Rapid one-step protein purification from plant material using the eight-amino acid StrepII epitope[*]

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Abstract

Beyond the rewards of plant genome analysis and gene identification, characterisation of protein activities, post-translational modifications and protein complex composition remains a challenge for plant biologists. Ideally, methods should allow rapid isolation of proteins from plant material achieving a high degree of purity. We tested three purification strategies based on the eight-amino acid StrepII, six-amino acid His$_6$ and 181-amino acid Tandem Affinity Purification (TAP) affinity tags for enrichment of a membrane-anchored protein kinase, *Nt*CDPK2, and a soluble protein, *At*SGT1b, from leaf extracts. Transiently expressed StrepII-tagged *Nt*CDPK2 was purified from *Nicotiana benthamiana* to almost complete homogeneity in less than 60 min and was directly suitable for enzymatic or mass-spectrometric analyses, allowing the identification of *in planta* phosphorylation sites. In contrast, purification of *Nt*CDPK2 via His$_6$ tag yielded partially oxidised protein of low purity. *At*SGT1b could be isolated after transient expression from *N. benthamiana* or from transgenic *Arabidopsis thaliana* as either TAP-tagged or StrepII-tagged protein. While StrepII-tag purification achieved similar yield and high purity as the TAP-tag strategy, it was considerably easier and faster. Using either tagging strategy, a protein was co-purified with *At*SGT1b from *N. benthaniana* and *A. thaliana* leaf extracts, suggesting that both the StrepII and TAP tags are suitable for purification of protein complexes from plant material. We propose that the StrepII epitope, in particular, may serve as a generally utilizable tag to further our understanding of protein functions, post-translational modifications and interaction dynamics in plants.

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; AP, alkaline phosphatase; CDPK, calcium dependent protein kinase; HA, haemaglutinin; IMAC, immobilised metal affinity chromatography; Q-TOF MS, quadrupole time-of-flight mass spectrometry; TAP tag, tandem affinity purification tag

Introduction

An increasing interest in analysing the biochemical functions, post-translational modifications and interacting partners of plant proteins demands improved purification techniques. Efraim Racker's motto "Don't waste clean thinking on dirty enzymes" adopted by Arthur Kornberg in his "ten commandments" (Kornberg, 2003) may serve as a guide. But how do we best get "dirty" proteins clean? This is greatly facilitated by the

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use of affinity tags. These polypeptides or small proteins are fused to the protein of interest and allow purification via an affinity matrix. A wide variety of affinity tags is currently available (Terpe, 2003). The ideal tag is small and allows rapid and flexible purification from a complex mixture, achieving high yield and purity. A small tag is generally less likely to interfere with the biological function of a protein. Rapid purification aids in maintaining protein integrity and post-translational modifications and increases the probability of co-purifying transiently bound interactors. Flexibility ensures that starting materials and buffers as well as buffer additives are not limited by the particular requirements of the tag. High yield and purity facilitate biochemical characterisation and make it easier to distinguish interactors from background.

In purifying two different proteins from leaf crude extracts, we tested the performance of the StrepII (WSHPQFEK), the His_6 and the tandem affinity purification-(TAP) tags. The StrepII tag binds to a streptavidin derivative termed Strep Tactin. It mimics structurally biotin and can be eluted from the StrepTactin matrix by washing with biotin or desthiobiotin containing buffers (Skerra and Schmidt, 2000; www.iba-go.com). The His_6 tag, comprising a stretch of six consecutive histidines, allows purification via immobilised metal affinity chromatography (IMAC). Nickel or Cobalt chelating resins are used (Chaga, 2001). The TAP tag has an IgG-binding protein A moiety separated by a TEV protease cleavage site from a calmodulin-binding peptide. The tagged protein is first bound via its protein A to an IgG column, cleaved off using TEV protease and then bound to a calmodulin column in the presence of calcium and eluted with EGTA (Rigaut et al., 1999).

In this study we were aiming to find methods for rapid isolation of proteins in high purity for subsequent mass-spectrometric analyses. We purified the membrane-associated calcium-dependent protein kinase NtCDPK2 from tobacco (Romeis et al., 2001), transiently expressed in N. benthamiana, and the soluble protein AtSGT1b from A. thaliana (Austin et al., 2002) either transiently expressed in N. benthamiana or stably expressed in transgenic A. thaliana. NtCDPK2-StrepII purification was compared to NtCDPK2-His_6 purification. For AtSGT1b we compared purifications via the StrepII tag and the TAP tag. These experiments demonstrate that the StrepII tag is vastly superior to His_6 in terms of flexibility and purity and offers key advantages over the TAP tag in terms of size, speed of purification and flexibility. We conclude that the StrepII tag is a valuable tool for rapid, easy and high quality protein purification from plant material.

Materials and methods

Vectors, bacterial strains, plant material and growth conditions

The following binary vectors derived from pam-PAT-MCS (accession number AY436765) were used in this study: pXCS-GFP, pXCS-HisHA, pXCS-HAHAT, pXCS-HAStrep (accession number AY457636), pXCSG-Strep and pXCSG-TAP (see below for construction details). These were introduced into Agrobacterium tumefaciens GV3101::pMP90RK (Koncz and Schell, 1986) carrying gentamycin, kanamycin and rifampicin resistances and conferred an additional carbenicillin resistance to transformed bacteria. The binary vector 35S:p19 conferring kanamycin resistance and carrying the gene for the p19 silencing suppressor from tomato bushy stunt virus was present in A. tumefaciens C58C1 (possessing rifampicin resistance) which carried the helper plasmid pCH32 (tetracyclin resistance) for T-DNA transfer (Voinnet et al., 2003). Agrobacterium strains were grown at 28 °C in YEB medium. Antibiotics were added to the media at the following final concentrations: carbenicillin, 75 μg/ml (solid), 50 μg/ml (liquid); gentamycin, 15 μg/ml; kanamycin, 50 μg/ml; rifampicin, 100 μg/ml, tetracyclin 10 μg/ml. Agrobacteria were grown first on solid media and densely inoculated from fresh plates into liquid media to achieve sufficient growth within 16 to 20 h. Longer growing times led to reduced expression in planta.

Arabidopsis Landsberg erecta sgt1b-3 null mutant plants (Austin et al., 2002) were grown at 21 °C, 10 h light period at 150–200 μEinsteins·m^{-2}·sec^{-1} (intensity) and 60% humidity. N. benthamiana were grown in late summer in a climatised greenhouse at 21 °C and 16 h light.

Generation of the pXC-tag derivatives

To generate pXCS-HAStrep (accession number AY457636), the adapter composed of CCC GGGGTTATCCATACGATGTTCCAGATTAT GCTGTCGGCGCCGGTTGGTCTCATCCTCA ATT TGAAAAATAAGTCTAGA (*Xma*I and *Xba*I sites underlined) was ligated into pamPAT-MCS (accession number AY436765) digested by *Xma*I and *Xba*I. Similarly, to obtain pXCS-HisHA, the adapter composed of CCCGGGATAATGGC TCATCACCATCACCATCATGGCGCCTATC CATACGATGTTCCAGATTATGCTG TCT AGA was inserted into pamPAT-MCS. *NtCDPK*2 was amplified from cloned cDNA (Romeis *et al.*, 2001) with primers GAATTCAAAATGGGGAAC ACTTG and CCCGGGAAGTCTTAGAGCCTC and cloned directionally into the *Eco*RI and *Xma*I sites of either pXCS-HAStrep or pXCS-HisHA, resulting in pXCS-NtCDPK2-HAStrep and pXCS-NtCDPK2-HisHA. Cloning details for pXCS-NtCDPK2-HAHAT (*NtCDPK*2 with HA and HAT tags) are available upon request.

To generate pXCS-GFP, the *smGFP* ORF was amplified from psmGFP (accession number U70495; Davis and Vierstra, 1996) using the primers ATCCCCGGGATGAGTAAAGGAGA AGAACTTTTC and AGTCTAGAGCTCTTAT TTGTATAGTTCATCCATG and cloned directionally into the *Xma*I and *Xba*I sites of pXCS-HAStrep. To construct pXCS-TAP, the TAP ORF was amplified from pBS1479 (Puig *et al.*, 2001) using the primers GCCCCGGGTTAG CAGAAGCTAAAAAGCTA and TCTCTAGA ATCAAGCTTCAGGTTGACTTC and cloned directionally into the *Xma*I and *Xba*I sites of pXCS-HAStrep. pXCSG-TAP and pXCSG-Strep were generated by ligation of the GATEWAY reading frame B (rfB; Blunt *Eco*RV fragment, Invitrogen, Carlsbad, CA) into pXCS-TAP digested with *Sma*I and pXCS-HAStrep, digested by *Sfo*I/*Sma*I, respectively. *AtSGT1b* cDNA from the L*er* ecotype was amplified from pCA134 (Azevedo *et al.*, 2002) using CAC-CATGGCCAAGGAATTAGCAG and ATA-CTCCCACTTCTTGAGCTCCATG, cloned directionally into pENTR/D-TOPO (Invitrogen) and recombined by LR reaction following the manufacturer's instructions into pXCSG-TAP and pXCSG-Strep giving pXCSG-SGT1b-TAP and pXCSG-SGT1b-Strep, respectively.

Transient protein expression in N. benthamiana

Experiments were performed as described (Romeis *et al.*, 2001). *Agrobacterium* cells in late exponential phase (<20 h growth in liquid medium) were harvested by centrifugation and resuspended in 10 mM MES–KOH (pH 5.6) containing 10 mM $MgCl_2$ and 150 μM acetosyringone to an OD_{600nm} of ~ 0.9. These cells were mixed with a dense solution of C58C1 (pCH32 35S:p19) expressing the silencing suppressor p19 of tomato bushy stunt virus (Voinnet *et al.*, 2003) so that the final density of the C58C1 cells was about 0.6. Bacteria were incubated for 2 h at room temperature and then injected into young fully expanded leaves of 4-weeks-old *N. benthamiana* plants. Leaves were harvested after 4 days, frozen in liquid nitrogen and stored at -80 °C.

Stable transformation of Arabidopsis

The Landsberg *erecta sgt1b-3* null mutant plants (Austin *et al.*, 2002) were transformed by flower dipping as described (Clough and Bent, 1998). Transformants were selected by spraying seven-day-old seedlings with 0.1% Basta (Aventis). Basta-resistant lines expressing detectable amounts of the *At*SGT1b-tag variants were self pollinated to produce T2 seeds. Single insertion lines were selected by segregation analysis of the Basta resistance in the T2 population. Leaves from two- to three-weeks-old Basta-resistant T2 plants were harvested, frozen in liquid nitrogen and stored at -80 °C.

Purification via the StrepII tag

Leaf material (0.25 g and 0.75 g for *N. benthamiana* and *A. thaliana*, respectively) was ground in liquid nitrogen and thawed in 0.75 ml Ex-strep buffer (100 mM Tris, pH 8.0; 5 mM EGTA; 5 mM EDTA; 150 mM NaCl; 10 mM DTT; 0.5 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride); 5 μg/ml aprotinin; 5 μg/ml leupeptin; plant protease inhibitor cocktail (Sigma P9599, Taufkirchen, Germany), diluted 1:200; 0.5% Triton X-100; and 100 μg/ml avidin). The slurry (about 0.8 ml) was placed in a 2 ml microfuge tube, transferred for 30 s into a sonication bath and then centrifuged for 10 min at 4 °C (21 000 g). The supernatant was

transferred to a new microfuge tube, sampled (lanes 1 of Figures 2A and 6A), and 150 μl slurry of StrepTactin Sepharose or StrepTactin Macroprep (IBA GmbH, Göttingen, Germany) was added. The two resins differ in the matrix to which the functional StrepTactin groups are attached: the Sepharose matix is based on Sepharose 4FF with a bead size of 45–165 μm while the Macroprep matrix consists of polymethacrylate beads with a diameter of 50 μm. All samples taken for electrophoretic analysis were mixed with a $3 \times$ SDS-loading buffer and heated for 3 min to 90 °C prior to loading. Binding was performed by incubation in an end-over-end rotation wheel for 30 min at 4 °C. The slurry was transferred into a micro spin column (BioRad 732-6204, Hercules, CA) and the flow-through collected and sampled (lanes 2). The resin was washed twice with 1 ml and four times with 0.5 ml W-strep buffer (50 mM Tris, pH 8.0; 2.5 mM EDTA; 150 mM NaCl; 2 mM DTT; 0.05% Triton X-100). To allow withdrawal of a sample before elution (lanes 3), the resin was resuspended in 0.8 ml W-strep buffer (same volume as original extract to facilitate comparisons). After taking a sample of the slurry (buffer including beads), the W-strep buffer was drained off again. For elution, 80 μl of E-buffer (10 mM Tris pH 8.0; 10 mM desthiobiotin; 2 mM DTT; 0.05% Triton X-100) representing the void volume of the system were carefully applied to the resin but not recovered. Four times 100 μl E-buffer were passed through and collected in two pools of 200 μl each (lanes 4 and 5). The resin was washed with 0.5 ml W-strep buffer and resuspended in 0.8 ml W-strep buffer for taking a slurry sample after elution (lanes 6). In case of subsequent direct Q-TOF MS analysis Triton X-100 needed to be removed completely. To achieve this, at least three washes without detergent were performed prior to elution. If Macroprep resin was used, one volume of washed beads was resuspended in one volume of $2 \times$ SDS-gel loading buffer and heated to 90 °C for 6 min. The liquid phase (without beads) was loaded on SDS gels. For assessment of protein kinase activity, 1 μl kinase of the first elution pool was incubated with 10 μM syntide-2 and 10 μM [γ^{32}P] ATP (10 μCi nmol^{-1}) in the presence of 1 mM CaCl$_2$ or 2 mM EGTA for 5 min at 30 °C (Romeis *et al.,* 2001). The phosphorylated substrate was quantified by scintillation counting.

Purification via His$_6$ or HAT tag

The leaf material (0.25 g per purification) was ground in liquid nitrogen and thawed in 0.75 ml Ex-His buffer A (100 mM NaPO$_4$, pH 7.0; 2 mM EGTA; 2 mM EDTA; 150 mM NaCl; 2 mM DTT; 0.5 mM AEBSF; 5 μg/ml aprotinin; 5 μg/ml leupeptin; plant protease inhibitor cocktail (Sigma P9599), diluted 1:200). The slurry (about 0.8 ml) was placed in a 2 ml microfuge tube and centrifuged for 10 min at 4 °C (21 000 g). The pellet was resuspended in 0.75 ml Ex-His buffer B (as buffer A but lacking EGTA, EDTA, DTT and inhibitor cocktail) and centrifuged for 5 min at 4 °C. The pellet was resuspended in 0.75 ml Ex-His buffer B containing 0.5% Triton X-100, transferred for 30 s into a sonication bath and centrifuged for 5 min at 4 °C. The supernatant was recovered, sampled (lane 1 of Figure 2B), and 150 μl 50% Talon slurry (BD Biosciences Clontech), previously washed with Ex-His buffer B, was added. The slurry was incubated for 30 min at 4 °C on a rotation wheel, transferred into a micro spin column and the flow-through was collected and sampled (lane 2). All following steps were performed as described for the purification via StrepII tag, except that other buffers were used. The wash buffer consisted of 50 mM NaPO$_4$, pH 7.0; 300 mM NaCl; and 0.05% Triton X-100. 150 mM imidazole was added to the wash buffer for elution.

Purification via TAP tag

Leaf material (0.25 g and 0.75 g for *N. benthamiana* and *A. thaliana*, respectively) was ground in liquid nitrogen and thawed in 0.75 ml Ex-strep buffer without avidin. The slurry (about 0.8 ml) was placed in a 2 ml microfuge tube, transferred for 30 s into a sonication bath and then centrifuged for 10 min at 4 °C (21 000 g). Purification was essentially performed as described (Rigaut *et al.*, 1999). The supernatant was transferred in a new centrifuge tube, sampled (lane 1 of Figure 6B), and 150 μl of washed rabbit IgG agarose beads (Sigma A2909) was added followed by an incubation for 1 h at 4 °C on an end-over-end rotation wheel. The slurry was transferred into a micro-spin column and the flow-through was sampled (lane 2). The resin was washed twice with 1 ml and four times with 0.5 ml W-TAP buffer

(50 mM Tris, pH 8.0; 5 mM EDTA; 5 mM EGTA; 150 mM NaCl; 2 mM DTT; 0.05% Triton X-100). To withdraw a sample before elution (lane 3), the resin was resuspended in 0.8 ml W-TAP buffer. (same volume as original extract to facilitate comparisons). After taking a sample of the slurry, the W-TAP buffer was drained off again. The column was first equilibrated with 1 ml and two times 0.5 ml TEV buffer (50 mM Tris, pH 8.0; 0.5 mM EDTA; 150 mM NaCl; 2 mM DTT; 0.05% Triton X-100). TEV protease digest was performed at 16 °C for 2 h in 500 μl TEV buffer with 100U TEV protease (Invitrogen). The flow-through was collected (lane 4) and added to 3 μl 1 M CaCl$_2$, 1.5 ml Cam Binding Buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 10 mM β-mercapto-ethanol; 1 mM Mg-acetate; 1 mM imidazole; 2 mM CaCl$_2$; 0.05% Triton X-100) and 150 μl washed calmodulin agarose beads (Stratagene #214303, Amsterdam-Zuidoost, The Netherlands). After 1 h at 4 °C on a rotation wheel, the slurry was transferred into a micro spin column (flow-through, lane 6). To collect a sample before elution, the resin was resuspended in 0.8 ml Cam Binding Buffer (lane 7) and the buffer was drained off after sampling. The final steps were performed as described for the StrepII-tag elution using the Cam E-buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 10 mM β-mercaptoethanol; 1 mM Mg-acetate; 1 mM imidazole; 2 mM EGTA; 0.05% Triton X-100).

SDS-gel electrophoresis, Western blot analysis and protein stainings

Proteins were separated by SDS–PAGE on 10% acrylamide gels and transferred onto nitrocellulose membranes by tank blotting (Tris-glycine buffer, pH 8.3; 20V/cm[1]; 1 h). Membranes were blocked in 5% milk powder in TBS buffer containing 0.1% Tween 20 (TBS-T buffer) for 30 min. Prior to the incubation with the StrepTactin-alkaline phosphatase (AP) conjugate, the blots were rinsed several times in TBS-T to remove free biotin originating from the milk powder, followed by a 10 min pre-incubation with TBS-T containing 2 μg/ml avidin (Fluka 11368, Buchs, Switzerland) to block biotinylated proteins on the blot. StrepTactin-AP conjugate was then added to the pre-incubation solution. For detection with the Streptavidin-AP conjugate, the blots were also

rinsed with TBS-T but not blocked with avidin. Antibodies and alkaline phosphatase conjugates were used in the following dilutions: StrepTactin-AP conjugate (IBA 2-1503-001, Göttingen, Germany) 1:4 000; Streptavidin-AP conjugate (Calbiochem OR04L, San Diego, CA) 1:2 000; rat anti-HA antibody (Roche 1 867 423, Mannheim, Germany) 1:4 000; rat anti-SGS antibody (raised against *At*SGT1a SGS domain; S. Betsuyaku *et al.*, unpublished); goat anti-rat IgG AP conjugate (Sigma A-9654) 1:10 000. First antibodies and conjugates were incubated at room temperature for 1–2 h or o/n at 4 °C followed by three 10 min washes with TBS-T and a 1 h incubation with the secondary antibody where applicable. After three 10 min TBS-T washes, staining was performed with p-nitro blue tetrazolium.

Coomassie stains were performed with 0.25% Coomassie R in 45:45:10 water, methanol, acetic acid. Silver staining compatible with mass spectrometry was performed as described (Shevchenko *et al.*, 1996).

Mass spectrometry

Tryptic digestion of StrepII-purified proteins was either performed directly in solution or in-gel after SDS-PAGE using a protocol based on Shevchenko *et al.* (2000). Digests were desalted for electrospray MS with a C18 reverse phase resin (ZipTip, Millipore, Bedford, MA). A Micromass Q-Tof-2 mass spectrometer operating with nano-spray and the Masslynx software (version 3.5) were used for analysis.

Results

Construction of pXC binary vectors for expression of C-terminally tagged proteins in planta

A family of binary vectors based on the pamPAT-MCS backbone (accession number AY436765) was generated to allow convenient and rapid *in planta* expression of proteins with various tags or promoters (see Materials and methods for cloning details). These vectors are high copy number in *E. coli* and confer ampicillin and car-benicillin resistance to *E. coli* and *A. tumefaciens*, respectively. The *pat* gene confers Basta herbicide resistance allowing rapid selection of transgenic
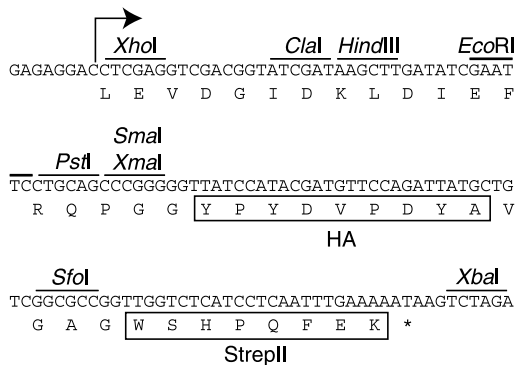
Figure 1. Polylinker map of pXCS-HAstrep. The nucleotide sequence of pXCS-HAStrep (accession number AY457636, base pairs 5292 to 94) is shown with the relevant unique restriction sites and coding frame. Boxed amino acids indicate the HA and StrepII epitopes. The 35S transcription start is represented by an arrow.

plants on soil. A series of derivatives of this vector with different tags, promoters and other features were generated and the following nomenclature adopted: "pX" (backbone); "C or N" (position of the tag relative to the protein sequence, here only C); "S" (2 × 35S promoter); "G" (GATEWAY-compatible); "tags". As an example, the complete sequence of pXCS-HAStrep has been deposited in the DNA database (Figure 1; accession number AY457636). In this study, we used pXCS-HAstrep and pXCS-HisHA for cloning of *NtCDPK2* and the GATEWAY-compatible vectors pXCSG-Strep and pXCSG-TAP for cloning of *AtSGT1b*. The pXCS-GFP vector was used as control.

StrepII-tag and His$_6$-tag purifications of Nt*CDPK2*

CDPKs are serine/threonine protein kinases unique to plants that bind calcium at a C-terminal calmodulin-like domain. N-terminal myristoylation and palmitoylation leads to the association of many CDPKs with cellular membranes (Cheng et al., 2002). *NtCDPK2* was found to play an essential role in plant defence response (Romeis et al., 2001).

NtCDPK2-HA-StrepII was isolated from crude *N. benthamiana* leaf extracts to high purity using StrepTactin Sepharose (Figure 2A). Starting from the crude extract (lane 1), the purification was performed in less than 60 min, yielding a single band in Coomassie-stained or silver-stained gels (lanes 4 and 7, respectively). The purified protein was active, possessing calcium-dependent
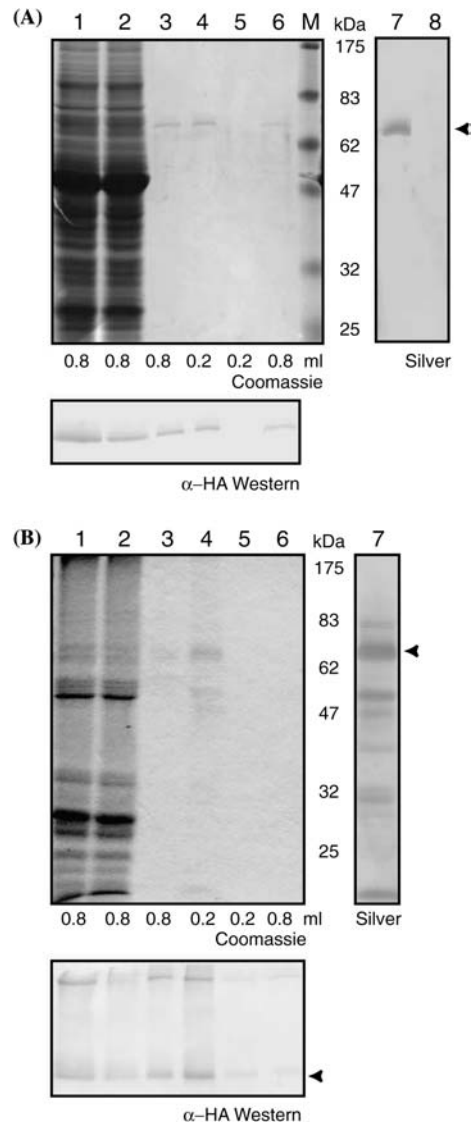

Figure 2. *NtCDPK2* purification from *N. benthamiana* via StrepII or His$_6$ tag. The different fractions collected during purification of *NtCDPK2*-HA-StrepII (A) or *NtCDPK2*-His$_6$-HA (B) on StrepTactin Sepharose or TALON resin, respectively, were separated by SDS-PAGE and analysed by Coomassie and silver stains or Western blot (anti-HA antibody). Marker proteins (M) are indicated with their molecular masses in kDa. 10 μl of each fraction were applied per lane. To allow quantitative comparisons, the total volume of each fraction (in ml) are given at the bottom of the Coomassie-stained gels. Arrows indicate the purified *NtCDPK2* protein. Lanes 1, extract before the addition of resin (input); lanes 2, extract after the incubation with resin (unbound protein); lanes 3, resin after washes, before elution (bound protein); lanes 4, affinity eluted protein, pool 1 (purified protein); lanes 5, affinity eluted protein, pool 2; lanes 6, resin after elution (protein remaining on resin); lanes 7, as 4 but silver-stained; lanes 8, affinity eluted protein (pool 1) from a control purification of GFP expressed in *N. benthamiana*.

(A)

(B)

(C)

N G L F Q $\boxed{S_{18}}$ V $\boxed{S_{20}}$ A A M W R
13 12 11 10 9   8   7   6   5 4 3 2 1   y-ions
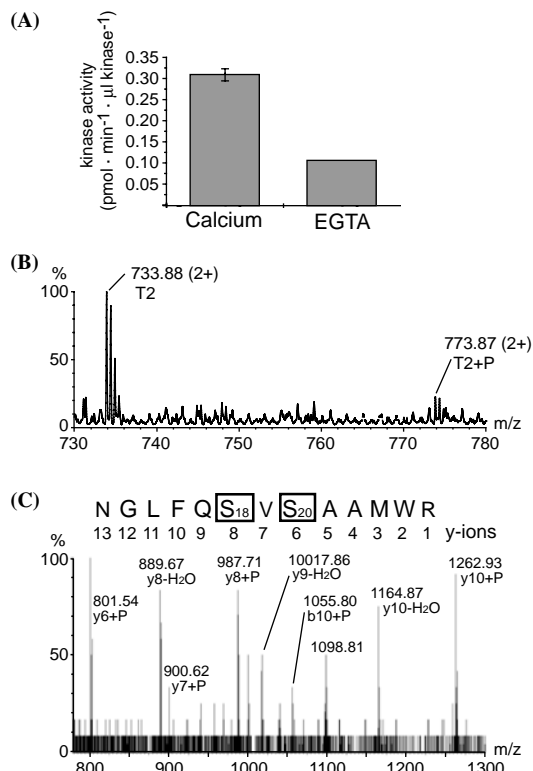
Figure 3. Enzymatic activity of NtCDPK2-HA-StrepII and identification of in planta phosphorylation sites. NtCDPK2-HA-StrepII was affinity purified from N. benthamiana using StrepTactin Sepharose. (A) Kinase activity was assayed by incorporation of ^{32}P into syntide-2 in presence of 1 mM calcium or 2 mM EGTA and quantified by scintillation counting. (B) MS spectrum from 730 to 780 m/z displaying the doubly charged tryptic peptide T2 (amino acids 13–25) in either non-phosphorylated (T2; 733.88 m/z) or phosphorylated (T2+P; 773.87 m/z) form. (C) MS/MS spectrum from 780 to 1300 m/z displaying fragment ions of the phosphorylated peptide T2P (773.87 m/z). Masses are labeled in reference to the expected fragment masses for the non-phosphorylated peptide T2 with "-H2O" indicating a loss of 18 mass units and " + P" a gain of 80 mass units characteristic for phosphorylation. The sequence of T2 is given on top and the numbers of possible y-ions are indicated underneath. The potential phosphorylation sites S18 and S20 are marked by a box. Phosphorylation at S20 is indicated by the presence of y-ions 6 + P and 7 + P.

kinase activity when syntide-2, a peptide substrate generally used for CDPK characterisation, was used in the assay (Figure 3A). Proteolytic digest and subsequent nanospray quadrupole time-of-flight mass-spectrometry (Q-TOF MS) analysis could be performed directly on the purified protein without the need for further electrophoresis and in-gel digestion. Due to the high degree of
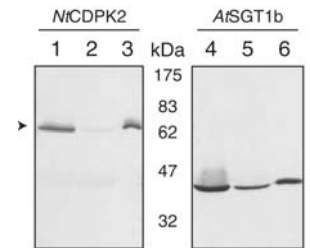
Figure 4. Improved binding yield of NtCDPK2 and AtSGT1b using the StrepTactin-Macroprep resin. NtCDPK2-HA-StrepII (left panel) and AtSGT1b-StrepII (right panel) were expressed in N. benthamiana and purified using StrepTactin-Macroprep resin. Western blots were probed with StrepTactin-alkaline phosphatase conjugate. Molecular masses of marker proteins are indicated in kDa. Lanes 1 and 4, extract before addition of the resin (input); lanes 2 and 5, extract after the incubation with resin (unbound protein); lanes 3 and 6, protein on resin after washes (boiled off in SDS buffer). Of each fraction (total volume 0.8 ml, respectively) 10 μl were applied per lane.

purity, mass spectra were dominated by peptides of NtCDPK2 which facilitated the search for post-translational modifications. An example is given in Figure 3B showing a mass spectrum that contains peaks of a tryptic NtCDPK2 peptide (amino acids 13–25) present in non-phosphorylated (733.88 m/z) and phosphorylated (773.87 m/z) form. Tandem mass-spectrometric analysis of the phosphorylated peptide clearly showed in planta phosphorylation of serine 20 demonstrated by the presence of phosphorylated y-fragment ions y6 + P and y7 + P in the spectrum (Figure 3C). As a negative control for purification specificity, extracts from plants expressing smGFP were subjected to the same purification scheme. Nonspecific protein purification was not observed (Figure 2A, lane 8). Western analysis of extracts taken before and after incubation with the affinity matrix revealed that only part of the protein was bound to the StrepTactin resin (compare lanes 1 and 2). The bound fraction is estimated in lane 3 (protein boiled off the resin in SDS buffer after washes). Binding was not significantly increased by extending the incubation time or binding at higher temperatures (data not shown). Affinity elution using desthiobiotin was possible (lane 4) though not quantitative (lanes 5 and 6). Binding of StrepII-tagged proteins was greatly improved if instead of StrepTactin Sepharose an alternative matrix termed Strep Tactin Macroprep (IBA GmbH, Göttingen, Germany) was used (Figure 4, left panel, lanes 1

and 2). Bound protein could be boiled off the column in SDS buffer (lane 3) but affinity elution from this alternative matrix was not possible in our hands. However, enzymatic activity of NtCDPK2 was retained on the Macroprep resin and MS-analysis after gel-electrophoresis and in-gel digest yielded spectra with a high density of peptide signals specific to the isolated protein (data not shown).

Despite widespread application of the His tag in Escherichia coli there are few reports on its use in plants (Terpe, 2003). One of the major drawbacks is its incompatibility with reductants and chelators. While chelators are generally used to block metal-proteases and may be dispensable, reductants like dithiothreitol (DTT) or β-mercaptoethanol are absolutely required while preparing a protein extract from most plant species to avoid rapid oxidation leading to enzyme inactivation (e.g. Witte and Medina-Escobar, 2001) and aggregation. To avoid these problems, extracts containing NtCDPK2-His$_6$-HA were first prepared in the presence of small amounts of DTT and chelators. Since the kinase is mostly associated with the microsomal pellet these compounds could then be washed away before extracting the pellet with detergent-containing buffer to solubilise the enzyme (see Materials and methods). For soluble proteins (such as AtSGT1b) a desalting step (dialysis or gel filtration) would be necessary after extraction with reductants and chelators before the extract could be chromatographed on an IMAC column. Although the microsomal pellet fraction applied to the IMAC column (TALON resin) was less complex than the total extract applied to the StrepTactin matrix (compare Figure 2A and B, lane 1) purity attained after IMAC purification was poor compared to the enrichment after StrepTactin chromatography (compare Figure 2A and B, lanes 4 or 7). Despite initially extracting in the presence of DTT partial oxidation could not be avoided, leading to the appearance of NtCDPK2 aggregates visible as high molecular species of NtCDPK2 on the Western blot (Figure 2B). Binding yield to the IMAC column is comparable to that obtained with the StrepTactin Sepharose column (compare lanes 1–3 of Figure 2A and B).

Purification of NtCDPK2 with the TAP-tag system was not attempted because we reasoned that the calmodulin-like domain of the CDPK would probably bind to the calmodulin-binding domain of the TAP tag and therefore interfere with the purification. This notion is supported by the finding that calmodulin can functionally replace the calmodulin-like domain in a hybrid CDPK (Yoo and Harmon, 1996).

Use of avidin prevents co-purification of biotinylated proteins on StrepTactin

StrepTactin is a streptavidin derivative which, like streptavidin, also binds free biotin and biotinylated proteins. Therefore, biotinylated proteins are co-purified with StrepII-tagged proteins (Figure 5, third panel, lanes 1 and 2: signals marked with asterisk) and binding sites on the StrepTactin matrix may be blocked by free biotin. This may be of particular relevance when working with plant extracts because free cytosolic biotin levels have been estimated at 11 μM (in pea leaves), three orders of magnitude higher than in bacteria (Tissot et al., 1997). However, free and protein-bound biotin can be captured by inclusion of avidin in the extraction buffer, taking advantage of the fact that the StrepII tag does not bind to avidin (Skerra and Schmidt, 2000). We observed that this procedure blocked co-purification of biotinylated proteins (Figure 5, third panel) but did not diminish the binding of NtCDPK2-HA-StrepII to StrepTactin (Figure 5, all panels). Additionally, one-step
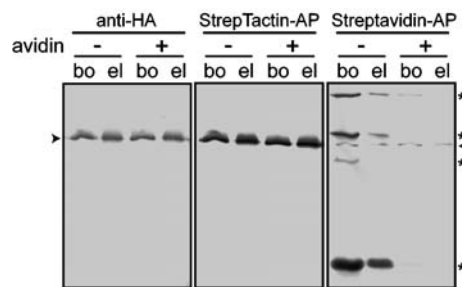
Figure 5. Effect of avidin on NtCDPK2 purification. Western blots of NtCDPK2-HA-StrepII purified on StrepTactin Sepharose in the presence (+) or absence (−) of avidin. Detection was performed either with anti-HA primary antibody and anti-rat IgG-alkaline phosphatase conjugate (left panel), StrepTactin-alkaline phosphatase conjugate (middle panel) or with Streptavidin-alkaline phosphatase conjugate (right panel). bo: protein bound to the resin after washes, directly before elution (total fraction size 0.8 ml); el: affinity eluted protein (total fraction size 0.2 ml). An amount of 10 μl of each fraction were applied per lane. Arrows indicate the purified NtCDPK2-StrepII and asterisk designate biotinylated proteins.

detection of the StrepII tag with StrepTactin-alkaline phosphatase conjugate (Figure 5, middle panel) produced an equivalent signal intensity on a Western blot compared to the conventional two-step HA-tag detection with primary and secondary antibodies (left panel).

Purification of AtSGT1b from N. benthamiana *using a StrepII tag or a TAP tag*

*At*SGT1b is a soluble protein with features of molecular co-chaperones that has been implicated in disease resistance and auxin responses of plants (Hubert *et al.*, 2003; Muskett and Parker, 2003). *At*SGT1b-StrepII complemented auxin insensitivity of the *Ler sgt1b-3* mutant (Gray *et al.*, 2003) in

a root growth inhibition assay and therefore appears to possess biological activity (data not shown). An *in vitro* test for activity is currently not available.

As for *Nt*CDPK2, *At*SGT1b-StrepII expressed in *N. benthamiana* was purified to near homogeneity (Figure 6A, lanes 4 and 7). However, a protein of ~ 70 kDa was co-purified that was absent in the GFP control (lane 8) and therefore represents a possible *At*SGT1b interactor. Affinity elution from the StepTactin-Sepharose matrix was marginally more efficient for *At*SGT1b than for the *Nt*CDPK2 (compare lanes 4–6 of Figures 2A and 6A). Similar to our findings with *Nt*CDPK2, binding yield of *At*SGT1b could be improved using a StrepTactin-Macroprep resin (Figure 4, right panel).

The TAP tag was first used to purify protein complexes from yeast (Rigaut *et al.*, 1999). A
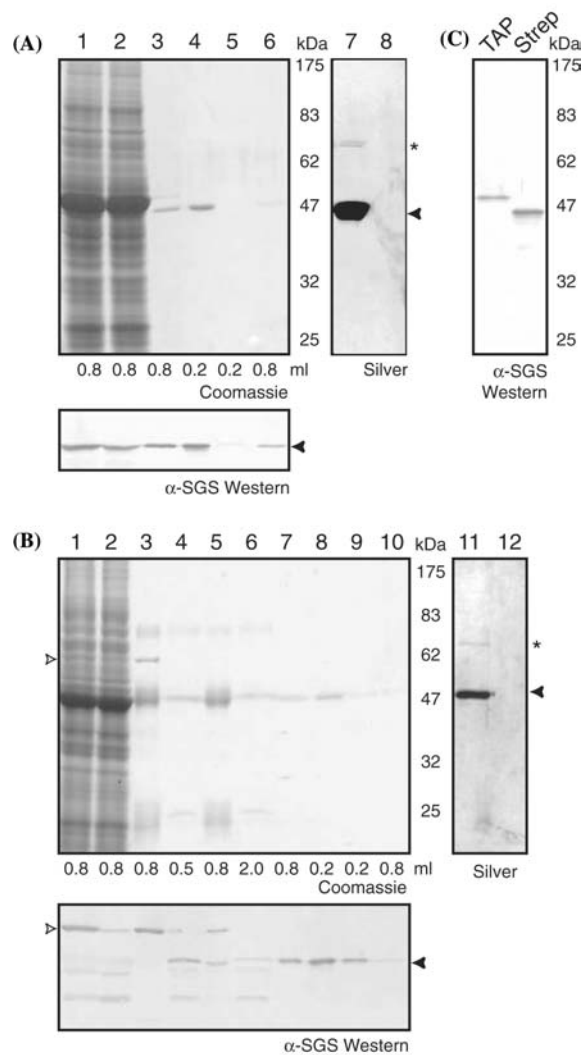

Figure 6. Purification of *At*SGT1b from *N. benthamiana* via StrepII tag and TAP tag. The different fractions collected during purification of *At*SGT1b-StrepII using StrepTactin Sepharose (A) or *At*SGT1b-TAP (B) were separated by SDS-PAGE and analysed by Coomassie and silver stains or Western blot (anti-SGS antibody). Molecular masses of marker proteins are indicated in kDa. An amount of 10 μl of each fraction were applied per lane. To allow quantitative comparisons, the total volume of each fraction (in ml) is given at the bottom of the Coomassie-stained gels. White and black arrows indicate full-length *At*SGT1b-TAP and purified *At*SGT1b, respectively. Note that TEV protease cleavage leads to a reduction in protein size. The asterisk designates a protein that co-purified with *At*SGT1b. (A) Lane 1, extract before the addition of resin (input); lane 2, extract after the incubation with resin (unbound protein); lane 3, resin after washes, before elution (bound protein); lane 4, affinity eluted protein, pool 1 (purified protein); lane 5, affinity eluted protein, pool 2; lane 6, resin after elution (protein remaining on resin); lane 7, as lane 4 but silver-stained; lane 8, affinity eluted protein (pool 1) from a control purification of GFP expressed in *N. benthamiana*. (B) Lane 1, extract before the addition of IgG resin (input); lane 2, extract after the incubation with IgG resin (unbound protein); lane 3, IgG resin after washes, before elution (bound protein); lane 4, TEV-eluted protein; lane 5, IgG resin after elution (protein remaining on resin); lane 6, extract after the incubation with calmodulin resin (unbound protein); lane 7, calmodulin resin after washes, before elution (bound protein); lane 8, EGTA-eluted protein, pool 1 (purified protein); lane 9, eluted protein, pool 2; lane 10, calmodulin resin after elution (protein remaining on resin); lane 11, as lane 8 but silver stained; lane 12, affinity eluted protein, pool 1, from a control purification of GFP expressed in *N. benthamiana*. C. *At*SGT1b-TAP (lane 1, marked TAP) and *At*SGT1b-StrepII (lane 2, marked Strep) protein amounts in purified fractions were compared by Western analysis (anti-SGS antibody).

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recent report describes the use of a modified TAP
tag for the purification of proteins from plants
(Rohila *et al.*, 2004). In our hands, the TAP tag
allowed isolation of *At*SGT1b to the same degree
of purity as observed for the StrepII-tagged pro-
tein (Figure 6B, lanes 8 and 11), with similar
overall yield (Figure 6C). However, starting from
the crude extract StrepII-tag purification took less
than 60 min while 5 h were needed for a TAP-tag
purification. As for the *At*SGT1b-StrepII purifi-
cation, a protein of ~ 70 kDa was co-purified (lane
11 of Figure 6B). Binding to the IgG column was
almost quantitative (compare lanes 1–3 of Fig-
ure 6B, Western). During cleavage with the TEV
protease (2 h at 16 °C) some *At*SGT1b was de-
graded (Figure 6B, lane 4, Western), presumably
by the action of co-purified proteases from the
plant extract. Not all *At*SGT1b-TAP was released
from the IgG column (lane 5). Binding of the
eluted *At*SGT1b to the calmodulin column was
nearly complete (compare lanes 4 and 6, Western;
and see binding yield in lane 7) and the protein
could be eluted quantitatively (lanes 8–10).

Purification of *At*SGT1b using a His tag was
not attempted due to the soluble nature of the
protein. Removal of chelators and reducing agents
prior to affinity chromatography would have re-
quired additional steps like desalting gel filtration
or dialysis. This was not compatible with our aim
to find an efficient purification method.

Purification of At*SGT1b via StrepII tag or TAP tag
from transgenic* A. thaliana *plants*

We also investigated the use of the StrepII and
TAP tags for *At*SGT1b purification from trans-
genic *A. thaliana*. Non-transformed *Ler sgt1b-3*
mutant served as control. Purification of
*At*SGT1b-StrepII and *At*SGT1b-TAP from
A. thaliana was achieved to high purity (Figure 7)
similar to our results obtained with *N. benthami-
ana*. Q-TOF MS analysis of purified *At*SGT1b-
StrepII covering 73% of the whole protein did not
provide evidence for any post-translational
modification (data not shown). With both tagged
variants, a protein of ~ 70 kDa was co-purified at
sub-stoichiometric levels and, using protein puri-
fied via the StrepII tag, was identified after in-gel
digestion by nanospray Q-TOF MS analysis
(L.D. Noël *et al.*, unpublished). A higher amount
of *At*SGT1b-TAP (and co-purifying 70 kDa
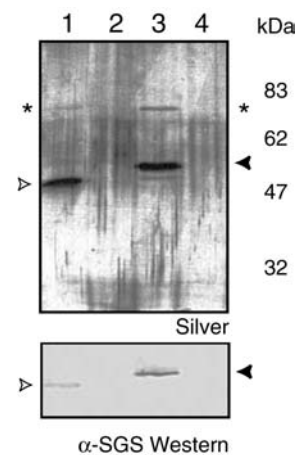
Figure 7. Purification of *At*SGT1b and a potential interacting
protein from *A. thaliana* extracts via StrepII tag and TAP tag.
Extracts from *Ler sgt1b-3* expressing *At*SGT1b-StrepII (lane 1)
and *At*SGT1b-TAP (lane 3) and non-transgenic L*er sgt1b-3*
(control, lanes 2 and 4) were subjected to the respective puri-
fication schemes. Purified proteins were analysed by
SDS-PAGE and detected by silver staining (upper panel) or
Western blot (anti-SGS antibody, lower panel). An amount of
10 μl of the final elution fractions (pool 1) were loaded. White
and black arrows indicate the purified *At*SGT1b-StrepII and
*At*SGT1b-TAP proteins, respectively. The asterisk indicates the
position of a protein that co-purified with *At*SGT1b. Molecular
masses of marker proteins are indicated in kDa.

protein) than *At*SGT1b-StrepII was obtained
(Figure 7) which may have been due to different
amounts of tagged proteins in the corresponding
crude extracts. An in-depth characterization of
*At*SGT1b biological function and interactions is
currently being undertaken and will be presented
elsewhere. In summary, our data suggest that the
TAP- as well as the StrepII-epitope are suitable for
the purification of protein complexes from plants.

Discussion

The pace of plant gene discovery and transcription
analyses has increased massively in recent years,
affording new insights to the roles of genes and
their products. As a first resort, plant biologists
generally attempt production of recombinant plant
proteins in *E. coli* or yeast cells for biochemical
analysis. Such studies can provide important
information about intrinsic biochemical properties.
There is, however, an increasing need to unravel the
nature of *in vivo* regulatory controls and modifi-
cations influencing protein function. Often these

questions can only be addressed by isolation of proteins and protein complexes from their natural plant source. Ideally such isolation should be rapid and yielding protein of high purity.

In this study we report on the successful use of the StrepII and TAP tags for protein purification from plant extracts. The utility of a modified TAP tag for plant work has been shown recently (Rohila *et al.*, 2004). Both tags allowed the isolation of *Nt*CDPK2 and *At*SGT1b to high purity (Figures 2A and 6). In contrast, several plant proteins appeared to co-purify during isolation of *Nt*CDPK2-His$_6$ on an IMAC column (Figure 2B), presumably because they contained metal-binding or natural His-tag sequences like, for example, those observed in urease accessory protein G (Witte *et al.*, 2001). Purity achieved by the StrepII-tag system was further increased by the inclusion of avidin in the extraction buffer to suppress co-purification of biotinylated proteins (Figure 5). Mass spectra of the StrepII-isolated proteins were dominated by peptides of either *At*Sgt1b or *Nt*CDPK2 which facilitated the search for post-translational modifications. Serine 20 of *Nt*CDPK2 was identified as an *in planta* phosphorylation site (Figure 3C). Tandem mass spectra were also consistent with the possibility that a minor part of peptide T2 may be alternatively phosphorylated at S18 as previously reported for recombinant kinase produced in *E. coli* (Glinski *et al.*, 2003). There are, to our knowledge, very few examples where *in vivo* phosphorylation was directly demonstrated for a particular plant protein under study. For eukaryotic organisms it was estimated that about one-third of all proteins are subject to phosphorylation (Mann *et al.*, 2002) and the regulatory role of such post-translational modifications is widely appreciated. Unravelling the locations and functions of these post-translational events in plant proteins may be facilitated by the protocols described here.

Purification via the TAP tag required approximately 5 h due to the combination of two purification steps and the need for an intermediate protease cleavage which alone takes 2 h (without reaching completion; Figure 6B). In contrast, the StrepII purification procedure could be performed in less than 60 min, likely reducing the risk of protein degradation, loss of interacting proteins or alteration of informative post-translational modifications. Additionally, the shorter processing time for StrepII-tagged proteins allowed us to proceed from the plant leaf to the MS analysis in one day.

The StrepII tag comprises only eight amino acids and has no particular overall charge, unlike the positively charged His$_6$ tag. The TAP tag codes for 181 amino acids. In principle, the StrepII tag is therefore least likely to interfere with the biological function of the tagged protein. This may be a particular advantage if the aim is to isolate protein complexes in a non-mutant background since proteins with bulky tags may be less efficient in competing with the endogenous protein for binding partners. This problem has been realised for the TAP tag and an approach employing double stranded RNA interference to deplete endogenous proteins in combination with the expression of TAP-tagged forms was proposed (Forler *et al.*, 2003).

In terms of flexibility the StrepII tag has few obvious limitations. In principle, any buffer or buffer additive that suits protein stability may be used (with the exception of biotin). In contrast, IMAC is incompatible with chelators and reducing agents (Chaga, 2001) that are indispensable for extracting proteins from many plant species (Gegenheimer, 1990). The TAP tag limits the use of protease inhibitors that block TEV cysteine protease activity. The absence of inhibitors may cause degradation problems during the two-hour incubation with TEV protease at 16 °C (see Figure 6B, lane 4) presumably due to the action of co-purified plant proteases. However, a recent report indicated that the inclusion of the cysteine protease inhibitor E-64 improved the stability of a TAP-tagged protein during TEV protease cleavage (Rohila *et al.*, 2004). Whether this inhibitor is of general utility for different proteins and for purifications from plant sources other than *Arabidopsis* remains to be shown. During TAP-tag purification, chelating agents are needed for the elution from the calmodulin column. Thus, purification of metal-containing enzymes is problematic because metal ions needed for biological function may be stripped from the purified protein.

Overall yield (amount of tagged protein present in the crude extract versus amount of tagged protein recovered in the purified fraction) remains a hurdle for all the tested tagging systems including the StrepII system. Using a different StrepII-matrix (Macroprep instead of Sepharose) may be a

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solution in some cases (Figure 4). Although we could not achieve affinity elution from the Macroprep resin, MS-analysis is still possible when the protein is boiled off from the resin in SDS buffer, electrophoresed and digested in-gel. Due to the high binding yield on the Macroprep resin this procedure even facilitates MS analysis because protein concentrations are higher. Furthermore, strong binding may be desirable for applications using immobilised protein. Overall yield of TAP-tagged *At*SGT1b protein appeared to be slightly inferior to that of StrepII-tagged *At*SGT1b when purified from *N. benthamina* (Figure 6C). The situation was reversed for purification of these tagged *At*SGT1b derivatives from *A. thaliana* (Figure 7). Such yield differences may reflect differences in the amount of tagged protein present in the respective crude extracts. This is likely for extracts obtained from *A. thaliana* since distinct transgenic lines expressing the respective tagged proteins were used. Due to the presence of protein A in the TAP tag it was not possible to compare accurately amounts of StrepII-tagged versus TAP-tagged protein in the corresponding crude extracts by Western blot analysis. Taken together, our data indicate that yield from the TAP-tag and the StrepII-tag system is similar. Losses in the TAP-tag system do not appear to result from low binding yield to the two resins, rather from degradation during TEV protease cleavage and incomplete release of protein from the IgG column (Figure 4B). The combination of two purification steps, each associated with some losses, also reduces overall recovery.

In the course of this study we also tested the HAT tag (BD biosciences Clontech, Palo Alto, CA), a 19 amino acid tag for use in IMAC chromatography (*Nt*CDPK2-HA-HAT construct) and the results obtained were similar to those with the His$_6$ tag (data not shown). In addition, preliminary experiments demonstrated that the 15-amino acid S tag (Raines *et al.*, 2000) and the chitin-binding-domain (CBD) tag (52 aa; Xu *et al.*, 2000) also allowed purification of *Nt*CDPK2 from crude plant extracts. However, elution of S-tagged protein was only possible with high amounts of salt (3M MgCl$_2$) while affinity elution of CBD-tagged protein was not possible (C.-P. Witte and T. Romeis, unpublished). Tags employing recognition by antibodies were not tested due to the high cost and limited stability of antibody matrices

(Terpe, 2003). As StrepTactin is derived from Streptavidin, a protein of high stability (Bayer *et al.*, 1990), it is likely to be comparatively stable. According to the manufacturer, stripping and recycling the resin is possible. At the current price of the StrepTactin-Sepharose matrix, protein purification applying this system should be affordable to most laboratories and may be suitable for large-scale or high-throughput applications (Boettner *et al.*, 2002; Holz *et al.*, 2002).

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