The Plant Journal (2000) **22**(6), 553–560

TECHNICAL ADVANCE

Intron-tagged epitope: a tool for facile detection and purification of proteins expressed in *Agrobacterium*-transformed plant cells

Alejandro Ferrando, Rosa Farràs, Ján Jásik†, Jeff Schell and Csaba Koncz*
Max-Planck Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

Received 10 January 2000; revised 13 March 2000; accepted 20 March 2000.
*For correspondence (fax +49 221 5062 213; e-mail koncz@mpiz-koeln.mpg.de).
†Permanent address: Department of Plant Physiology, Comenius University, Mlynska dolina B2, 842 15 Bratislava, Slovakia.

Summary

Epitope tagging provides a useful tool for immunological detection and cellular localization of proteins *in vivo*. Using T-DNA-mediated transformation, the detection of epitope-tagged proteins *in planta* is currently feasible only in transgenic plants, because an artificial expression of cDNA and gene constructs driven by plant promoters in bacteria obscures an early detection of epitope-tagged proteins in *Agrobacterium*-infected plant cells. We have developed a method for labelling plant coding sequences with intron-tagged epitope-coding domains that are not processed in *Agrobacterium*. Here we show that the expression of HA-epitope-tagged constructs encoding β-glucuronidase and S-phase kinase-associated (AtSKP1/ASK1) proteins can be specifically and exclusively detected in cultured *Arabidopsis* cells as early as five days after *Agrobacterium* infection. This epitope-tagging approach offers an unlimited source of transformed material for purification and localization of proteins expressed individually or simultaneously in *Agrobacterium*-transformed plant cells.

Introduction

Labelling of coding sequences with epitope tags facilitates the physical identification and subcellular localization of proteins, as well as the isolation and characterization of large protein complexes. In addition, epitope tagging resolves the problem of genetic redundancy by permitting a separate study of immunologically closely related members of protein families. To label proteins with different epitopes for subsequent study of their expression, stability, localization, topology, transport and molecular interaction in yeast and animal cells, a wealth of versatile vectors and specific antibodies are available (for review see Jarvik and Telmer, 1998). Immunolocalization of different members of the plasma membrane proton pump family (DeWitt and Sussman, 1995) and monitoring the stability of a disease resistance gene product (Boyes *et al.*, 1998) provide good examples of the applicability of epitope tagging in plants. Nevertheless, wide-ranging exploitation of epitope labelling in plant proteomics calls for technical developments.

Transformation of plant protoplasts with DNA, used as a transient assay for immunological detection of modified proteins *in planta* (see e.g. Strizhov *et al.*, 1996), is handicapped by a limiting amount of initially available transformed cells, an undesired induction of stress signalling during cell-wall digestion and PEG treatment of protoplasts, and a rapid decline of transient expression levels. Therefore epitope-tagging approaches are mostly based on the production of transgenic plants by *Agrobacterium*-mediated transformation and propagation of seed progeny to yield a suitable amount of starting material for protein analysis. Assaying the expression of epitope-labeled proteins in plant cells or tissues immediately after *Agrobacterium* infection could dramatically increase the speed of biochemical and immunological studies, if artificial expression of cDNAs and genes driven by plant promoters in *Agrobacterium* did not obscure the immunoassays. To resolve this problem we have developed a technique for labelling plant protein-coding

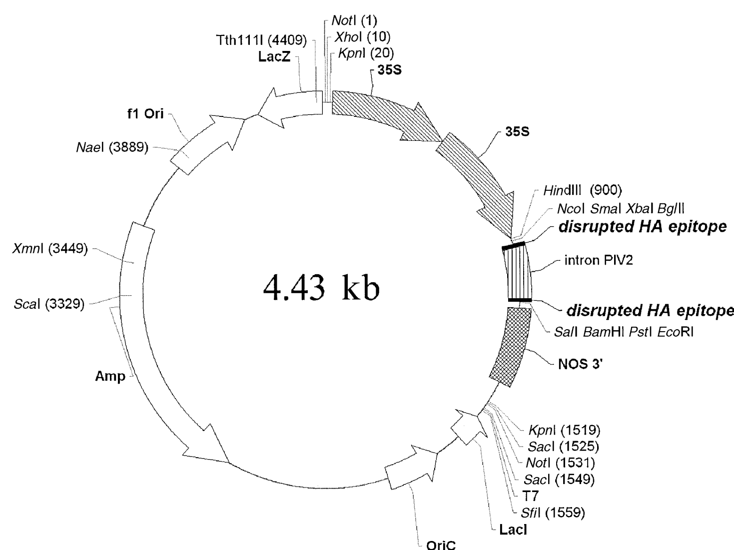
© 2000 Blackwell Science Ltd

domains with intron-tagged epitope sequences that are not processed in *Agrobacterium*. Our results, obtained with an intron-tagged HA epitope, illustrate that this technique is suitable for rapid and sensitive immunological detection of a stable reporter protein, such as β-glucoronidase, in *Agrobacterium*-infected plant cells. In addition, we show that epitope tagging also provides a simple tool for specific cellular localization and purification of modified plant proteins, such as the AtSKP1/ASK1 subunit of putative ubiquitin ligase complexes implicated in the control of auxin signalling, floral organ identity and male meiosis in *Arabidopsis* (Gray *et al.*, 1999; Samach *et al.*, 1999; Yang *et al.*, 1999; Zhao *et al.*, 1999).

Results and Discussion

Vectors for construction of intron-tagged epitope fusions

A PCR strategy generally applicable to any epitope modification was used for construction of an intron-tagged version of the human influenza virus hemagglutinin HA1-epitope. The HA coding sequence was split to four N-terminal and five C-terminal amino acid codons that were incorporated into two PCR primers to amplify intron IV2 of the potato gene ST-LS1, carried by the β-glucuronidase (*uidA*) gene in plasmid p35S GUS INT (Vancanneyt *et al.*, 1990). Suitable restriction endonuclease cleavage sites were placed 5′ upstream and 3′ downstream of the HA codons to allow an oriented cloning of modified HA sequences in the expression vector pPE1000 (Hancock *et al.*, 1997). Two different intron-tagged HA-epitope fusion vectors were constructed. In pPILY, the polylinker of pPE1000 was maintained to help the construction epitope fusions by insertion of coding sequences upstream or downstream of the IV2 intron-tagged HA coding domain (Figure 1). In pMENCHU the polylinker was modified to allow direct cloning of cDNAs from the yeast two-hybrid vector pACT2 (Durfee *et al.*, 1993) into a *BamH*I site placed in frame with the HA epitope (Figure 1). The HA epitope in both vectors was linked 5′ upstream to a CaMV35S promoter carrying a duplicated enhancer domain, and 3′ downstream to polyadenylation sequences of the nopaline synthase gene. To excise and clone this plant gene-expression cassette into *Agrobacterium* binary vectors for plant transformation, the vectors were provided with rare *Not*I cleavage sites in flanking positions, as well as a unique *Hin*dIII site in the polylinker for potential replacement of the CaMV35S promoter (Hancock *et al.*, 1997).



pPILY

H::A — *IV2* — H::A

*Hin*dIII *Nco*I *Sma*I *Xba*I *Bgl*II **Y P Y D** *intron* **V P D Y A** *Sal*I *Bam*HI *Pst*I *Eco*RI

CAAGCTTCCACC ATG GCG CCC GGG TCT AGA AGA TCT TAT CCA TAC GAT gt........ag GTT CCA GAT TAT GCT GAG TCG ACG GAT CCA CTG CAG TGA ATT C

*

pMENCHU

H::A — *IV2* — H::A

*Hin*dIII *Nco*I *Sma*I *Xba*I *Bgl*II **Y P Y D** *intron* **V P D Y A** *Sal*I *Bam*HI *Pst*I *Eco*RI

CAAGCTTCCACC ATG GCG CCC GGG TCT AGA AGA TCT TAT CCA TAC GAT gt........ag GTT CCA GAT TAT GCT GGT CGA CGG ATC CAC TGC AGT GAA TTC

Figure 1. Schematic map of intron-tagged epitope-fusion vectors pPILY and pMENCHU.
Restriction endonuclease cleavage sites and their positions are indicated on the map. Regions of functional importance are labelled by arrow-headed and stripped boxes. The nucleotide sequence of the IV2 intron-tagged HA epitope (H::A) coding region in the polylinker of the vectors is depicted below the map. The polylinker sequence in pPILY is identical to that of pPE1000 (Hancock *et al.*, 1997). In pMENCHU a sequence shift facilitates direct cloning of cDNAs from the yeast two-hybrid vector pACT2 into a *Bam*HI site in frame with the HA epitope-coding sequence. An asterisk indicates the presence of a stop codon at the *Eco*RI site in pPILY. Amp, ampicillin resistance gene; f1Ori and OriC, f1 phage and ColE1 plasmid replication origins, respectively; 35S, duplicated enhancer regions in the promoter of cauliflower mosaic virus 35S RNA gene (CaMV35S).

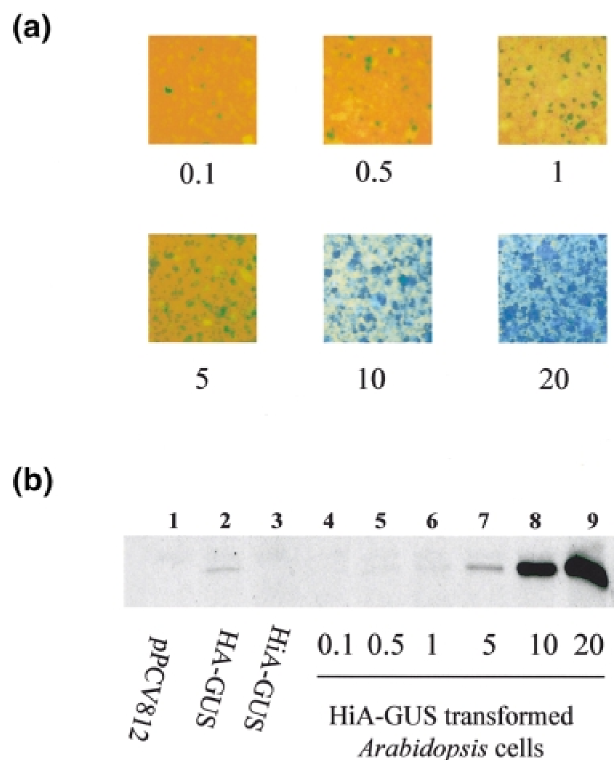


Figure 2. Detection of HiA-GUS expression in *Agrobacterium*-infected *Arabidopsis* cells.
(a) Optimization of *Agrobacterium*-mediated transformation of *Arabidopsis* cell suspension. X-Gluc staining of *Arabidopsis* cells 5 days after co-cultivation with *Agrobacterium*. Aliquots of 0.1, 0.5, 1, 5, 10 and 20 ml from an *Agrobacterium* culture (1×10^9 cells ml^{-1}) were added to equivalent amounts of pelleted *Arabidopsis* cells followed by adjusting the volume to 50 ml during subculturing.
(b) Western immunoblot of protein extracts prepared from *Agrobacterium* strains carrying pPCV812, HA-GUS and HiA-GUS, respectively (lanes 1–3) and *Agrobacterium*-infected *Arabidopsis* cell cultures expressing HiA-GUS in the titration experiment (lanes 4–9). As in (a), numbers indicate the amount of *Agrobacterium* inoculum.

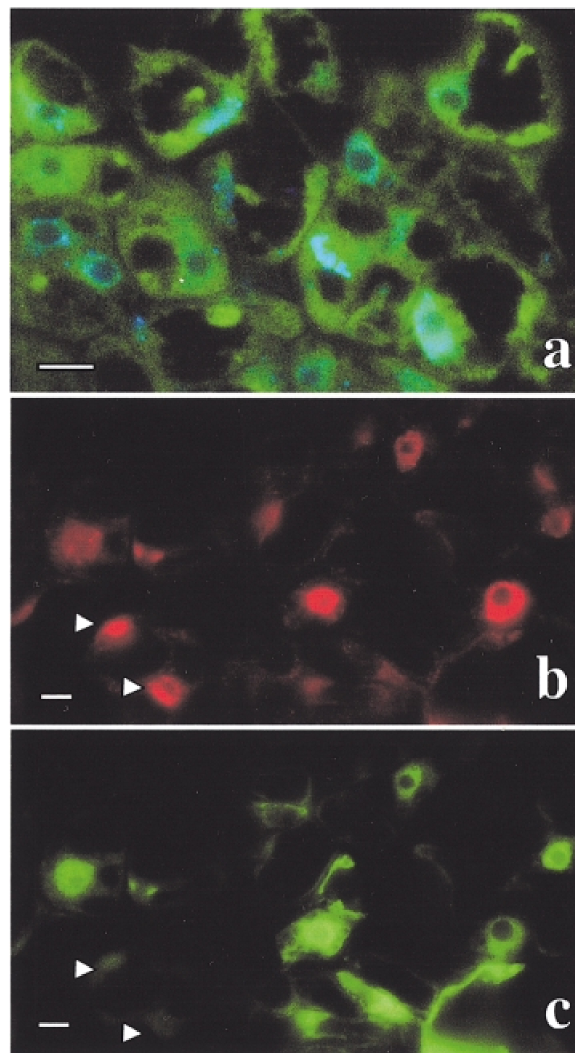


Figure 5. Immunodetection of HA epitopes in HiA-GUS- and SKP1-HiA-expressing *Arabidopsis* cells and localization of cellular SKP1-epitopes recognized by a polyclonal anti-AtSKP1 antibody.
(a) Immunofluorescence detection of HiA-GUS protein in the cytoplasm of transformed *Arabidopsis* cells with mouse anti-HA IgG traced by a FITC-labelled goat anti-mouse IgG. The nuclei were stained with 2 μg ml^{-1} DAPI. Bar = 10 μM.
(b) Cellular pattern of SKP1 epitopes resulting from the red fluorescence signal of Cy^{TM} 3-conjugated goat anti-rabbit IgG which detected the rabbit polyclonal anti-AtSKP1 IgG in *Arabidopsis* cells. The image was captured using an N2.1 filter (Leica). Bar = 10 μM.
(c) The section shown in (b) is viewed through an L4 filter detecting the green fluorescence of FITC-labelled anti-mouse IgG that traces the localization of mouse anti-HA antibody in *Agrobacterium*-transformed *Arabidopsis* cells expressing the SKP1-HiA protein. Note an absence of signal in the nucleus of some non-transformed cells marked by arrows. Bar = 10 μM.

Optimization of epitope immunoassays with a β-glucuronidase reporter protein using Agrobacterium*-mediated transformation of an* Arabidopsis *cell suspension*

To develop a procedure for rapid *in planta* detection of epitope-tagged proteins, a protocol for *Agrobacterium*-mediated transformation of *Arabidopsis* cell suspensions (Mathur and Koncz, 1998; Mathur *et al.*, 1998) was exploited in combination with the use of the β-glucuronidase (GUS) reporter protein (Jefferson, 1987). The *uidA*/*gusA* gene was moved as a *Sal*I-*Eco*RI fragment from pPE118-2 (a derivative of pPE120, Hancock *et al.*, 1997) into pPILY to create pHiA-GUS. The GUS expression cassette was then excised by *Not*I and inserted into the *Sma*I-*Sac*I sites of the binary vector pPCV812 (Koncz *et al.*, 1994) to yield the intron-tagged epitope construct HiA-GUS. The *uidA* gene in 5′ fusion with an HA epitope domain was moved in a similar manner from pPE118-2 into pPCV812 to obtain a control intronless epitope construct HA-GUS. Following introduction of binary vectors HiA-GUS and HA-GUS into *Agrobacterium* GV3101 (pMP90RK) (Koncz and Schell, 1986), different amounts of mid-log bacterial cultures were added to equal aliquots of *Arabidopsis*

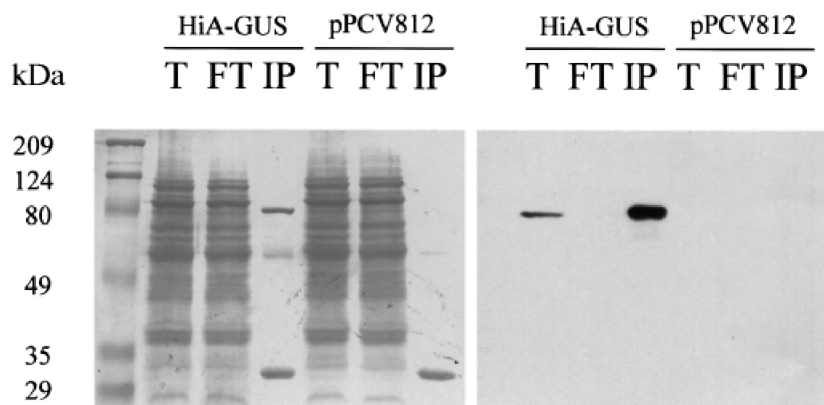


Figure 3. Immunoaffinity purification of HiA-GUS reporter protein.
Protein extracts prepared from 50 ml *Arabidopsis* cell cultures 5 days after infection with *Agrobacterium* strains carrying pHiA-GUS and pPCV812 were subjected to purification on an anti-HA.11 monoclonal IgG matrix. Samples from the total cell extract (T), column flow-through (FT), and immunoaffinity-purified (IP) protein fractions were separated on 10% SDS-PAGE and transferred onto a nitrocellulose filter. Ponceau staining of the filter is shown on the left; enhanced chemiluminescence (ECL) detection of the HiA-GUS protein after incubating the filter with a peroxidase-conjugated anti-HA antibody on the right.

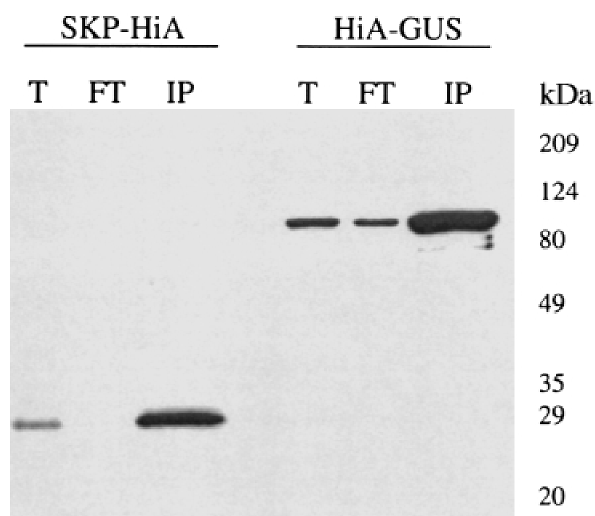


Figure 4. Purification of SKP1-HiA protein.
Arabidopsis cell suspensions were transformed with *Agrobacterium* strains carrying the SKP1-HiA and HiA-GUS constructs. Protein extracts prepared from *Arabidopsis* cells 5 days after *Agrobacterium*-infection were purified on an immobilized anti-HA IgG matrix. Protein samples from the total cell lysate (T), flow-through (FT), and immunoaffinity purified (IP) fractions obtained by elution with HA peptide were separated on 10% SDS-PAGE and transferred to a nitrocellulose filter for ECL detection with a peroxidase-conjugated anti-HA antibody. Due to overloading the affinity matrix, some HiA-GUS protein is also visible in the flow-through fraction.

(Col-0) cell suspensions to determine the optimal *Agrobacterium* titre that yields a maximal rate of transformation. A maximum in the frequency of transformed cells (70–80%) was detected by histochemical X-Gluc staining after 3 days, whereas a maximum in GUS activity was measured after 5 days of *Agrobacterium* infection (Figure 2a). To monitor the expression level of HA-tagged GUS proteins, cell extracts prepared from each *Agrobacterium*-infected *Arabidopsis* culture used in the titration experiment were subjected to Western immunoblotting with an anti-HA antibody. Protein extracts from *Agrobacterium* strains carrying the intron-tagged (HiA-GUS) and intronless (HA-GUS) epitope constructs, as well as an empty pPCV812 vector, served as controls. Expression of the intronless HA-GUS construct from the CaMV35S promoter was detectable in *Agrobacterium*, whereas no protein cross-reacting with the anti-HA antibody was found in bacterial cells carrying the intron-tagged HiA-GUS epitope construct (Figure 2b). The intensity of HiA-GUS immunodecoration detected in the infected *Arabidopsis* cells showed a perfect correlation with increasing amounts of *Agrobacterium* cells in the titration experiment. The results thus indicated that the intron-tagged epitope fusion technique is well suited for specific detection of an epitope-labeled protein in *Agrobacterium*-infected plant cells by a simple immunoassay that is not masked by artificial expression of the epitope-tagged protein in bacteria.

Agrobacterium infection of cell suspension provided an unlimited amount of transformed material to test whether the technique was also applicable to single-step immunoaffinity purification of the HA-IV2 epitope-tagged GUS protein from *Arabidopsis* cells. Protein extracts were prepared from *Arabidopsis* cultures 5 days after infection with *Agrobacterium* strains harbouring either the epitope construct HiA-GUS or the control plasmid pPCV812 (see Experimental procedures). The crude extracts (1–2 mg soluble protein) were pre-cleared with Sepharose-G and bound to HA.11 beads carrying monoclonal anti-HA IgG. Following stringent washes, the matrix-bound proteins were eluted, separated on SDS-PAGE together with the crude extracts and unbound protein fractions, and blotted to a membrane. As shown by Figure 3, the affinity-purified fraction from cells expressing the intron-tagged epitope construct contained a sufficiently high amount of HiA-GUS protein (2–5 μg) for detection with low-sensitivity Ponceau staining. A significant amount of IgG light chain and traces of IgG heavy chain found as contamination in this fraction

represented the only proteins recovered from the affinity matrix after binding of proteins from the control pPCV812-transformed cells. Western blotting with a peroxidase-conjugated anti-HA antibody failed to detect a cross-reacting protein in the flow-through fractions, indicating a quantitative recovery of HiA-GUS in the matrix-bound affinity-purified fraction (Figure 3).

Immunoaffinity purification and cellular localization of the ASK1 protein in Arabidopsis

The AtSKP1/ASK1 protein, encoded by a family of *SKP1-LIKE* genes in *Arabidopsis*, has recently been identified as a subunit of potential ubiquitin-ligase protein complexes implicated in the regulation of auxin signalling, floral organ identity and chromosome separation during male meiosis (Gray *et al.*, 1999; Samach *et al.*, 1999; Yang *et al.*, 1999; Zhao *et al.*, 1999). Characterization of diverse AtSKP1 complexes is thus an important task to understand the role played by ubiquitin-mediated proteolysis in plant cell differentiation and hormonal signalling (Del Pozo and Estelle, 1999). AtSKP1 was therefore chosen as an example to demonstrate that the intron-tagged epitope-labelling method is not only applicable to the stable β-glucuronidase reporter protein, but also allows the detection, purification and subcellular localization of a potentially less stable or abundant plant protein. The AtSKP1 cDNA was PCR-amplified and cloned in pPILY to construct a 3′ fusion with the HA-IV2 intron-tagged epitope. The expression cassette was moved by *Not*I into pPCV812, transferred into *Agrobacterium*, and used for expression of the SKP1-HiA protein in *Arabidopsis* cells. As an internal control, another identical batch of cells was transformed with the HiA-GUS construct. Five days after *Agrobacterium* infection protein extracts were prepared from the transformed cells and subjected to immunoaffinity purification as described above, except for that the HA-matrix-bound protein complexes were eluted using a synthetic HA-peptide (see Experimental procedures). The crude extract, flow-through and anti-HA IgG-bound fractions were separated by SDS-PAGE and analysed by immunoblotting with an anti-HA antibody. As shown in Figure 4, as with the control HiA-GUS protein, the procedure resulted in a single-step quantitative recovery of HA-epitope-labelled AtSKP1 protein by immunoaffinity purification.

AtSKP1 is one of 19 highly homologous SKP1-LIKE proteins in *Arabidopsis* (Samach *et al.*, 1999; Ökrész, unpublished results). Therefore expression of SKP1-HiA offered an excellent tool to compare the immunolocalization of epitope-tagged AtSKP1 protein, using anti-HA antibody, to that of homologous SKP1-LIKE proteins detected by an affinity purified polyclonal anti-AtSKP1 antibody in the same cells (see Experimental procedures). *Arabidopsis* cells transformed with SKP1-HiA and control HiA-GUS constructs were sectioned using a polyethylene glycol-embedding procedure. The control HiA-GUS protein was localized in the cytoplasm of cells after treating the sections with anti-HA IgG followed by incubation with FITC-conjugated goat anti-mouse IgG and staining the nuclei with DAPI (Figure 5a). Cells transformed with the SKP1-HiA construct were subjected to a simultaneous treatment with rabbit polyclonal anti-AtSKP1 and mouse anti-HA IgGs. Subsequently, the sections were incubated with a Cy^{TM} 3-conjugated anti-rabbit IgG, tracing the polyclonal anti-AtSKP1 IgG with red colour, as well as with a FITC-conjugated anti-mouse IgG, detecting the anti-HA IgG with green colour. The red fluorescence pattern seen in the cells with the polyclonal anti-AtSKP1 antibody perfectly matched with the SKP1-HiA-specific green fluorescence pattern, except for few cells that remained untransformed by *Agrobacterium* (Figure 5b,c). These staining patterns could, however, be selectively eliminated by pretreating the primary anti-AtSKP1 and anti-HA antibodies with either AtSKP1 or HA peptides, respectively, in control experiments (data not shown). The immunolocalization data obtained with the polyclonal anti-AtSKP1 IgG thus indicated that the AtSKP1 (ASK1) homologues are predominantly localized in and around the nucleus, and this pattern was faithfully replicated by specific detection of the SKP1-HiA protein with the anti-HA IgG.

Conclusions

This paper documents several applications of the intron-tagged epitope-fusion method. In its present form the technique is suitable for selective and sensitive immunodetection of epitope-tagged proteins in cells of an *Arabidopsis* suspension culture as early as 5 days after *Agrobacterium* infection. A sample of 50 ml *Agrobacterium*-infected cell culture yields 2–5 μg epitope-labelled protein purified by a single-step affinity chromatography that is detectable not only by sensitive Western immunoblotting, but also by Ponceau staining. Thus if an epitope-tagged protein is used for affinity purification of a protein complex, this amount should be sufficient not only for mass spectrometry analysis using MALDI-TOF (Blackstock and Weir, 1999), but also for analytical protein sequencing, even if further purification steps were involved. Whereas such applications remain to be worked out, e.g. with purified AtSKP1 complexes, the method already provides a sensitive tool for rapid subcellular localization of epitope-tagged proteins as shown for HiA-GUS and SKP1-HiA. There are two potential criticisms: overexpression of a protein by the CaMV35S promoter could lead to artefacts; and many proteins expressed in differentiated cell types could show artificial localization in quickly dividing cell suspensions. A solution to both

problems is presented: the CaMV35S promoter can readily be replaced by a regulated promoter of choice in the epitope-tagging vectors, whereas transgenic plants can easily be regenerated from the transformed *Arabidopsis* cells if required (Mathur *et al.*, 1998). The method is also applicable to labelling virtually any coding sequence with any intron-tagged epitope sequence that is amplified by PCR. This is an important facet for further applications aiming at an efficient confirmation of *in planta* association and co-localization of two proteins that are found to interact with each other, e.g. in the yeast two-hybrid system. To facilitate such applications by *Agrobacterium*-mediated co-transformation of plants cells with two different expression vectors (De Buck *et al.*, 1998), intron-tagged c-Myc-epitope vectors have been similarly constructed (Koncz-Kálmán, unpublished results). Other epitopes, such as the Strep-tag II peptide (Voss and Skerra, 1997), could provide alternatives for facile protein affinity purification. However, in each case it remains to be experimentally determined whether the corresponding antipeptide antibody provides such a low background in immunoassays with plant cells and proteins as is seen with anti-HA and anti-Myc IgGs (Boyes *et al.*, 1998; DeWitt and Sussman, 1995). As the intron-tagged epitope-labelling technique is applicable to any plant species transformable by *Agrobacterium*, further modifications, for example using different introns and transformation techniques, could extend its application widely. It remains a challenge for immunocytology to determine whether the intron-tagged epitope-labelling technique is sensitive enough to visualize the expression of proteins in different cell types of intact plants transformed by *Agrobacterium* infiltration, as described for detection of the expression of an intron-tagged β-glucuronidase gene *in planta* (Vancanneyt *et al.*, 1990).

Experimental procedures

Construction of intron-tagged epitope-fusion vectors

To PCR amplify intron IV2 of the potato gene ST-LS1 (Eckes *et al.*, 1986), a *Hin*dIII fragment of plasmid p35S GUS INT (Vancanneyt *et al.*, 1990) was used as a template in combination with the primers IHA1 5′-GGAGATCT**TATCCATACGAT**GTAAGTTTCTGCTTCTACCT-3′ and IHA2a 5′-GGGTCGACTC**AGCATAATCTGGAAC**CTGCACATCAACAAATTTTG-3′ carrying, respectively, four and five codons of the HA epitope-coding sequence (in bold). The PCR product was cloned as a *Bgl*II-*Sal*I fragment in pPE1000 (Hancock *et al.*, 1997) and confirmed by sequencing to yield the vector pPILY. Primer IHA2b 5′-GGGTCGACCAGCATAATCTGGAACCTGCACATCAACAAATTTTG-3′ with IHA1 was used to similarly amplify and clone a PCR fragment into pPE1000 to obtain pMENCHU. Plasmid pHiA-GUS was constructed by cloning the *uidA* gene as a *Sal*I-*Eco*RI fragment from pPE118-2 (Hancock *et al.*, 1997) in pPILY. A *uidA* gene in 5′ fusion with an intronless HA-coding sequence (HA-GUS) in pPE118-2 was used as a control. The AtSKP1 coding sequence was PCR amplified using the primers SKP-N 5′-GGGCCATGGTGTGATCCTCTGCGAAGAAGATTGTG-3′ and SKP-T 5′-GGGGAATTCTCAAGATCTTTCAAAGCCCATTGGTTCT-3′, subcloned as a *Nco*I-*Bgl*II fragment into pPILY, and verified by sequencing to yield pSKP1-HiA. Plant gene expression cassettes carrying the HA-GUS, HiA-GUS and SKP1-HiA constructs were cloned as *Not*I fragments into the *Sma*I and *Sac*I sites of pPCV812 (Koncz *et al.*, 1994) after filling in the sites with T4-DNA polymerase. The resulting binary vectors were introduced into *Agrobacterium* GV3101 (pPMP90RK) by electroporation (Maing *et al.*, 1995).

Agrobacterium *transformation of* Arabidopsis *cell suspension*

Agrobacterium strains carrying the binary vectors were grown in selective YEB medium (Koncz *et al.*, 1994) to OD_{600} 1.0, collected by centrifugation, and resuspended in 0.1 vol of cell culture CM-medium (1×10^{10} cells ml^{-1}). Following optimization of transformation with the HiA-GUS construct, 2 ml of *Agrobacterium* cells were routinely used for infection of 50 ml *Arabidopsis* suspension. After 3 days' co-cultivation, claforan (500 μg ml^{-1}, Hoechst, Germany) was added in order to arrest bacterial growth, and 2 days later the *Arabidopsis* cells were collected for GUS staining and preparation of protein extracts for immunoassays. To maintain stable transformed cell lines expressing the T-DNA-encoded hygromycin resistance marker and epitope labelled genes, 15 ml of *Arabidopsis* cells were subcultured weekly in 35 ml fresh CM medium containing 15 μg ml^{-1} hygromycin as well as claforan and plant hormones, as described (Mathur *et al.*, 1998). For histochemical GUS staining, cells from 1–2 ml of *Agrobacterium*-infected *Arabidopsis* culture were collected by centrifugation and incubated in 1–2 ml GUS buffer [1 g X-gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid cyclohexylammonium) dissolved in 2 ml DMSO and 1 ml Triton X-100 in 997 ml 0.1 m phosphate buffer pH 7.0; filter sterilized and stored at –20°C] at 26°C for 1 h before microscopy according to Mathur *et al.* (1998).

Immunoaffinity binding and Western blotting of epitope-tagged proteins

Protein samples from *Agrobacterium* were prepared by collecting cells from 1 ml culture (OD_{600}: 1.0) in 1× SDS sample buffer (Laemmli, 1970) followed by boiling the extracts for 5 min. To prepare plant protein extracts, cells from 50 ml culture were pelleted, homogenized to powder in liquid N_2 and resuspended on ice in 2 ml of 2× extraction buffer (100 mm Tris–HCl pH 7.6, 20% glycerol, 0.2% Igepal CA-630; 2 mm EDTA, 1 mm DTT, 1 mm PMSF, 2 mm benzamide, 4 μg ml^{-1} pepstatin and 1 μg ml^{-1} each of aprotinin and leupeptin). The cell lysate was cleared by centrifugation twice at 3000 ***g*** for 10 min, then at 100 000 ***g*** for 30 min at 4°C. Following the estimation of protein concentration in the cleared supernatant, the samples were further processed for immunoaffinity purification and Western blotting.

For immunoaffinity binding the extract (containing typically 1–2 mg protein) was supplemented with 150 mm NaCl, mixed with 50 μl Sepharose G beads (Sigma) equilibrated with IPW buffer (50 mm Tris–HCl pH 7.6, 150 mm NaCl, 0.1% Igepal CA-630), and incubated for 3 h at 4°C on a rocking platform. Subsequently the beads were removed by centrifugation (3000 ***g***, 30 sec) and the pre-cleared protein extract was incubated at 4°C on a rocking platform overnight at 4°C with 50 μl HA.11 monoclonal antibody affinity matrix (2 mg ml^{-1} purified anti-HA IgG beads, BabCO)

equilibrated with IPW buffer. After removing the supernatant, the matrix was transferred into a column and washed with 100 vol IPW buffer; 100 vol IPW buffer with 500 mm NaCl; and again with 100 vol IPW buffer. To recover the immunopurified HiA-GUS protein, the matrix was resuspended in one bead volume of 1× SDS sample buffer and boiled for 5 min. In contrast, the SKP1-HiA protein was eluted from the immunoaffinity matrix by peptide competition. After washing with IPW buffers, the beads were resuspended in half volume of elution buffer containing 2.5 mg ml^{-1} HA peptide (Genosys) and incubated for 15 min at 30°C. After collecting the supernatant, the elution was repeated and both eluted fractions were combined. An additional elution was performed by incubating the beads with 100 mm glycine (pH 2.9). The affinity HA beads were reconstituted by neutralization with TBS (25 mm Tris–HCl pH 8.0, 150 mm NaCl, 0.1% Tween 20) and stored at 4°C in TBS with 0.1% NaN_3.

For Western blot analysis, the protein samples were separated by SDS-PAGE, blotted to nitrocellulose filters (Protran, Schleicher & Schuell) and, after incubation with a peroxidase-coupled monoclonal anti-HA antibody (Boehringer Mannheim), the epitope-tagged proteins were detected using an enhanced chemiluminescence system (ECL, Amersham) as described (Ausubel *et al.*, 1999).

Immunofluorescence microscopy

Arabidopsis cells were fixed in 4% formaldehyde (freshly prepared from paraformaldehyde (Sigma) in stabilizing buffer: 50 mm PIPES, 5 mm $MgSO_4$, 5 mm EGTA pH 6.9) for 1 h, then washed three times with stabilizing-buffer for 10 min on ice and once with PBS (6.5 mm Na_2HPO_4 $2H_2O$, 1.5 mm KH_2PO_4, 2.5 mm KCl, 0.14 m NaCl pH 7.1). The samples were dehydrated in a graded ethanol/PBS series on ice and embedded in polyethylene glycol 400 distearate : 1-hexadecanol (10 : 1 mixture, Aldrich) at 37°C according to Vitha *et al.* (1997). After hardening the wax at room temperature, sections 4–5 µm thick were cut using a rotary microtome, and the ribbons were placed on slides coated with either glycerol-albumin or poly L-lysine, expanded by adding a drop of distilled water, and dried overnight at room temperature. To remove the wax, the dried samples were treated with decreasing ethanol/PBS series and washed with PBS. The samples were incubated with a mouse monoclonal anti-HA antibody (diluted 1 : 400 in stabilizing buffer containing 2% BSA, fraction V, fatty acid-free, Boehringer) for 1 h at room temperature in a moisture chamber, then washed three times with PBS. Subsequently the samples were similarly treated with FITC-conjugated goat anti-mouse IgG (Sigma, F-0257, dilution 1 : 200) for 1 h, washed three times with PBS and mounted with SlowFade^{TM} (Molecular Probes) to visualize the HA antigen in HiA-GUS-transformed cells. For double labelling, samples of SKP1-HiA-transformed cells were treated in a similar manner with a rabbit polyclonal anti-AtSKP1 antibody (dilution 1 : 250) and mouse anti-HA IgG, then incubated with FITC-conjugated goat anti-mouse IgG (1 : 200) in combination with a Cy^{TM} 3-conjugated anti-rabbit IgG (H + I, Jackson ImmunoResearch Laboratories, 1 : 400) as described above. The rabbit polyclonal anti-AtSKP1 antibody was raised against a C-terminal AtSKP1 peptide (KNDFTPEEEEEVRRE) that was marked by an N-terminal cysteine facilitating its conjugation to carrier proteins. This AtSKP1 peptide shared at least nine amino-acids with other known members of the SKP1-LIKE protein family in *Arabidopsis.* The polyclonal anti-AtSKP1 antibody was affinity purified using the AtSKP1 peptide linked to Affi-Prep 10 support (Bio-Rad) as described (Harlow and Lane, 1988). In control experiments, the antibodies were either independently or sequentially applied to the cell sections in order to examine and exclude a possible cross-reaction between monoclonal and polyclonal IgGs. The specificity of immunodetection was further tested by omitting the treatments with primary anti-HA and anti-AtSKP1 antibodies before the application of FITC-and Cy^{TM} 3-conjugated second antibodies. In competition experiments the primary polyclonal anti-AtSKP1 and monoclonal anti-HA antibodies were blocked with an excessive amount of SKP1 and HA peptides, respectively, overnight at 4°C before applying them onto the sections to confirm that these treatments abolish the signal in immunodetection. Fluorescence images were examined with a Leica DMRB epifluorescence microscope and photographed with a Hitachi HV-C20 camera controlled by a diskus microscopic computer programme (Carl H. Hilgers, Technisches Büro, Köln, Germany).

Acknowledgements

We thank Drs Paul Ealing and Kerrie Hancock for kindly providing the vectors pPE1000 and pPE118-2. This work was supported by a grant (Ko 1483/3-1) from the Deutsche Forschungsgemeinschaft and a stipend for J.J. by the Alexander von Humboldt Stifftung.

References

Ausubel, F., Brent, R., Kingston, R.E., Moore, J.G., Seidman, J.G., Smith, J.A. and Struhl, K. (1999) *Current Protocols in Molecular Biology*. New York: John Wiley & Sons.

Blackstock, W.P. and Weir, M.P. (1999) Proteomics: quatitative and physical mapping of cellular proteins. *Trends Biotech.* **17**, 121–127.

Boyes, C.D., Nam, J. and Dangl, J.L. (1998) The *Arabidopsis thaliana RPM1* disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. *Proc. Natl Acad. Sci. USA*, **95**, 15849–15854.

De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. (1998) *Agrobacterium tumefaciens* transformation and cotransformation frequencies in *Arabidopsis thaliana* root explants and tobacco protoplasts. *Mol. Plant-Microbe Interact.* **11**, 449–457.

Del Pozo, C.J. and Estelle, M. (1999) Function of the ubiquitin-proteosome pathway in auxin response. *Trends Plant Sci.* **4**, 107–112.

DeWitt, N.D. and Sussman, M.R. (1995) Immunocytological localization of an epitope-tagged plasma membrane proton pump (H^+-ATPase) in phloem companion cells. *Plant Cell*, **7**, 2053–2967.

Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A.E., Lee, W.-H. and Elledge, S.J. (1993) The retinoblastoma protein associates with the protein phosphatase 1 catalytic subunit. *Genes Devel.* **7**, 555–569.

Eckes, P., Rosahl, S., Schell, J. and Willmitzer, L. (1986) Isolation and characterization of a light-inducible, organ-specific gene from potato and analysis of its expression after tagging and transfer into tobacco and potato shoots. *Mol. Gen. Genet.* **205**, 14–22.

Gray, W.M., del Pozo, C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H. and Estelle, M. (1999) Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Devel.* **13**, 1678–1691.

Hancock, K.R., Phillips, L.D., White, D.W.R. and Ealing, P.M. (1997)

pPE1000: a versatile vector for the expression of epitope tagged foreign proteins in transgenic plants. *Biotechniques*, **22**, 861–865.

Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Jarvik, J.W. and Telmer, C.A. (1998) Epitope tagging. *Annu. Rev. Genet.* **32**, 601–618.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the *GUS* gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.

Koncz, C. and Schell, J. (1986) The promoter of T_L-DNA gene 5 controls the tissue specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**, 383–396.

Koncz, C., Martini, N., Szabados, L., Hrouda, M., Bachmair, A. and Schell, J. (1994) Specialized vectors for gene tagging and expression studies. In *Plant Molecular Biology Manual*, Vol. B2 (Gelvin, S.B., Schilperoort, R.A. and Verma, D.P.S., eds). Dordrecht: Kluwer, pp. 1–22.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T3. *Nature*, **227**, 680–685.

Maing, G.D., Reynolds, S. and Gartland, J.S. (1995) Electroporation protocols for *Agrobacterium*. In *Methods in Molecular Biology, Agrobacterium Protocols*, Vol. 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Humana Press, pp. 405–412.

Mathur, J. and Koncz, C. (1998) Establishment and maintenance of cell suspensions. In *Methods in Molecular Biology*, Vol. 82 *Arabidopsis Protocols* (Martínez-Zapater, J. and Salinas, J., eds). Totowa: Humana Press, pp. 27–30.

Mathur, J., Szabados, L., Schaefer, S., Grunenberg, B., Lossow, A., Jonas-Straube, E., Schell, J., Koncz, C. and Koncz-Kálmán, Z. (1998) Gene identification with sequenced T-DNA tags generated by transformation of *Arabidopsis* cell suspension. *Plant J.* **13**, 707–716.

Samach, A., Klenz, J.E., Kohalmi, S.E., Risseeuw, E., Haughn, G.W. and Crosby, W.L. (1999) The *UNUSUAL FLORAL ORGANS* gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem. *Plant J.* **20**, 433–445.

Strizhov, N., Keller, M., Mathur, J., Koncz-Kálmán, Z., Bosch, D., Prudovsky, E., Schell, J., Sneh, B., Koncz, C. and Zilberstein, A. (1996) A synthetic *cryIC* gene, encoding a *Bacillus thuringiensis* δ-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco. *Proc. Natl Acad. Sci. USA*, **93**, 15012–15017.

Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L. and Rocha-Sosa, M. (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol. Gen. Genet.* **220**, 245–250.

Vitha, S., Baluska, F., Mews, M. and Volkmann, D. (1997) Immunofluorescence detection of F-actin on low melting point wax sections from plant tissues. *J. Histochem. Cytochem.* **45**, 89–95.

Voss, S. and Skerra, A. (1997) Mutagenesis of a flexible loop in streptavidin leads to higher affinity for the *Strep*-tag II peptide and improved performance in recombinant protein purification. *Protein Engineering*, **10**, 975–982.

Yang, M., Hu, Y., Lodhi, M., McCombie, R. and Ma, H. (1999) The *Arabidopsis SKP1-LIKE1* gene is esential for male meiosis and may control homologue separation. *Proc. Natl Acad. Sci. USA*, **96**, 11416–11421.

Zhao, D., Yang, M., Solava, J. and Ma, H. (1999) The *ASK1* gene regulates development and interacts with the *UFO* gene to control floral organ identity in *Arabidopsis*. *Dev. Genet.* **25**, 209–223.