a significantly higher score in the severity of synovitis (lines; 07, 10, 25 and 36) ($p < 0.05$ vs. C3H/lpr) and pannus formation (lines; 10, 25 and 36) ($p < 0.05$ vs. C3H/lpr) while others such as lines 51

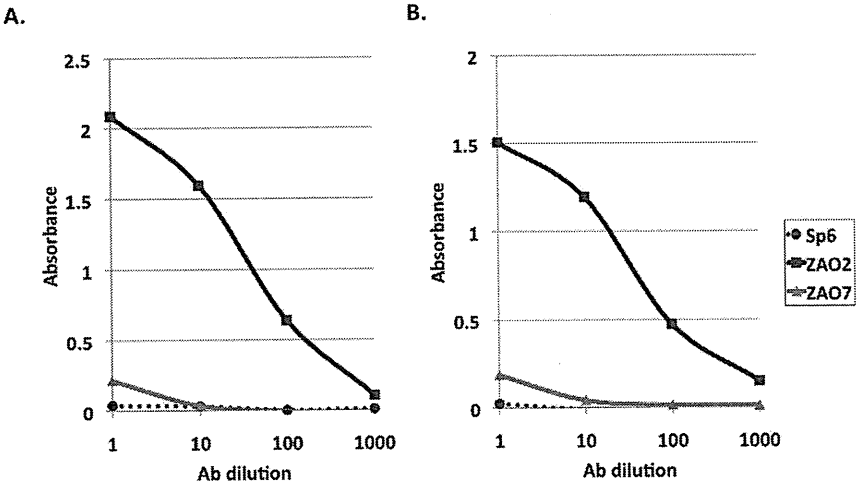

Fig. 3. Reactivity of monoclonal RF on the capture ELISA and the conventional ELISA systems. The culture supernatant of the hybridomas producing IgM monoclonal RF (clones; Zao2 and Zao7) and IgM monoclonal anti-TNP antibodies (clone; Sp6) was reacted on the capture ELISA (A) and the conventional ELISA (B) systems in a serial dilution. These antibodies showed almost the same reactivity between both ELISA systems. Absorbance indicates the mean value of OD_{405}-OD_{510} in triplicate samples.