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Activation of SIPK in response to UV-C irradiation: utility of a glutathione-S transferase–tagged plant MAP kinase by transient expression with agroinfiltration

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Abstract Salicylic acid (SA)-induced protein kinase (SIPK) in *Nicotiana tabacum* L. is activated in response to various stimuli. We assessed the response of SIPK in *N. benthamiana*. leaves to ultraviolet-C (UV-C) and SA stress. Infiltration of *Agrobacterium tumefaciens* EHA105 cells containing pBE2113-GST-SIPK into leaves gave transient production of glutathione-S transferase (GST)-fused SIPK. Irradiation of agroinfiltrated leaves with UV-C (254 nm) activated GST-SIPK and endogenous protein kinases with a molecular mass of about 45 kDa, close to that of SIPK simultaneously. These results suggest that SIPK is involved in stress signalling in *Nicotiana* plants under UV-C irradiation.

Key words: MAPK, UV irradiation, *Nicotiana*, *Agrobacterium tumefaciens*.

Salicylic acid (SA)-induced protein kinase (SIPK) is a mitogen-activated protein kinase (MAPK) in *Nicotiana tabacum* L. that is activated by multiple extracellular stresses (e.g., SA, H_2O_2, TMV infection; Zhang and Klessig 1997). To characterise stress-activated MAPKs, including SIPK, in higher plants, we used a transient expression assay based on *Agrobacterium*-mediated infiltration (agroinfiltration; Yang et al. 2000) of *N. benthamiana* leaves *in planta* with a binary plasmid encoding a glutathione-S transferase (GST)-fused SIPK. We used *N. benthamiana* in preference to *N. tabacum* because leaves of *N. benthamiana* are more easily agroinfiltrated with *Agrobacterium* suspension, and *N. benthamiana* showed more efficiency in expression levels of GST-SIPK after agroinfiltration than *N. tabacum* cv. Samsun and Xanthi-nc in preliminary experiments (data not shown).

We examined the effects of UV-A (>320 nm) and UV-C (<280 nm) irradiation on activity of SIPK. It is well known that UV-C induces DNA damage, imposing a genotoxic stress. Ulm et al. (2002) reported that in *Arabidopsis* a mutation of MAPK phosphatase, which would normally downregulate MAPK activity, produced a severe genotoxic-sensitive phenotype. In early studies of MAPK in mammals, UV irradiation of fibroblast cells stimulated Ras-Raf-MEK-ERK signalling (Engelberg et al. 1994) and c-Jun kinase cascades (Kyriakis et al. 1994), resulting in apoptosis. These observations suggest that intracellular signalling pathways of MAPKs and programmed cell death stimulated by DNA damage are highly conserved in eukaryotic organisms. However, the involvement of stress-activated MAPKs in *Nicotiana* species under UV irradiation and/or genotoxic stress remains to be clarified.

To make pBE2113-GST we used two vectors: pBE2113Not (Mitsuhara et al. 1996), which expresses polypeptides in higher plants after infiltration via *Agrobacterium tumefaciens* EHA105, and pEBG (Yuasa et al. 1998), which we used as a source of a GST open reading frame. An *Xba*I–*Xba*I fragment containing GST cDNA from pEBG was subcloned into an *Xba*I site of pBE2113Not. SIPK cDNA was amplified by polymerase chain reaction (PCR) from a cDNA library of tobacco BY-2 suspension-cultured cells with Vent DNA polymerase and a primer set (5′-CGC GGA TCC AAA ATG GAT GGT TCT GGT CAG CAG ACG GAC-3′ and 5′-GGG AAT TCA AAG CTT CAT ATG CTG GTA TTC AGG ATT AAA TGC-3′; Proligo USA Co.) for SIPK. The PCR product of SIPK was digested with *BamH*I. The fragment of the SIPK open reading frame was subcloned into *BamH*I–*Sma*I sites of pBE2113-GST to make pBE2113-GST-SIPK (Figure 1A).

Abbreviations: dpi, days after infiltration; GST, glutathione-S transferase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; SA, salicylic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SIPK, salicylic acid-induced protein kinase; TBS, Tris-buffered saline; UV, ultraviolet.

An agroinfiltration assay was performed according to the methods of Van de Hoorn et al. (2000) and Yang et al. (2000) with minor modification. *A. tumefaciens* EHA105 cells were grown overnight at 28°C in 10 ml of growth medium (10 g l^{-1} bactotryptone, 1 g l^{-1} yeast extract, 5 g l^{-1} sucrose, 2 mM $MgSO_4$, 10 mM MOPS-KOH [pH 7.0], 30 mg l^{-1} kanamycin, 0.25 mM acetosyringone). The culture was harvested by centrifugation at 3000×*g* for 15 min. The pellet was resuspended in 10 ml of MMS (10 mM MES-KOH [pH 5.5], 10 mM $MgSO_4$, 2% [w/v] sucrose, 0.25 mM acetosyringone) and then centrifuged at 3000×*g* for 15 min. Washing was repeated three times. Finally, OD_{600} was adjusted to 0.5 in MMS. The *Agrobacterium* suspension was infiltrated with a 1-ml syringe into *N. benthamiana* leaves pin-holed with a no. 23 needle, and then plants were covered with transparent plastic bags and incubated in a growth chamber at 25°C under 16-h light/8-h dark for 48 h.

Plants received one of three treatments. For UV-C treatment, plants were irradiated at 254 nm with a UV illuminator (Fluo link, Vilber Lourmat). For UV-A treatment, plants were irradiated at 320–380 nm with a UV illuminator (Stratagene). For SA treatment, leaves were placed in 10 mM MES-KOH buffer (pH 5.5) containing 0.5 mM SA for 10 min. At intervals after each treatment, the leaves were cut off and frozen in liquid nitrogen, and then stored at −80°C.

Frozen leaves were homogenised on ice in an equal weight of lysis buffer containing 20 mM HEPES-NaOH, 50 mM Na_3β-glycerophosphate (pH 7.6), 5 mM EDTA, 5 mM EGTA, 30 mM NaF, 5 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 0.1% β-mercaptoethanol, 1 mM PMSF, 5 mM *n*-aminocaproic acid, 1 mM benzamidine, and 1 mM Na-bisulfite. After centrifugation at 20,000×*g* for 20 min at 4°C, supernatant containing 250 μg protein was added to 30 μl of a slurry suspension of 50% (v/v) glutathione Sepharose CL-4B beads (Amersham Pharmacia LKB) equilibrated with Tris-buffered saline (TBS) for a pull-down assay. For alkaline treatment of GST-fusion proteins, 2 M Tris·base solution was added to the supernatant and glutathione beads to adjust the pH to 9.5, and the mixture was incubated on ice for 30 min. Then, 2 M Tris·HCl (pH 6.8) was added to the mixture to adjust the pH to 8.0. The suspension was rotated for 2 h at 4°C. The glutathione beads were precipitated by centrifugation at 10,000×*g* for 1 min at 4°C and were then washed in 1 ml of washing buffer containing 25 mM Tris·HCl (pH 7.4), 1 M NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Triton X-100, and 0.1% β-mercaptoethanol. After three washes, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the glutathione beads, and the mixture was incubated at 95°C for 3 min. After brief centrifugation, the resultant supernatant was subjected to SDS-PAGE for an in-gel protein kinase assay as described below. For immunoprecipitation assay, 0.5 μl of a slurry suspension of anti-GST antibody and 20 μl of 50% (v/v) protein A-Sepharose beads were added to the 20,000×*g* supernatant of the leaf extracts, and the mixture was rotated at 4°C for 2 h. After centrifugation, all other procedures were carried out as for the pull-down assay. Crude extracts (20 μg protein) were diluted in SDS-PAGE sample buffer without alkaline treatment, and then subject to SDS-PAGE.

An in-gel protein kinase assay was carried out as described by Kameshita and Fujisawa (1989) with a minor modification (Yuasa et al. 2001) in 10% polyacrylamide gels containing 0.2 mg ml^{-1} myelin basic protein (MBP) (Sigma, St. Louis, MO, USA). After the assay, radioactive signals of ^{32}P-phosphorylated polypeptides in the gels were visualised with a BAS2500 image plate scanner (Fuji Photo Film Co., Ltd).

Immunoblot analysis was carried out with anti-GST polyclonal antibody (Sigma) diluted to 1/5,000 (v/v), and then with 1/20,000 (v/v)-diluted horseradish peroxidase-conjugated anti-rabbit IgG goat polyclonal antibody in TBS containing 3% skim milk and 0.05% Tween 20. The immuno-decorated protein bands were visualised with an ECL Plus kit (Amersham Pharmacia LKB) and Kodak (Rochester, NY, USA) X-ray film.

Immunoblot analysis of leaf extracts with anti-GST antibody showed that the antibody cross-reacted with GST-SIPK with a relative molecular mass of 75 kDa, and with endogenous polypeptides (minor signals) (Figure. 1B, left), and with the precipitates bound to the glutathione beads (Figure 1B, right).

A profile of expression levels of GST-SIPK at various intervals by transient expression analysis is critical for studies of stress signalling in plants. Immunoblot analysis showed that expression levels of GST-SIPK gradually increased at 1, 2, and 3 days after infiltration (dpi), remained high between 3 and 7 dpi, but declined slightly at 10 dpi (Figure 1C). Minor signals appeared at lower molecular masses at 3 to 7 dpi, indicating that some proteolysis produces GST polypeptides. The difference in expression levels between 2 and 10 dpi was marginal when compared with that between 1 and 2 dpi. Accordingly, in the following experiments, we harvested leaves at 2 days (48 h) after agroinfiltration.

In immunoblot analysis, infiltration with *Agrobacterium* suspension at a density of 0.57 and 1.2 OD_{600} gave higher expression of GST-SIPK than at 0.14 and 1.9 OD_{600} in 2 independent experiments (Figure 1D). It is conceivable that too high density of *Agrobacterium* suspension on its own can stress plants, decreasing the expression of GST-fused polypeptides. Therefore, we adjusted the *Agrobacterium* suspension to 0.5 OD_{600} in the following agroinfiltration assays.

To analyse the activity of GST-SIPK specifically, one

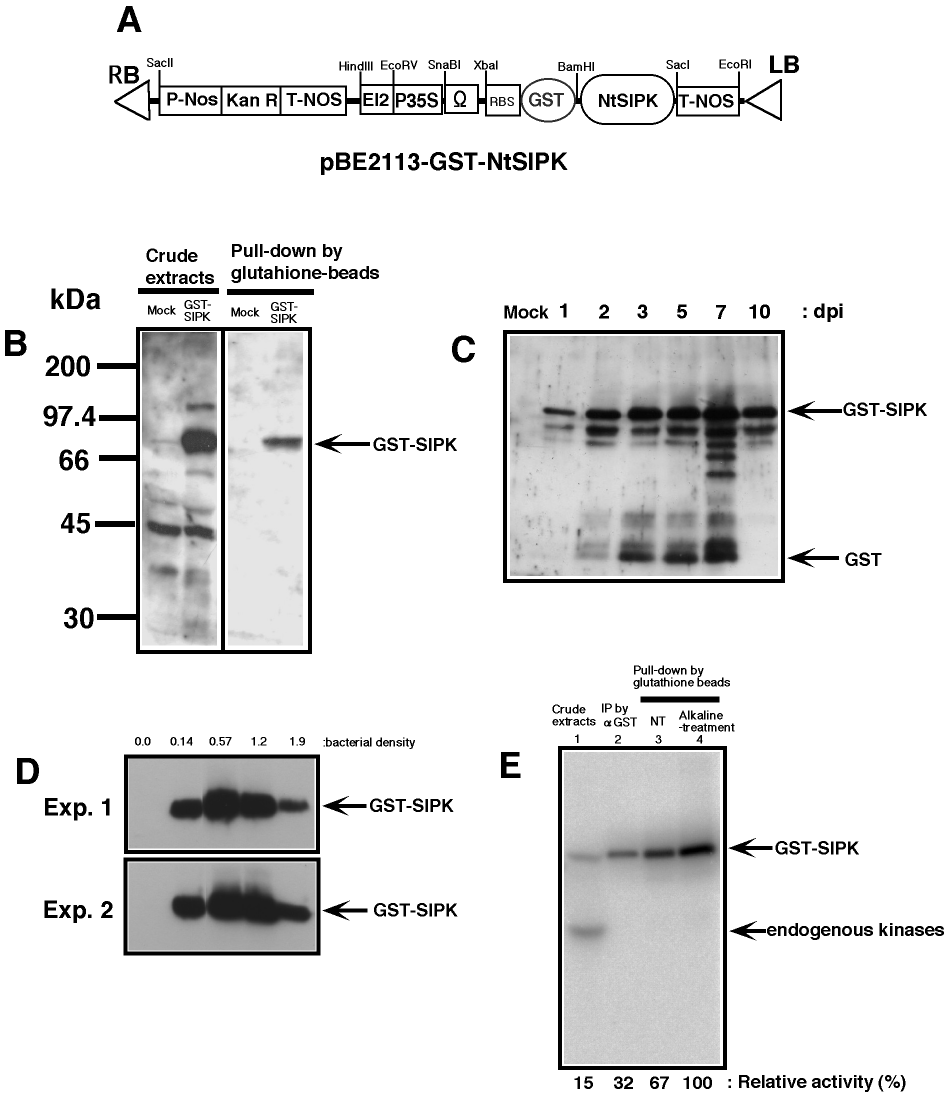

Figure 1. Transient expression of GST-SIPK in *Nicotiana benthamiana* leaves by agroinfiltration system.

(A) Schematic representation of the T-DNA region-derived binary plasmid for expression of GST-SIPK. (B) Immunoblot analysis of the leaf extracts with anti-GST antibody. *N. benthamiana* leaves were agroinfiltrated with *Agrobacterium* suspension containing pBE2113-GST-SIPK, and harvested at 48 h after agroinfiltration. GST-fusion proteins in crude extracts were purified using glutathione beads. The crude leaf extracts (left) and purified GST-fusion proteins bound on glutathione beads (right) were subject to immunoblot analysis using anti-GST-antibody. Immuno-detected signals of GST-SIPK are indicated by the arrow (about 75 kDa). (C) Time course of expression profiles of GST-SIPK in *N. benthamiana* leaves after agroinfiltration. Agroinfiltrated *N. benthamiana* leaves were harvested at the indicated days after agroinfiltration with *Agrobacterium*. (D) Expression of GST-SIPK on various densities of *Agrobacterium* suspensions (OD_{600}=0.0, 0.14, 0.57, 1.2, and 1.9) in leaves harvested at 48 h after agroinfiltration. (E) Pull-down assay of GST-SIPK with anti-GST antibody and glutathione beads. Lane 1, crude extracts of agroinfiltrated leaves (crude extracts); lane 2, immunoprecipitates by anti-GST antibody and protein A-Sepharose from crude extracts (IP by (GST); lane 3, pulled-down by glutathione beads from crude extract (no treatment, NT); lane 4, pulled-down by glutathione beads after alkaline treatment (alkaline treatment). *N. benthamiana* leaves were treated with salicylic acid (0.5 mM, 10 min) at 48 h after agroinfiltration. The extracts were immunoprecipitated with anti-GST antibody and protein A-Sepharose beads or pulled-down by glutathione beads after alkaline treatment, and then subjected to in-gel kinase assay. Immunodetection was done with anti-GST antibody and horseradish peroxidase-conjugated anti-rabbit IgG goat antibody. Six- to 8-week-old plants with four to six fully expanded leaves were agroinfiltrated.

would normally use an immunoprecipitation assay with a specific antibody (Figure 1E, lane 2) or a pull-down assay with glutathione beads. However, the conventional pull-down procedure with glutathione beads from leaf extracts without pre-treatment (NT) recovered less GST-SIPK (Figure 1E, lane 3). This lower recovery can be explained by the facts that higher plants accumulate glutathione and glutathione conjugates for anti-oxidant/redox mechanisms in the cytoplasm and vacuoles (Rea et al. 1998; Fricker and Meyer 2001), and that GST-fused polypeptides bind tightly to such endogenous glutathione and derivatives when leaf extracts are prepared. To improve the recovery of GST-fused polypeptides with glutathione beads in transient

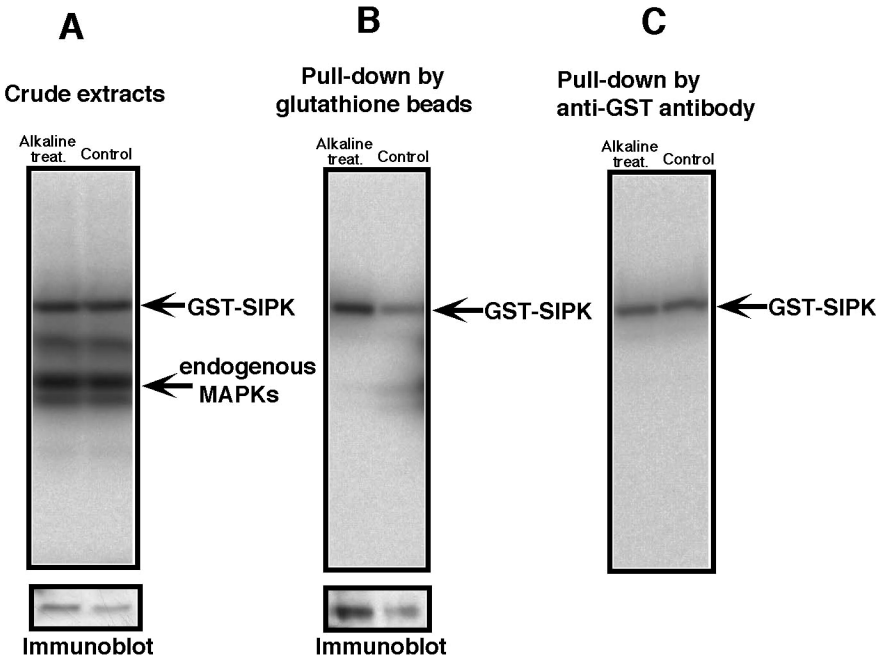

Figure 2. Effects of alkaline treatment on endogenous protein kinases and GST-SIPK.

(A) Activities of protein kinases in crude extracts. (B) Activities of GST-SIPK bound on glutathione beads. (C) Activities of GST-SIPK bound to anti-GST antibody and protein A Sepharose beads. *Nicotiana benthamiana* leaves were agroinfiltrated with *Agrobacterium* suspension containing pBE2113-GST-SIPK. At 48 h after agroinfiltration, plants were treated with salicylic acid (0.5 mM, 10 min). Alkaline treatment was carried out by adding 1/4 volume of 2 M Tris·base to samples, and incubating samples on ice for 5 min. The resultant sample was neutralised by adding the 1/5 volume of 2 M Tris-Cl (pH 6.0) to the alkaline-treated samples (left lanes). Control samples received 2 M Tris-Cl (pH 8.0) and were then incubated on ice for 5 min (right lanes). Amounts of GST-SIPK in crude extracts (A, lower) and samples pulled-down with glutathione beads (B, lower) were confirmed by immunoblot analysis with anti-GST antibody. In-gel kinase assay used MBP-gel.

expression assay, we transiently increased the pH of the leaf extracts before incubation with the glutathione beads, because high pH enhances the rate of exchange of glutathione between solution and GST (Ortiz-Salmer et al. 2001). The in-gel kinase assay showed that the recovery of GST-SIPK by glutathione beads was significantly improved by alkaline treatment (Figure 1E, lane 4) when compared with the conventional pull-down assay by glutathione beads (lane 3) and immunoprecipitation by anti-GST antibody (lane 2).

Furthermore, immunoblot analysis also showed that the immuno-detected signal of GST-SIPK bound on glutathione beads (Figure 2B, lower, left lane) was stronger than that of a control sample (lower, right lane), as the in-gel kinase assay showed (Figure 2B, upper). Alkaline treatment had little effect on protein kinase activity in crude extracts (Figure 2A) or in immunoprecipitates (Figure 2C). Therefore, we used alkaline treatment and pull-down assay with glutathione beads to detect the activity of GST-SIPK in leaves. Because GST has a relatively large molecular mass compared with those of two other epitope tags, Flag (Zhang and Liu 2001) and Hisx6 (Yang et al. 2001), signals of GST-tagged SIPK and endogenous MAPK-like stress-activated kinases in in-gel kinase assay were easily distinguished by their relative molecular masses (Figures 1E, 2B, C). Therefore, GST-SIPK and endogenous MAPK-like protein kinases can be distinguished by in-gel kinase assay even with crude extracts.

We examined whether the system of transient expression of GST-SIPK in *N. benthamiana* by agroinfiltration can be used to study plant stress signalling and whether ectopically expressed GST-SIPK and endogenous stress-induced MAPKs are activated in parallel under stress treatments. *N. benthamiana* leaves expressing GST-SIPK were treated with SA (Figure 3). In-gel kinase assay of crude extracts showed that activities of the SA-stimulated endogenous 45-kDa kinase and GST-SIPK were strongly stimulated at 10 min after SA treatment, but declined slightly at 30 min (Figure 3A). In-gel kinase assay of pull-down samples showed that the activity of glutathione-bound GST-SIPK altered in parallel with the signals in the crude extract (Figure 3B). These results suggest that the ectopically expressed GST-SIPK was stimulated in parallel with the endogenous 45-kDa MAPK.

Next, we examined the activation of GST-SIPK and the endogenous 45-kDa MAPK after UV-C irradiation. To minimise the effect of overexpression of ectopically expressed GST-SIPK on *N. benthamiana*, we used a low-density *Agrobacterium* suspension (OD_{600}=0.15). In-gel kinase assay indicated that GST-SIPK (Figure 4A) and

endogenous 45-kDa kinase (Figure 4B) were rapidly activated at 2 min after UV-C irradiation, and that activation of both kinases peaked at 5 min (lane 5) and then remained high from 5 to 90 min (lane 12). The activation profiles were almost parallel.

This study demonstrated that agroinfiltration-based transient expression of epitope-tagged protein kinases is a useful and convenient method for studies of signal transduction in higher plants. In a transgenic tobacco expressing Flag epitope-tagged SIPK, the tagged SIPK and the endogenous SIPK were similarly activated in response to various stresses (Liu et al. 2003). When GST-SIPK was transiently expressed, 0.5 mM SA treatment stimulated the endogenous 45-kDa kinase and the ectopically expressed GST-SIPK (Figures 3A, B; 4B, lane 2). Therefore, it is reasonable to assume that the responses of ectopically expressed GST-SIPK reflect those of the endogenous stress-activated MAPKs, such as SIPK. This suggests that by transient expression with agroinfiltration we can analyse ectopically expressed signalling molecules that reflect activities of related endogenous signalling molecules in intact leaves.

UV-C irradiation causes oxygen-independent damage to DNA, RNA, and proteins (Iordanov and Magun 1999). When *Arabidopsis* T-87 suspension-cultured cells were irradiated with UV-A and UV-C, only UV-C irradiation stimulated activity of ATMPK6, a SIPK ortholog in *Arabidopsis* (Yuasa, T., unpublished data). In addition, UV-C-induced DNA damage and genotoxic reagents

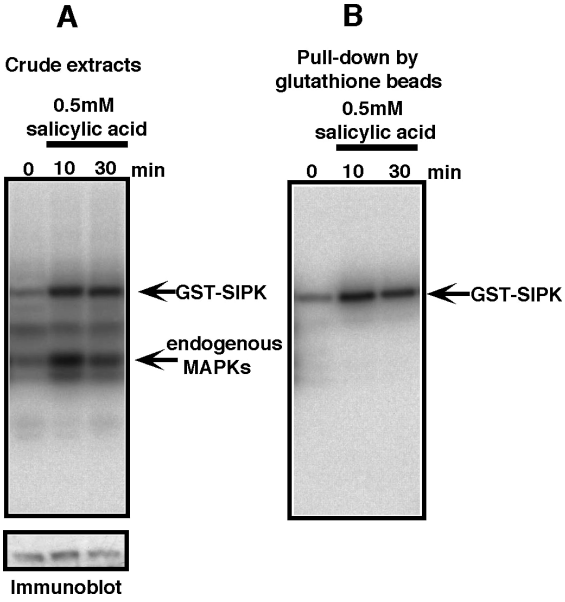

Figure 3. Activation of endogenous 45-kDa kinase and GST-SIPK in *Nicotiana benthamiana* by salicylic acid treatment.

(A) Activities of protein kinases in the crude extracts. (B) Activities of GST-SIPK bound on glutathione beads and (lower) immunoblot analysis. *N. benthamiana* leaves were agroinfiltrated with *Agrobacterium* suspension containing pBE2113-GST-SIPK. At 48 h after agroinfiltration, plants were treated with salicylic acid (0.5 mM). The leaves were harvested at 0 (control), 10, or 30 min after stress treatment. The extracts were subjected to glutathione pull-down assay after alkaline treatment. Expression of GST-SIPK was confirmed by immunoblot analysis with anti-GST antibody. In-gel kinase assay used MBP-gel.

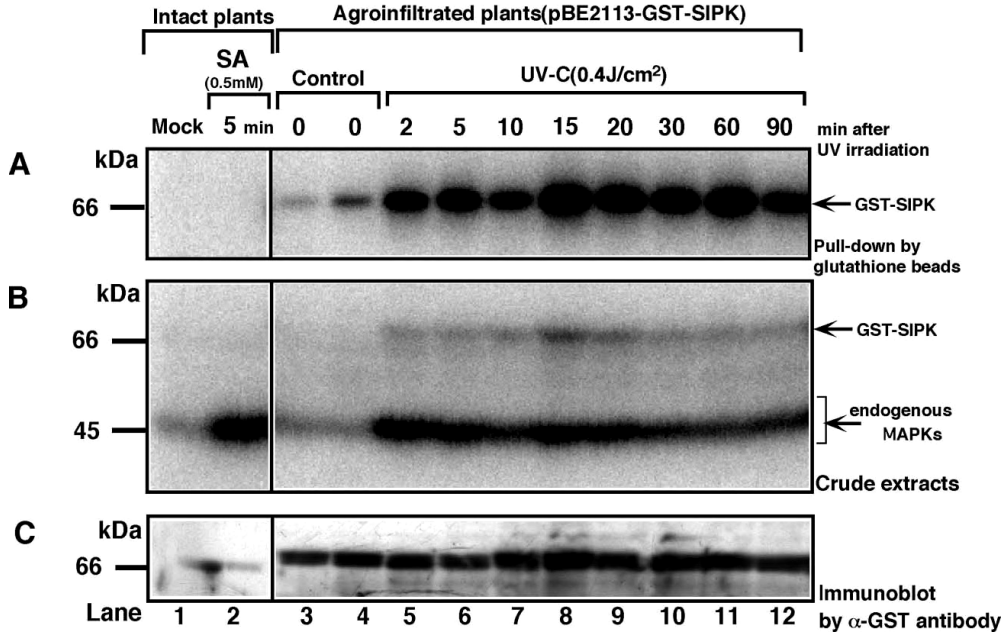

Figure 4. Profile of activation of GST-SIPK after UV irradiation.

(A) Activities of GST-SIPK bound on glutathione beads. (B) Activities of protein kinases in the crude extracts. (C) Immunoblot analysis. *Nicotiana benthamiana* leaves were agroinfiltrated with *Agrobacterium* suspension containing pBE2113-GST-SIPK (lanes 3–12). At 48 h after agroinfiltration, intact plants were treated with salicylic acid (0.5 mM, 5 min) (lane 2). Leaves were UV-C irradiated (0.4 J cm^{-2}) (lanes 5–12). The leaves were harvested at 2, 5, 10, 15, 20, 30, 60, or 90 min after stress treatment. The extracts were subjected to glutathione pull-down assay after alkaline treatment. Expression of GST-SIPK was confirmed by immunoblot analysis. In-gel kinase assay used MBP-gel.

activated ATMPK6 in *Arabidopsis* leaves (Ulm et al. 2003). The effect of UV-C on ATMPK6 activity is consistent with our observation that only UV-C stimulates SIPK. Therefore, it is reasonable to assume that activation of SIPK in agroinfiltrated leaves under UV-C irradiation results from UV-induced DNA damage.

We showed recently that ATMPK6 in *Arabidopsis* suspension-cultured T-87 cells is activated by reactive oxygen species (ROS; i.e., H_2O_2 and O_2^-) and that photo-oxidative stress by methylviologen results in stimulation of ATMPK6 under light in *Arabidopsis* leaves but not in T-87 cells (Yuasa et al. 2001). SIPK is also activated by ROS (Samuel et al. 2000). Therefore, it is conceivable that UV-induced damage to intracellular macromolecules and ROS-induced stress are partly sensed in a common intracellular signalling pathway in *Arabidopsis* and *Nicotiana* plants, leading to activation of stress-activated MAPKs.

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