Phytosulfokine (PSK), an endogenous 5-amino-acid-secreted peptide in plants, affects cellular potential for growth via binding to PSKR1, a member of the leucine-rich repeat receptor kinase (LRR-RK) family. PSK interacts with PSKR1 in a highly specific manner with a nanomolar dissociation constant. How-

er ever, it is not known which residues in the PSKR1 extracellular domain constitute the ligand binding pocket. Here, we have identified the PSK binding domain of carrot PSKR1 (DcPSKR1) by photoaffinity labeling. We cross-linked the photoactivatable PSK analog \([^{125}\text{I}]\text{-N}^\bullet\text{-}(4\text{-azidosalicyl})\text{Lys}^5\text{PSK}\) with DcPSKR1 using UV irradiation and mapped the cross-linked region using chemical and enzymatic fragmentation. We also established a novel “on-column photoaffinity labeling” methodology that allows repeated incorporation of the photoaffinity label to increase the efficiency of the photoaffinity cross-linking reactions. We purified a labeled DcPSKR1 tryptic fragment using anti-PSK antibodies and identified a peptide fragment that corresponds to the 15-amino-acid Glu\(^{503}\)-Lys\(^{517}\) region of DcPSKR1 by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Deletion of Glu\(^{503}\)-Lys\(^{517}\) completely abolishes the ligand binding activity of DcPSKR1. This region is in the island domain flanked by extracellular LRRs, indicating that this domain forms a ligand binding pocket that directly interacts with PSK.

Cell-to-cell communication is essential for growth and development of multicellular organisms throughout their life. In plants, hormones, including small lipophilic compounds and secreted peptides, comprise a large group of signaling molecules that are central to intercellular communication. They elicit biological activity by binding to cell surface receptors that have kinase activity or by directly interacting with intracellular proteins.

Phytosulfokine (PSK)\(^2\) is a 5-amino-acid-secreted peptide that has been identified in the medium of plant cell cultures, based on the results of assays of the growth-promoting activity of cultured cells (1). The addition of chemically synthesized PSK to culture medium, even at nanomolar concentrations, significantly promotes the proliferation of callus and suspension cells. PSK also promotes tracheary element differentiation (2), somatic embryogenesis (3, 4), adventitious bud formation (5), adventitious root formation (6), and pollen germination in vitro (7). PSK is produced from \(\approx\)80-amino-acid precursor peptides via post-translational sulfation of tyrosine residues and proteolytic processing (8). Genes encoding PSK precursors are redundantly distributed in the genome and are expressed in cultured cells and in a variety of tissues, including leaves, stems, flowers, and roots (9, 10).

PSK binds the membrane-localized PSK receptor PSKR1, which is a leucine-rich repeat receptor kinase (LRR-RK) that has been purified from solubilized carrot microsomes by ligand-based affinity chromatography (hereafter referred to as DcPSKR1) (11). The extracellular domain of DcPSKR1 contains 21 tandem copies of LRR interrupted by a 36-amino-acid island domain rich in hydrophilic and charged amino acid residues. Disruption or overexpression of the Arabidopsis ortholog of PSKR1 (AtPSKR1) significantly alters cellular longevity and potential for growth without interfering with primary morphogenesis of plants (10). PSK appears to activate the basic potential for cellular growth rather than directly determining cell fate and thereby exerts a pleiotropic effect on individual cells in response to environmental hormonal conditions.

Ligand binding generally causes a receptor protein to undergo a conformational change that directly activates the receptor so that it can interact with another cellular molecule and/or exert intrinsic enzyme activities such as kinase activity. PSK interacts with DcPSKR1 in a highly specific manner, with a high affinity dissociation constant of \(K_D = 4.2 \text{ nm}\) (11). However, it is not known which amino acids in the DcPSKR1 extracellular domain constitute the ligand binding pocket.

Photoaffinity labeling is one of the most useful methods for analyzing ligand-receptor interactions. Identification of the labeled amino acid residues can yield valuable information about the ligand binding domain of the receptor. However, it is often quite difficult to identify the cross-linked residues by MS analysis due to the low efficiency of cross-linking reactions. These difficulties are further compounded by low concentrations of binding proteins, especially in the case of transmembrane receptors and channels that cannot be functionally over-expressed in bacteria. In fact, most known cross-linked regions of membrane-localized proteins have been identified by analyzing chemical and enzymatic fragmentation patterns of labeled
proteins using radioactive photoaffinity ligands rather than direct MS analysis of purified peptide fragments cross-linked with photoaffinity ligands (12–14).

In the present paper, we have reported identification of the PSK binding site of DcPSKR1 by SDS-PAGE mapping of the cross-linked fragments generated by chemical and enzymatic fragmentation of the photoaffinity-labeled ligand-receptor complex and by direct analysis of the purified cross-linked fragments by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). We have also reported the usefulness of a novel solid-phase photoaffinity labeling technology, “on-column photoaffinity labeling,” which allows repeated incorporation of a photoaffinity label to increase the efficiency of the photoaffinity cross-linking reactions.

**EXPERIMENTAL PROCEDURES**

**Vector Construction and Transformation of Tobacco BY-2 Cells**—For construction of plasmids pBI121-DcPSKR1-His6 and pBI121-DcPSKR1-ΔKD-His6, PCR was performed using primers PSKR-5f (5′-GCTctagaATTTGCTTGGTTTGGTGG-3′) and PSKR-3r (5′-CTAGTTGGTGTGTGGTTGTTG-3′) for DcPSKR1-His6, using primers PSKR-5f and PSKR-3r (5′-CTAGTTGGTGTGTGGTTGTTG-3′) for DcPSKR1-ΔKD-His6, (His6 coding regions are underlined; restriction sites are in lowercase), and using DcPSKR1 cDNA as a template. The amplified fragments were digested with XbaI and inserted into the binary vector pBI121, which had been digested with XbaI and SalI (blunted). For the construction of plasmid pBI121-DcPSKR1-ΔID[Glu503-Lys517], the PCR-ligation-PCR method was used (15). Two independent PCRs were performed 1) using primers PSKR-5f and DcR1ID-3d (5′-GGAGACAAGGCTCTGTAAACTGG-3′) and 2) using primers DcR1ID-5e (5′-AAAGACGGAATGTTCCCCAGG-3′) and PSKR-3p (5′-AATGACAGGCAATGCTACTGAC-3′). The two PCR products were then phosphorylated and blunt-ligated. A second series of PCRs were performed to amplify the ligated DcPSKR1-ΔID[Glu503-Lys517] using primers PSKR-5f and PSKR-3p. Finally, the product was purified, digested with XbaI, and inserted into the binary vector pBI121, which had been digested with XbaI and SalI (blunted). For construction of pBI121-DcPSKR1-ΔID[Lys518-Ile538], two independent PCRs were performed 1) using primers PSKR-5f and DcR1ID-3d (5′-TTTTTCTTTGAAAAATGGAAAATCGCTG-3′) and 2) using primers DcR1ID-5d (5′-ATAGACGTTATTAATATCCCGG-3′) and PSKR-3p. The PCR products were phosphorylated, blunt-ligated, and subjected to a second PCR using primers PSKR-5f and PSKR-3p. The products were purified, digested with XbaI, and inserted into the binary vector pBI121. The resultant recombinant plasmids were used to transform *Agrobacterium tumefaciens* strain C58C1 (mpMP90) using electroporation. Tobacco BY-2 cells were co-cultivated with *Agrobacterium* for 2 days, and the resultant transformed BY-2 cells were selected on modified MS agar medium containing 200 mg/liter kanamycin and 500 mg/liter carbenicillin for 3–4 weeks until transformed cells were formed (16). Selected cell lines were transferred into MS liquid medium containing 100 mg/liter kanamycin to initiate suspension culture and were used for subsequent analysis. Transgenic BY-2 cells were maintained in both liquid and solid culture by subculturing once a week.

**Ligand Binding Assay**—The ligand binding assay of plant microsomal fractions and affinity-purified DcPSKR1 was performed using [3H]PSK and following a protocol described previously (11, 17).

**Affinity Purification of DcPSKR1-ΔKD-His6**—Transformed BY-2 microsomal membranes (1,800 mg of protein prepared from 6-day-old culture) were solubilized in 320 ml of buffer containing 20 mM HEPES-KOH (pH 7.5), 50 mM KCl, and 1% Triton X-100. Solubilized materials were centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatants were applied to a [Lys5]PSK-Sepharose column (5.0-ml column) at a flow rate of 0.5 ml/min using the AKTA prime chromatography system (Amersham Biosciences) (11). After washing with 50 ml of buffer containing 20 mM HEPES-KOH (pH 7.5), 50 mM KCl, and 0.1% Triton X-100 (wash buffer), the column was eluted with 15 ml of buffer containing 20 mM HEPES-KOH (pH 7.5), 500 mM KCl, and 0.1% Triton X-100 (elution buffer). The eluates were added to a 1.0-ml Macro-Prep ceramic hydroxyapatite Type I column (Bio-Rad) at a flow rate of 0.5 ml/min at 4 °C. The column was washed with 20 ml of wash buffer and eluted with an 18-ml gradient of KH2PO4 (0–400 mM) in wash buffer. Aliquots of active fractions (1.0 ml), as determined by [3H]PSK binding assay, were concentrated by ultrafiltration (Ultrafree-MC MWCO 30,000; Millipore) and analyzed by SDS-PAGE using 7.5% gels for confirmation of purity. Protein bands in SDS-polyacrylamide gels were visualized using the fluorescent dye SYPRO Red (Molecular Probes). The immobiloblot of the active fraction (20 μl) was probed with anti-DcPSKR1 antibodies raised against the DcPSKR1 100-amino-acid N-terminal region (11) and was visualized using ECL Advance (Amersham Biosciences) according to the manufacturer’s protocol.

**PNGase F Treatment of Purified DcPSKR1-ΔKD-His6**—Affinity-purified DcPSKR1-ΔKD-His6 (10 μl) was added to PNGase F buffer containing 100 mM Tris-HCl buffer (pH 8.6) and 0.1% SDS and was incubated at 95 °C for 3 min. The denatured sample was then incubated with 2 milliliters of peptide N-glycosidase F (PNGase F; Takara Bio, Shiga, Japan) in the presence of Nonidet P-40 (1.0% final solution) at 37 °C for 16 h at a total volume of 25 μl. After deglycosylation, samples were mixed with SDS-PAGE sample buffer and were then analyzed by SDS-PAGE and immunoblotting, as described above.

**Preparation of Photoactivatable Analog of PSK**—Preparation of the photoactivatable PSK analog [N*- (4-azidosalicyl)Lys5]PSK (ASA-PSK) and radioactive [125I]ASA-PSK was performed as previously described (18). The specific radioactivity of [125I]ASA-PSK was estimated to be 210 Ci/mmol.

**In-solution Photoaffinity Labeling**—Aliquots of purified DcPSKR1-ΔKD-His6 (10 μl) were incubated with 1 μM [125I]ASA-PSK for 10 min at 4 °C. Thereafter, the samples were irradiated with a UV lamp (model ENF-260C/1, 365 nm; Spectronics Co., Ltd., NY) for 10 min on ice at a distance of <1 cm. SDS-PAGE sample buffer was added to each of the samples, which were then heated at 95 °C for 5 min. The samples were loaded onto NuPage 12% BisTris gel (Invitrogen) and separated

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**Ligand Binding Site of Phytosulfokine Receptor**

JOURNAL OF BIOLOGICAL CHEMISTRY

JANUARY 5, 2007 • VOLUME 282 • NUMBER 1
according to the manufacturer’s protocol. The dried gels were exposed to a bio-imaging plate (Fujifilm, Tokyo, Japan) for 16 h at room temperature and were analyzed using a bio-imaging analyzer (BAS 2000, Fujifilm).

**Chemical Cleavage and Protease Digestion**—[\(^{125}\)I]ASA-PSK-labeled DcPSKR1-\(\Delta\)KD-His\(_6\), with or without PNGase F treatment, was used for chemical cleavage and protease digestion. Cyanogen bromide (CNBr) cleavage of cross-linked proteins (100 \(\mu\)l) was performed in 75\% formic acid containing 0.5 mg/ml CNBr under a nitrogen atmosphere. After this digestion was incubated for 16 h at room temperature, it was diluted 10-fold with distilled water and lyophilized. For trypsin digestion, 10 \(\mu\)l of labeled DcPSKR1-\(\Delta\)KD-His\(_6\) was treated with 100 pmol of TPCK-treated trypsin (Sigma) in 50 mM endoproteinase Asp-N (TakaraBio, Shiga, Japan) in 50 mM Tris-HCl (pH 7.6), 10 mM CaCl\(_2\), and 10\% CH\(_3\)CN in a total volume and was incubated for 16 h at a total volume of 20 \(\mu\)l. For Asp-N digestion, 10 \(\mu\)l of labeled DcPSKR1-\(\Delta\)KD-His\(_6\) was treated with 100 pmol of endoproteinase Asp-N (TakaraBio, Shiga, Japan) in 50 mM sodium phosphate buffer (pH 8.0) at 37 °C for 16 h at a total volume of 20 \(\mu\)l. All of these samples were analyzed by SDS-PAGE using the NuPage 12% BisTris gel and autoradiography.

**On-column Photoaffinity Labeling and Trypsin Digestion**—Affinity-purified DcPSKR1-\(\Delta\)KD-His\(_6\) (9 ml) was immobilized on Ni\(^{2+}\)-loaded HiTrap chelating HP-Sepharose (bed volume 200 \(\mu\)l; Amersham Biosciences) by recycling three times. After the column was washed with 1 ml of the above-described wash buffer, 1 ml of 10 \(\mu\)l [\(^{125}\)I]ASA-PSK or 1 \(\mu\)M cold ASA-PSK (dissolved in the wash buffer) was loaded onto the column, which was then left to stand for 5 min for ligand binding. After the column was washed with 600 \(\mu\)l of the wash buffer to remove unbound ligand, the bound ligand was cross-linked to DcPSKR1-\(\Delta\)KD-His\(_6\) by directly irradiating the column with UV light (365 nm) for 10 min. Urease-modified ligand was removed by washing the column with 1 ml of the wash buffer, and the labeling cycle was then repeated up to 3 times for [\(^{125}\)I]ASA-PSK and 10 times for ASA-PSK. For trypsin digestion, labeled DcPSKR1-\(\Delta\)KD-His\(_6\) on Sepharose was suspended in 200 \(\mu\)l of digestion buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM CaCl\(_2\), and 10\% CH\(_3\)CN in a total volume and was then digested by 100 pmol of TPCK-treated trypsin (Sigma) at 37 °C for 16 h. Sepharose was then removed by filtration, and liberated peptides were used for the following analysis.

**Gel Filtration of the Labeled Tryptic Peptides**—Gel filtration analysis was performed using Sephadex G-25 Superfine (1.5 cm inner diameter \(\times\) 35 cm; Amersham Biosciences) at a flow rate of 0.2 ml/min. Column equilibration and chromatography were performed using buffer containing 20 mM HEPES-KOH (pH 7.0) and 150 mM NaCl. On-column tryptic digests of [\(^{125}\)I]ASA-PSK-labeled DcPSKR1-\(\Delta\)KD-His\(_6\) (200,000 counts/min eq.) were applied to the column, and 1.0-ml fractions were analyzed for radioactivity using an autowell \(\gamma\) system (ALOKA). Blue dextran (Amersham Biosciences) and cyanocobalamin were used for molecular mass calibration.

**Immunopurification of the Labeled Tryptic Peptides**—For the purification of the anti-PSK antibodies, anti-PSK antiserum (5 ml) (19) was loaded onto a [Lys\(^{5}\)]PSK-Sepharose column (1.0-ml column). After the antiserum was recycled three times, the column was washed with phosphate-buffered saline, and the bound antibodies were then eluted with 0.1\% formic acid (pH 2.6) and immediately neutralized with NaHCO\(_3\). Purified antibodies were coupled with HiTrap NHS-activated HP-Sepharose (Amersham Biosciences) for 16 h at 4 °C. The immobilized anti-PSK antibodies were stored in phosphate-buffered saline until use. On-column tryptic digests of ASA-PSK-labeled DcPSKR1-\(\Delta\)KD-His\(_6\) were incubated at 95 °C for 10 min to inactivate trypsin and were then loaded onto the immunopurification column (20-\(\mu\)l column). After the column was washed with 200 \(\mu\)l of the trypsin digestion buffer and 200 \(\mu\)l of distilled water, bound fragments were eluted with 1\% trifluoroacetic acid (v/v). The eluate was used for MALDI-TOF MS analysis.

**MALDI-TOF MS Analysis**—The eluate from the immunopurification column was concentrated by evaporation, desalted using Zip Tip C18™ pipette tips (Millipore), and mass-profiled with a 4700 proteomics analyzer (Applied Biosystems) using \(\alpha\)-cyano-4-hydroxycinnamic acid as the matrix. A pulsed nitrogen laser (337 nm) was used to induce desorption/ionization, and mass spectra were obtained using the reflector mode. Each representative mass spectrum shown in the present figures was the smoothed average of 100,000 laser shots.

**RESULTS**

**Overexpression of DcPSKR1 in Tobacco BY-2 Cells**—To obtain the relatively large amount of DcPSKR1 protein required for the direct identification of the PSK binding domain by photoaffinity labeling, we first overexpressed DcPSKR1 in BY-2 suspension cells, which grow rapidly and can be harvested weekly. For DcPSKR1 expression, BY-2 cells were transformed using two constructs, 35S::DcPSKR1-His\(_6\), as a positive control for functional expression of full-length DcPSKR1 in heterologous cells, and the 35S::DcPSKR1-\(\Delta\)KD-His\(_6\) construct, in which the coding region for the kinase domain of DcPSKR1 had been removed (Fig. 1A). The latter construct was used to test whether the extracellular domain of DcPSKR1 is sufficient for interaction with PSK. The His\(_6\) tag was introduced to immobilize these proteins on the nickel column to perform on-column photoaffinity labeling (described below).

Northern blot and immunoblot analysis of the membrane fractions of each transformed BY-2 cell clone revealed that both DcPSKR1-His\(_6\) and DcPSKR1-\(\Delta\)KD-His\(_6\) proteins were successfully overexpressed in BY-2 cells (Fig. 1B). Both proteins migrated, with apparent molecular sizes of 150 and 120 kDa, respectively. The theoretical molecular masses of these proteins, without the signal sequence, are 110.2 and 78.8 kDa, respectively, indicating that both proteins are post-translationally modified by the addition of an \(\sim\)40-kDa moiety, most likely by glycosylation.

The ligand binding assay using [\(^{3}\)H]PSK confirmed a significant increase in PSK binding activity in the membrane fractions derived from both transformants, compared with fractions derived from untransformed BY-2 cells (Fig. 1C). These results indicate that DcPSKR1 is functionally expressed in BY-2 cells and that the intracellular kinase domain of DcPSKR1 is not essential for PSK binding. Scatchard analysis of the binding of [\(^{3}\)H]PSK to membranes expressing DcPSKR1-\(\Delta\)KD-His\(_6\) showed that the dissociation
FIGURE 1. Overexpression of DcPSKR1-KD-His<sub>6</sub> and DcPSKR1-KD-His<sub>6</sub>-KD-His<sub>6</sub>. A, schematic structures of DcPSKR1 and its derivatives. DcPSKR1 is a 1021-amino-acid LRR-RK containing 21 tandem copies of LRR, a 36-amino-acid island domain between the 17th and 18th LRR, a single transmembrane domain, and a cytoplasmic kinase domain. B, Northern blot and Western blot of transgenic BY-2 cells overexpressing DcPSKR1-His<sub>6</sub> and DcPSKR1-KD-His<sub>6</sub>. C, Scatchard plot of the [<sup>3</sup>H]PSK binding data of DcPSKR1-KD-His<sub>6</sub>. Total proteins purified using a PSK-Sepharose column and hydroxypatite column were concentrated by ultrafiltration, separated by SDS-PAGE, and visualized by SYPRO red (left panel). Purified DcPSKR1-KD-His<sub>6</sub> was deglycosylated by PNGase F, separated by SDS-PAGE, and visualized by immunoblot analysis with anti-DcPSKR1 antibodies (right panel). D, Chemical Fragmentation and Enzymatic Digestion of [<sup>125</sup>I]ASA-PSK-labeled DcPSKR1-KD-His<sub>6</sub>-Extracellular domain of DcPSKR1 contains 21 LRRs with a 36-amino-acid island domain between the 17th and 18th LRR. To roughly map the cross-linked region within DcPSKR1-KD-His<sub>6</sub>, we chemically and enzymatically fragmented the [<sup>125</sup>I]ASA-PSK-labeled protein and estimated the apparent molecular size of the radioactive fragments. E, SDS-PAGE analysis and deglycosylation of the affinity-purified DcPSKR1-KD-His<sub>6</sub>. Total proteins purified using a PSK-Sepharose column and hydroxypatite column were concentrated by ultrafiltration, separated by SDS-PAGE, and visualized by SYPRO red (left panel). Purified DcPSKR1-KD-His<sub>6</sub> was deglycosylated by PNGase F, separated by SDS-PAGE, and visualized by immunoblot analysis with anti-DcPSKR1 antibodies (right panel).
Ligand Binding Site of Phytosulfokine Receptor

FIGURE 2. Photoaffinity labeling of DcPSKR1-ΔKD-His₆. A, structure of [125I]ASA-PSK. B, photoaffinity labeling of DcPSKR1-ΔKD-His₆, and fragmentations of labeled proteins. Purified DcPSKR1-ΔKD-His₆ was incubated with 10 nM [125I]ASA-PSK in the absence (−PSK) or presence (+PSK) of excess unlabeled PSK and was then irradiated with UV light (365 nm). Cross-linked proteins were analyzed by SDS-PAGE and autoradiography. Fragmentations of labeled proteins by CNBr, Asp-N, and TPCK-treated trypsin were analyzed by SDS-PAGE and autoradiography. The decrease in apparent molecular size after PNGase F treatment indicates the presence of N-linked glycans. C, theoretical digestion map of DcPSKR1-ΔKD-His₆, after treatment with CNBr, Asp-N, and trypsin. Lengths of fragments are proportional to the number of amino acids. Highlighted bars with molecular weights indicate the possible fragments labeled by the photoaffinity ligand. Met-Thr sequences were resistant to CNBr treatment due to the conversion of Met to homoserine without cleavage.

On-column Photoaffinity Labeling of DcPSKR1-ΔKD-His₆—To confirm the location of the cross-linking site of ASA-PSK by mass spectrometry, we performed large scale photoaffinity labeling followed by trypsin digestion. The main difficulty in identifying photoaffinity-labeled peptide fragments contained in the complex enzymatic digests of the labeled protein is that the relative abundance of the labeled fragment is extremely low due to the low efficiency of the photoaffinity cross-linking reaction. To overcome this limitation, we established a novel on-column photoaffinity labeling methodology that allows repeated incorporation of the photoaffinity label. We immobilized DcPSKR1-ΔKD-His₆ on nickel-chelating HiTrap™ HP-Sepharose beads using a His₆ tag and performed solid-phase photoaffinity labeling in a transparent narrow column by directly irradiating the column with UV light (365 nm). The crucial advantage of the on-column photoaffinity labeling is that the cross-linking reaction can be repeated after washing out the uncross-linked ligands that act as potential competitors of the newly added ligands in the next round of photoaffinity reaction. In addition, this system allows direct buffer exchange without dialysis upon enzymatic digestion.

We repeated sequential on-column photoaffinity labeling three times and confirmed the significant increase in cross-linking of [125I]ASA-PSK to immobilized DcPSKR1-ΔKD-His₆ (Fig. 3A). We also confirmed that labeled DcPSKR1-ΔKD-His₆ can be successfully digested by trypsin even if it is immobilized on nickel-chelating beads (Fig. 3A). Released labeled fragment was no longer detectable on SDS-PAGE after trypsin digestion, confirming that its molecular size was <3.5 kDa.

To gain more information about the molecular size of the labeled tryptic fragment, we chromatographed the tryptic digest of labeled DcPSKR1-ΔKD-His₆ on a gel filtration column. Autowell γ counting of each fraction revealed the presence of two clear peaks (Fig. 3B). High pressure liquid chromatography analysis of the larger peak fraction showed that radioactivity was not retained on the reverse-phase column, suggesting that the radioactive molecule contained in this fraction was free [125I] rather than labeled fragments (data not shown). Based on the separation range of Sephadex G-25 (Mr, 5000–8000) and the elution profiles of blue dextran (marker for Mr) and cyanocobalamin (marker for molecular mass separation, Mr, 1355), the apparent molecular size of the labeled peptide contained in the smaller peak was estimated to be between 1.5 and 5 kDa. Together with the data from the SDS-PAGE of the labeled tryptic peptide, we estimated the apparent molecular size of the labeled fragment to be between 1.5 and 3.5 kDa.

Immunoprecipitation and MALDI-TOF MS Analysis of the Labeled Peptide—To directly detect the peptide fragment cross-linked with ASA-PSK using MALDI-TOF MS, we purified the labeled fragment by immunoprecipitation using anti-PSK antibodies. We performed large scale on-column photoaffinity labeling of ~200 pmol of purified DcPSKR1-ΔKD-His₆ using non-radioactive ASA-PSK (repeated 10 times) and purified labeled fragments derived from the tryptic digest of the labeled protein using the immunoaffinity column. Using positive mode MALDI-TOF MS analysis, we identified two specific molecular ion peaks at m/z 1752.83 and 1880.87 in the eluate of the immunoaffinity column; these peaks were not detected in the control experiments using the tryptic digest of the unlabeled protein (Fig. 4A). Upon assignment of these peaks, we considered two residues (five sites within the Pro²⁹⁵-Met⁵³⁷ fragment) yielded fast migrating labeled peptides with an approximate size of 5 kDa, with or without PNGase F treatment (Fig. 2B), suggesting that the cross-linking site is in the C-terminal region of the CNBr fragment (Fig. 2C). In contrast, the labeled fragment obtained by trypsin digestion (18 sites within Pro²⁹⁵-Met⁵³⁷ fragment) was no longer detectable on the gel, indicating that the molecular size of the labeled fragment was <3.5 kDa (Fig. 2B). These results indicate that the cross-linking site is confined to one tryptic fragment within the island domain. The most likely location of the cross-linking site in DcPSKR1-ΔKD-His₆ is the region Glu⁵⁰³-Lys⁵¹⁷ (Fig. 2C).
Ligand Binding Site of Phytosulfokine Receptor

Of the two above-described molecular ion peaks, the peak at \(m/z\) 1752.83 perfectly matched the calculated mass value for the free form of the tryptic peptide fragment (Glu\(^{503}\)-Lys\(^{517}\)) of DcPSKR1-\(\Delta K D\)-His\(_{6}\) (calculated exact mass of ENAVEEPSPDFPFFK (in protonated form), 1752.80) (Fig. 4B). This domain corresponds to the N-terminal side of the 36-amino-acid island domain of DcPSKR1. Similarly, the peak at \(m/z\) 1880.87 matched the miscleaved tryptic peptide fragment (Glu\(^{503}\)-Lys\(^{518}\)) derived from the same region of DcPSKR1-\(\Delta K D\)-His\(_{6}\) (calculated exact mass of ENAVEEPSPDFPFKKK (in protonated form), 1880.89) (Fig. 4B). Because the synthetic peptide ENAVEEPSPDFPFKKK alone exhibited no interaction with the immunoaffinity column (data not shown), it is likely that these fragments were immunoprecipitated with the cross-linked ASA-PSK and were generated by the cleavage of cross-linked sites presumably due to the high energy from the laser beam during MALDI-TOF MS analysis. This possibility is supported by the following three observations. (i) Negative mode MALDI-TOF MS of the same sample detected an ion peak at \(m/z \) 900.36, which corresponds to the \([M-H–SO_3]^–\) ion of the cleaved ligand (Fig. 4A). (ii) The appearance of the peak at \(m/z\) 1880.87, which corresponds to the miscleaved tryptic peptide fragment (Glu\(^{503}\)-Lys\(^{518}\)), is consistent with Lys\(^{517}\) being a cross-linking site that is resistant to trypsin digestion due to modification of its side chain. (iii) The calculated mass of the adduct form (2741.10) is within the estimated range of the molecular size obtained from gel filtration experiments. Because negatively charged peptides, such as phosphorylated and sulfated peptides, are often resistant to ionization in positive mode MALDI-TOF MS analysis, it is possible that the level of ionization of the adduct form was below the limit of detection.

Deletion of Glu\(^{503}\)-Lys\(^{517}\) of DcPSKR1 Abolishes Ligand Binding Activity—To confirm that the 15-amino-acid Glu\(^{503}\)-Lys\(^{517}\) region within the island domain of DcPSKR1 is involved in ligand binding, we generated a deletion mutant of DcPSKR1 that lacks Glu\(^{503}\)-Lys\(^{517}\) (DcPSKR1-\(\Delta ID\)(Glu\(^{503}\)-Lys\(^{517}\))) (Fig. 5A). The ligand binding assay using \([^3\text{H}]\text{PSK}\) showed that PSK binding activity in DcPSKR1-\(\Delta ID\)(Glu\(^{503}\)-Lys\(^{517}\)) membranes decreased to background levels despite successful expression of truncated proteins detected by immunoblot analysis, indicating that the region Glu\(^{503}\)-Lys\(^{517}\) is necessary for ligand binding (Fig. 5, B and C). We also prepared a deletion mutant of DcPSKR1 lacking Lys\(^{518}\)-Ile\(^{538}\), which is a region adjacent to Glu\(^{503}\)-Lys\(^{517}\) within the island domain. The ligand binding assay showed that DcPSKR1-\(\Delta ID\)(Lys\(^{518}\)-Ile\(^{538}\)) also completely lacked PSK binding activity, suggesting that the entire island domain is necessary for ligand recognition.

We determined that the ligand contact domain of DcPSKR1 is located within the 15-amino-acid Glu\(^{503}\)-Lys\(^{517}\) region of the island domain and that Glu\(^{503}\)-Lys\(^{517}\) and several adjacent residues together form a functional ligand binding pocket.

**DISCUSSION**

Based on the present results of on-column photoaffinity labeling, MALDI-TOF MS analysis, and fragmentation of labeled DcPSKR1-\(\Delta K D\)-His\(_{6}\) by CNBr and endoproteinase Asp-N, we conclude that the 15-amino-acid Glu\(^{503}\)-Lys\(^{517}\) region located on the N-terminal side of the 36-amino-acid region located on the N-terminal side of the 36-amino-acid.
island domain is the ligand contact domain of DcPSKR1. This region is also highly conserved in Arabidopsis PSK receptor AtPSKR1 (10). On-column photoaffinity labeling allows repeated cross-linking, thus increasing labeling efficiency without interference by uncross-linked photolyzed ligands, which act as potential competitors of the newly added photoaffinity ligands in the next round of the photoaffinity reaction. This system is also compatible with enzymatic digestion after changing to the appropriate buffer and enables us to use MALDI-TOF MS to directly analyze labeled fragments derived from small quantities of natural receptor preparations.

It has been reported that the brassinosteroid (BR) receptor BRI1, which also belongs to the LRR-RK family, directly interacts with photoactivatable BRs and that the 90-amino-acid region containing the island domain and an adjacent single LRR can recognize BRs even when they are bacterially expressed as a glutathione S-transferase fusion (25). These findings indicate that this region can, on its own, form a functional binding pocket for recognition of BRs. However, in the present study, recombinant glutathione S-transferase fusion proteins containing the island domain of DcPSKR1 and several adjacent LRRs did not bind to PSK (data not shown), suggesting that this domain is not sufficient for the formation of a stable ligand binding pocket. The 70-amino-acid island domain of BRI1 contains two cysteine residues, which may form a disulfide bond that stabilizes the ligand binding pocket. In contrast, there are no cysteine residues within the 36-amino-acid island domain of DcPSKR1 or the several LRRs adjacent to it. Although further experiments are required to demonstrate how extracellular 21 tandem LRRs of DcPSKR1 contribute to the formation of a stable ligand binding pocket, we speculate that the global ternary structure of the DcPSKR1 extracellular domain, rather than a local sequence motif within the island domain, defines the specific conformation of the island domain by which DcPSKR1 recognizes PSK with high affinity and specificity.

The theoretical structural model of Cf-9, a membrane-localized LRR-receptor-like protein of tomato cells (Lycopersicon pimpinellifolium) involved in the resistance response to the fungal pathogen Cladosporium fulvum, shows that its 38-amino-acid island domain forms a unique “loop-out” structure that is isolated from the adjacent LRR loops (26).

Secondary structure prediction of DcPSKR1 using the multivariate linear regression combination software (27) suggests that its 36-amino-acid island domain has no characteristic structural motif, such as α-helix and β-strand, which are highly conserved in the LRR domain (data not shown). We propose that the island domain acts as a flexible hinge-like region that modulates the relative conformation of the two surrounding LRR regions upon ligand binding. An island domain has been found in several LRR-RKs, including BRI1 (28) and tBRI1/SR160 (29), and in some LRR-receptor-like proteins including CLV2 (30), Cf-9 (31), and LeEIX (32); each of these island domains has a unique and specific amino acid sequence that is distinct from that of the highly conserved LRR motif (33). There are also many LRR-RKs and receptor-like proteins that have no island domain, such as FLS2 (34), Xa21 (35), and TMM (36). In a recent report, photoaffinity labeling indicates that flagellin, an elicitor-active structural component of bacterial flagella,
Ligand Binding Site of Phytosulfokine Receptor

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