An improved toolbox to unravel the plant cellular machinery by tandem affinity purification of *Arabidopsis* protein complexes

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Tandem affinity purification coupled to mass spectrometry (TAP-MS) is one of the most advanced methods to characterize protein complexes in plants, giving a comprehensive view on the protein-protein interactions (PPIs) of a certain protein of interest (bait). The bait protein is fused to a double affinity tag, which consists of a protein G tag and a streptavidin-binding peptide separated by a very specific protease cleavage site, allowing highly specific protein complex isolation under near-physiological conditions. Implementation of this optimized TAP tag, combined with ultrasensitive MS, means that these experiments can be performed on small amounts (25 mg of total protein) of protein extracts from *Arabidopsis* cell suspension cultures. It is also possible to use this approach to isolate low abundant protein complexes from *Arabidopsis* seedlings, thus opening perspectives for the exploration of protein complexes in a plant developmental context. Next to protocols for efficient biomass generation of seedlings (~7.5 months), we provide detailed protocols for TAP (1 d), and for sample preparation and liquid chromatography-tandem MS (LC-MS/MS; ~5 d), either from *Arabidopsis* seedlings or from cell cultures. For the identification of specific co-purifying proteins, we use an extended protein database and filter against a list of nonspecific proteins on the basis of the occurrence of a co-purified protein among 543 TAP experiments. The value of the provided protocols is illustrated through numerous applications described in recent literature.

INTRODUCTION

Well-organized, complex networks of interacting proteins are the basis of all cellular processes, and they are involved in nearly all aspects of plant growth and development. The elucidation of these PPIs is thus of utmost importance to define functions to protein-encoding genes and to gain more insight into the macromolecular protein complexes that regulate cellular functions.

Two major classes of experimental approaches are used to map PPIs: *in situ* analysis of protein complexes by affinity purification coupled to MS (AP-MS) and analysis of direct interactions between two proteins by binary approaches such as yeast two-hybrid (Y2H) or bimolecular fluorescence complementation (BiFC)[1]. Both approaches are complementary, exploring different subspaces of the whole protein interactome[2], and ideally they should be used in parallel. Recently, a comprehensive network of 6,200 PPIs was built for *Arabidopsis* by Y2H, analyzing ~30% of all protein-encoding genes[3]. Nevertheless, in contrast to most other model species, genome-wide PPI studies are lagging behind in plants.

In the past, protein complexes were classically isolated through immunoprecipitation with specific antibodies recognizing the bait protein[1,4]. These antibodies were often not available, especially for plant-specific proteins, which hindered high-throughput analysis of plant protein complexes. This problem was addressed by fusing an affinity handle to the protein of interest, allowing the development of generic and standardized AP protocols and increasing throughput[5,6]. One-step AP-MS protocols, however, suffer from a high false positive rate through co-purification of sticky or high-abundance contaminating proteins. This often demands integration of complex quantitative tools to discriminate the true interactors from false-positive interactions[7]. Moreover, detection of low-abundance proteins during MS is often masked by interference of peptides derived from these contaminating proteins[8]. In TAP, the bait is translationally fused to a double affinity tag[9–11]. By using this affinity handle, bait and interactors (preys) are purified in two consecutive purification steps under physiological conditions and with high specificity. Next, co-purifying proteins are digested and identified by MS. When TAP was compared with single-step AP[9], the specificity of the isolated protein complexes was improved markedly, lowering the false negative rate. By using TAP, genome-wide interactomes were mapped for several model species[12–16].

The strength of AP-MS analysis lies in the proteome-wide elucidation of interactions for a protein of interest (bait), both direct and indirect, without the need for intensive genome-wide screening of a whole library of open reading frames. Over the past decade, we have developed a successful TAP technology platform for *Arabidopsis* cell suspension cultures[10,17,18] (**Box 1**), and we discovered protein complexes involved in a plethora of basic cellular processes and explored different cellular compartments.

As described below, our TAP platform is used for many applications, including functional analysis of proteins or whole protein complexes, discovery of new proteins functioning in a defined molecular pathway and mapping of protein networks. The function of a protein can be derived by the nature of its interacting

Box 1 | Cell culture TAP protocol

Cell cultures are an ideal system to isolate protein complexes involved in basic cellular pathways, out of a developmental context, offering a fast and unlimited supply of biomaterial. After cell culture transformation through cocultivation with *Agrobacteria* containing a TAP expression vector, transformed cells are directly selected in liquid medium and gradually scaled up, as previously described[17]. During TAP, 25 mg of total protein extract is used as input. After streptavidin elution (step 23 in this Box), analyze the sample as described in the main PROCEDURE starting from protein separation and staining (Step 50).

ADDITIONAL MATERIALS

Additional materials specifically used for TAP on cell cultures are listed below; if not specified, all other materials used are similar as described for TAP on seedlings.

EQUIPMENT

- Tube, 12 ml (Greiner, cat. no. 187261)
- Tube, 15 ml (Corning, cat. no. 430791)
- Dispersing probe S 25 N (VWR International, 10 gauge, cat. no. 431-2452; 25 gauge, cat. no. 431-2456)
- GF prefilter (Sartorius, cat. no. 17824)
- Homogenizer, T 25 digital Ultra-Turrax (VWR International, cat. no. 431-2881)
- Syringe filter RC25, 0.45 µm (Sartorius, cat. no. 17765)

REAGENT SETUP

- The *A. thaliana* cell suspension cultures are derived from ecotype Landsberg erecta, and they can be ordered at the *Arabidopsis* Biological Resource Center (ABRC). PSB-D (ABRC clone no. CCL84840) grows in darkness at 25 °C, and PSB-L (ABRC clone no. CCL84841) grows in a 16-h light/8-h dark cycle at 21 °C, both with shaking at 130 r.p.m. Approximately 15 g of transformed cells are collected from 1 liter of cell culture on a sintered glass pore filter 3 d after subculturing. Cells are frozen in liquid nitrogen and stored at –80 °C until further use. Cells can be stored for at least 1 year.

PROCEDURE

Protein extraction ● TIMING ~2 h

1. Grind 2.5 g of collected cell culture cells (see Reagent Setup in this box) in liquid nitrogen with a mortar and a pestle to a fine powder; transfer the powder to a precooled 12-ml tube and add 1.67 ml of ice-cold extraction buffer.

▲ **CRITICAL STEP** Typically, cells are collected 3 d after subculturing. However, depending on the bait, alternative collection times might be required. We always perform TAP on cell cultures in duplicate, starting from 5 g of cells with 3.33 ml of buffer in a 50-ml tube.

2. Add Benzonase to a final concentration of 0.1% (vol/vol).
3. Mix it 3 times for 30 s with an Ultra-Turrax homogenizer using a precooled dispersing probe (10 gauge for 2.5 g of cells; 25 gauge for 5–10 g of cells).

▲ **CRITICAL STEP** Cool the sample between each mixing step on ice for at least 30 s to prevent heating of the extract and protein degradation.

4. Incubate the mixture for 30 min on a tube rotator at 4 °C.
5. Divide the extract in 2-ml protein LoBind tubes, and centrifuge the tubes twice for 20 min at 20,800*g* in a centrifuge at 4 °C. Carefully transfer the supernatant to 1.5-ml precooled protein LoBind tubes in between the two centrifugation steps.
6. Collect the supernatant and filter it through a GF prefilter combined with a 0.45-µm syringe filter.
7. Determine the protein concentration via a standard Bradford method[17].

Tandem affinity purification ● TIMING ~4 h

8. *IgG-based AP (steps 8–17)*. Equilibrate 25 µl of IgG-Sepharose beads by washing the beads three times with 250 µl of extraction buffer. Centrifuge the beads in between washing steps for 1 min at 450*g* at 4 °C, and carefully remove the supernatant.
9. Add 25 mg of total protein extract to the equilibrated IgG-Sepharose beads in a 2-ml protein LoBind tube.

▲ **CRITICAL STEP** If the bait fusion accumulation level is very low, or if no bait or interactors are identified after performing Steps 80 and 81, start this procedure again using a higher input of 100 mg or 200 mg of total protein extract derived from 10 g or 20 g of cell suspension cells. For extract preparation, the volume of the extraction buffer used changes proportionally to the amount of cells used. For 100 mg or 200 mg of input, use 25 U or 50 U of rhinovirus 3C protease, respectively, both in step 14 and in step 15. However, all other components used during the TAP (e.g., amount of IgG beads, wash buffer and so on) remain the same.

10. Incubate the mixture for 1 h at 4 °C on a tube rotator.
11. Transfer the mixture stepwise (750 µl per step) to a Mobicol column (with 35-µm bottom filter) assembled on a vacuum manifold, and carefully remove the unbound fraction by applying vacuum.
12. Wash the beads five times with 750 µl of wash buffer (3.75 ml in total).
13. Remove the Mobicol column from the vacuum manifold and close it at the bottom with a plug.
14. Add 100 µl of wash buffer and 10 U rhinovirus 3C protease to the Mobicol column and close it with the Luer-lock cap.
15. Incubate the mixture for 1 h at 4 °C on a tube rotator. After 30 min, apply a second boost of 10 U.
16. Remove the plug and transfer the Mobicol column to a 1.5-ml protein LoBind tube. Collect the eluate by a 1-min centrifugation step at 450*g* at 4 °C.

(continued)

Box 1 | (continued)

17. Add 100 µl of wash buffer to the IgG beads in the Mobicol column and collect the eluate by repeating the centrifugation from step 16.
18. *Streptavidin-based affinity purification* (steps 18–26). Add the IgG eluate to 25-µL Streptavidin beads, equilibrated in wash buffer as previously described in step 8.
19. Incubate the mixture for 1 h at 4 °C on a tube rotator in a 1.5-ml protein LoBind tube.
20. Transfer the mixture to a Mobicol column assembled on a vacuum manifold, and carefully remove the unbound fraction by applying vacuum.
21. Slowly wash the beads three times with 833 µl of wash buffer (2.5 ml in total) by gravity, and repeat step 13.
22. Elute the complexes from the streptavidin beads by adding 30 µl of 1× NuPAGE sample buffer supplemented with 1× NuPAGE reducing agent and 20 mM desthiobiotin, and incubate for 10 min at room temperature with regular mixing.
23. Repeat step 16.

■ **PAUSE POINT** The protein sample can be stored at –80 °C for several weeks.

proteins, according to the 'guilt by association' principle. By using this approach, the E2F target gene *ETG1* was found to interact with members of the minichromosome maintenance replisome complex, and it was assigned a role in DNA replication[19,20]. TAP is also a very efficient protein discovery tool. By using known players of a certain pathway of interest as bait, new proteins are identified with a possible role in that pathway. This is elegantly demonstrated by the discovery of NINJA (novel interactor of JAZ) as a novel player in jasmonate signaling through differential TAP analysis on jasmonate-elicited cell cultures[21]. A more detailed view of an entire protein complex can be obtained when multiple TAP screens are performed on different members of the complex, starting with TAP of a protein of interest, followed by 'reverse' TAP experiments using the co-purified proteins as bait. In doing so, it becomes feasible to delineate the different protein complexes that are present in a list of co-purifying proteins, because a bait protein can participate in multiple protein complexes. By adding binary PPI approaches such as Y2H or BiFC, direct interactions can be distinguished, further unraveling the molecular architecture of the complex.

Additional confirmation of the protein complex can be achieved through integration of, e.g., subcellular localization and mutant analyses, as members of a protein complex should reside in the same cellular compartment and often give rise to similar phenotypes after perturbation, respectively. Such a combinatorial approach was used to characterize the TPLATE complex, a robust eight-core-component protein complex that acts as an adapter module for clathrin-mediated endocytosis in plants[22]. A similar strategy was followed to characterize a protein complex that targets protein phosphatase 2A activity to microtubules, regulating the transition from the interphase to the premitotic microtubule array during cell division[23].

More comprehensive networks can be built through TAP studies on a whole set of proteins involved in a particular biological process, extending the network and identifying new links with other pathways. Recently, we published a first draft of the *Arabidopsis* cell cycle interactome using ~100 cell cycle proteins as bait[24]. Besides a central regulatory network of core complexes, a peripheral network was identified, which linked the core cell cycle machinery to upstream and downstream pathways. A whole set of new candidate cell cycle proteins were defined, of which some are already confirmed to have a pivotal role in cell proliferation *in planta*[25,26]. For example, ethylene-responsive transcription factor ERF115 (ERF115) was confirmed as a substrate of the anaphase-promoting complex (APC), controlling root quiescent center cell division and stem cell replenishment[27].

Since the publication of our previous detailed TAP protocols using cell cultures[17], we steadily continued optimizing the technology. Here we present our latest TAP protocols (**Fig. 1**), now using a highly specific improved GS^{rhino}-TAP tag (**Fig. 2**), a derivative of the previously described GS-TAP tag[10,28], consisting of the protein G tag and the streptavidin-binding peptide, but now separated by rhinovirus 3C protease cleavage sites instead of tobacco etch virus (TEV) cleavage sites. This GS^{rhino}-TAP tag is more suitable for studying unstable proteins with a high turnover. For flexible cloning, we offer a toolbox of multisite Gateway-compatible TAP vectors. Through integration of ultrasensitive MS, sensitivity was increased, allowing downscaling of the amount of input material from cell cultures (**Box 1**). Although we successfully isolated many protein complexes with TAP on cell cultures, we are aware that this system has to deal with some limitations when screening interactions involved in a developmental context. Therefore, we adapted our cell culture–based strategy[17] for TAP on *Arabidopsis* seedlings. This protocol has been successfully applied for AP of the APC, which is a huge E3 ligase involved in cell proliferation, and it confirmed previous results obtained in cell culture and identified SAMBA as a novel plant-specific APC regulator involved in early plant development[25]. Further confirmation of the plant TAP protocol was achieved through co-purification of a switch/sucrose nonfermenting (SWI/SNF) chromatin remodeling complex using ANGUSTIFOLIA3 (AN3), a transcriptional coactivator that stimulates cell proliferation during *Arabidopsis* leaf development, as bait[29]. To ensure highly reliable protein identification and full coverage, we built the TAIRplus database (TAIR, The Arabidopsis Information Resource), a repository of all *Arabidopsis* protein sequences present in TAIR10 (ref. 30), extended with Common Repository of Adventitious Proteins (cRAP) protein sequences (The Global Proteome Machine, http://www.thegpm.org/crap/) and other non-TAIR10 protein sequences commonly found in TAP purifications, in total containing 35,839 sequence entries. Finally, for efficient filtering of nonspecific proteins, we defined a comprehensive list of 760 nonspecific binders on the basis of protein occurrence in 543 GS-based TAP experiments using 115 different bait proteins.

Implementation of the improved GS^{rhino} tag for TAP

Many alternative versions of the original TAP tag are now available[31,32]. Introduction of the GS tag increased the purification

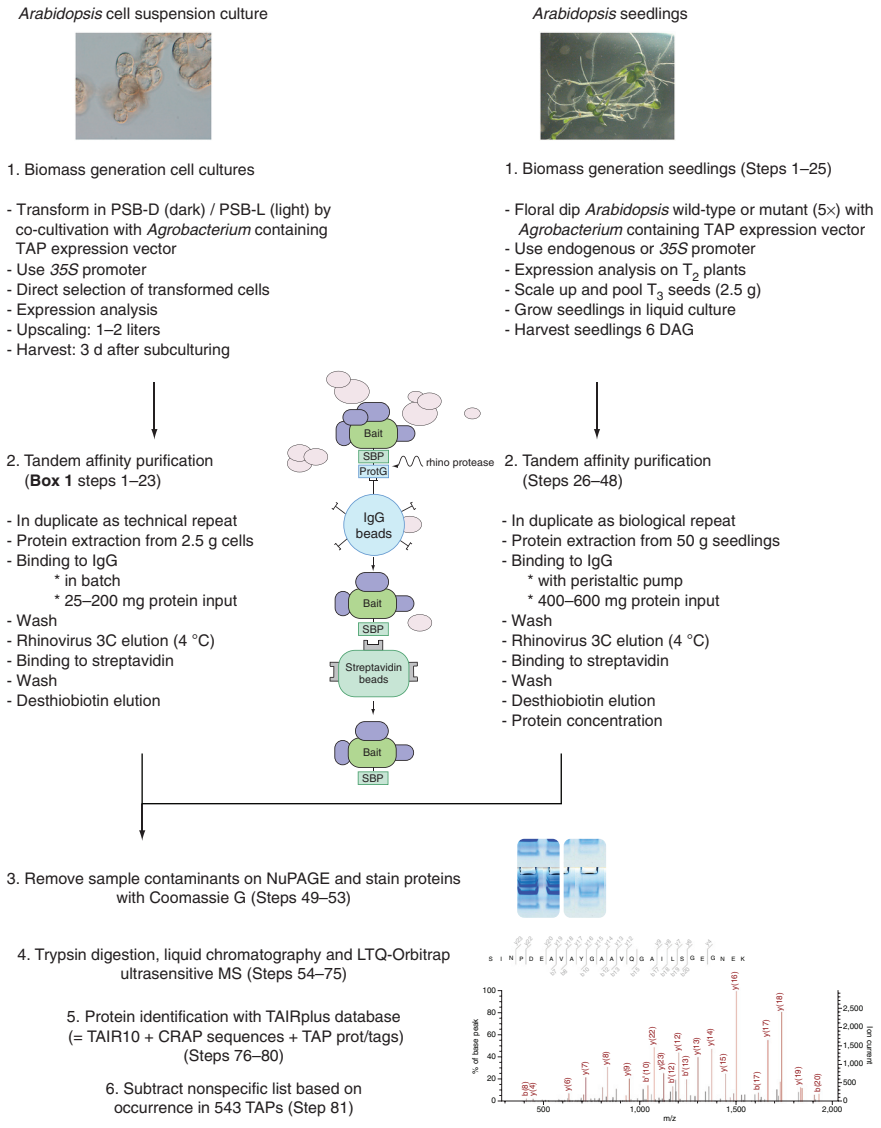

Figure 1 | Workflow of the TAP platform for *Arabidopsis* cell cultures and seedlings. PSB-D, cell suspension line that grows in the dark at 25 °C. PSB-L, cell suspension line that grows in a 16-h light/8-h dark regime at 21 °C. DAG, days after germination. *35S*, constitutive cauliflower mosaic virus promoter.

yield, the fusion protein accumulation levels and the specificity of the purification, thus lowering the amount of contaminating proteins[10,28,33], and in our hands it is currently the most optimal tag for TAP in plants. The GS tag combines a protein G tag with a streptavidin-binding peptide (SBP). In the first purification step, native protein complexes that incorporate the transgenic bait are isolated through high-affinity binding on an IgG resin. After a first washing step, gentle but specific elution is achieved through incubation with tobacco etch virus (TEV) protease, which will recognize its corresponding cleavage site present in tandem between the two tags. In the second purification step, the protein complexes of interest are trapped through binding to streptavidin-conjugated beads. Next, residual TEV protease and contaminating proteins are washed away. Despite the high affinity of the SBP-streptavidin interaction, the complexes can be easily released through competitive elution with buffer containing (desthio)biotin. To obtain an idea of the purity of the isolated protein complexes, proteins can be separated by 1D gel electrophoresis and stained with Coomassie G (**Fig. 3**). A mock TAP on wild-type cell culture extracts clearly reveals the high specificity of the GS-TAP protocol. The purity of the isolated complexes is shown for three different members of the AN3 chromatin remodeling complex and for the APC with SAMBA as bait. Comparison of two TAP eluates from a technical repeat further allows the visualization of the high reproducibility of the protocol. After TAP, the isolated protein complexes are processed for protein identification by MS. Alternatively, the complexes can be used for enzymatic assays[18], identification of post-translational modifications[34], or even for structure determination[35,36].

To reduce possible degradation of unstable proteins during the 1-h incubation at 16 °C with TEV protease, we incorporated a tandem repeat of the highly specific human rhinovirus 3C cleavage site in the GS tag[10,37] (**Fig. 2**), allowing efficient cleavage in 1 h at 4 °C. To compare both tags, we performed TAP analyses on cell cultures expressing the APC regulator SAMBA as bait, fused to either the GS^{TEV} or GS^{rhino} tag. In accordance with the lower temperature used during the first elution, more interactors were found with the GS^{rhino} tag (**Table 1**). This was further confirmed using the core cell cycle protein cyclin-dependent kinase A-1 (CDKA1) as bait (**Supplementary Table 1**).

Fast and flexible Gateway-compatible cloning

For efficient cloning of chimeric constructs encoding GS-TAP fusions, we offer a toolbox of recombination-based Gateway-compatible vectors, allowing both N- and C-terminal fusions (**Table 2** and **Fig. 4**). Because the 21.9-kDa GS^{rhino} tag might interfere with native complex assembly, typically both N- and C-terminal fusions are analyzed to increase the TAP success rate. To drive transcription of the transgene in cell cultures, we use the constitutively active *35S* cauliflower mosaic virus promoter. We also use this constitutive promoter for TAP on seedlings, because often the used endogenous promoter clone is not completely functional, leading to final bait accumulation levels that are too low for successful TAP-MS. The 'systematic analysis of *Arabidopsis* promoters' (SAP) repertoire, for example, contains Gateway-compatible promoter amplicons of ~2.0 kb upstream of the translation initiation codon[38], and because of this important distant transcription regulatory elements (e.g., enhancers) might be missed, sometimes giving rise to incomplete functional promoters. However, when a functional endogenous promoter clone is available, it can be used for the expression of the TAP fusion protein, ideally in the corresponding

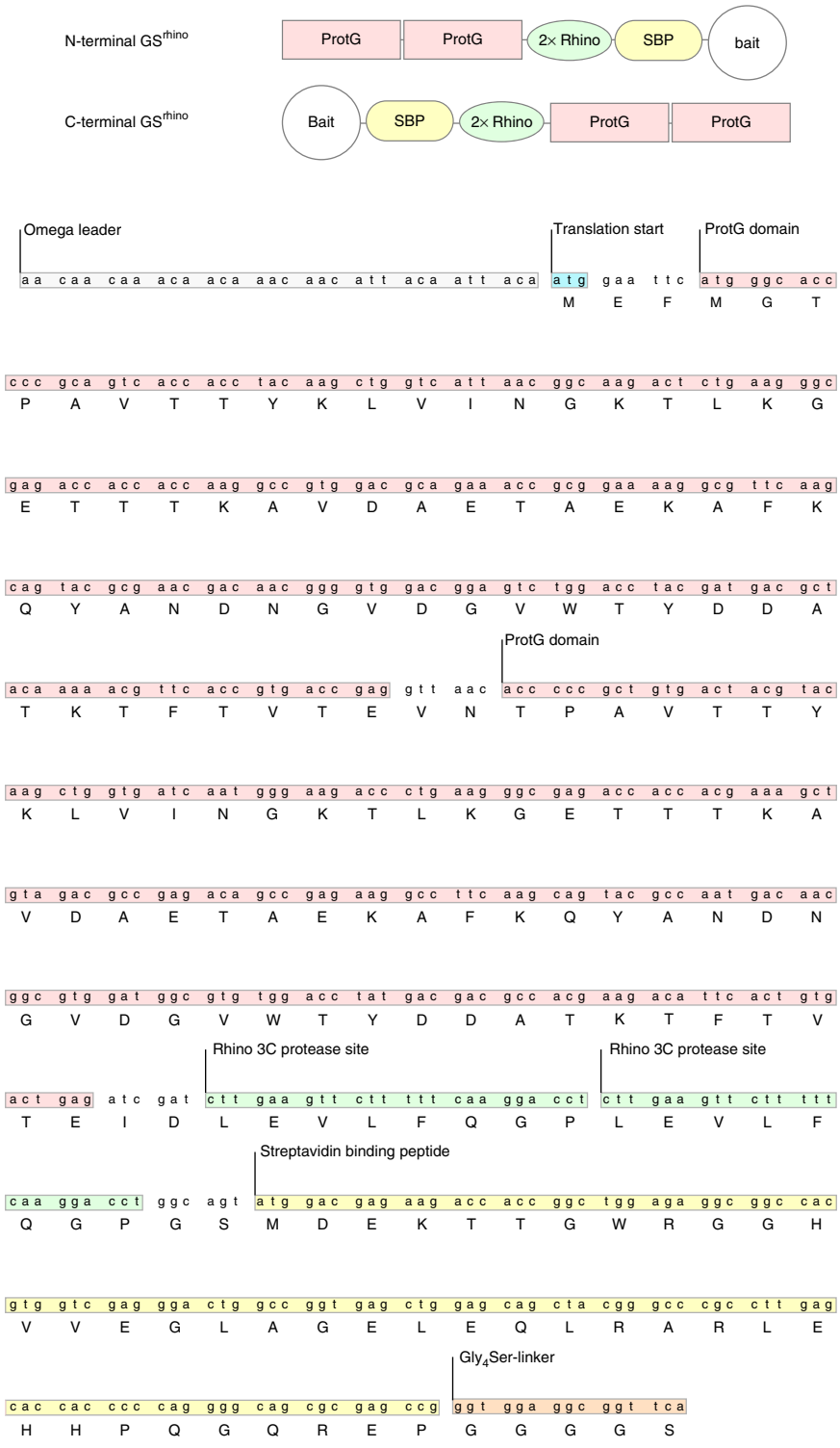

Figure 2 | Schematic representation of the GS^{rhino} tag fused to either the N or C terminus of the bait protein of interest. In addition, the nucleotide and amino-acid sequence of the N-terminal GS^{rhino} tag is shown, including annotation of the two protein G domains, the tandem repeat of the rhinovirus 3C protease cleavage site and the streptavidin-binding peptide (SBP). The start codon is preceded by a tobacco mosaic virus omega leader sequence to enhance translation. The Gly_4Ser-linker serves as a peptide linker connecting the tag to the bait protein.

mutant background. Our cloning strategy is compatible with multisite Gateway destination vectors containing either *npt*II (kanamycin), *hpt* (hygromycin) or *bar* (phosphinothricin) plant selection markers. Next to antibiotic- or herbicide-based selection, vectors harboring visual markers for screening are available. A major advantage of this Gateway-compatible vector set is the high flexibility, which allows fast cloning of alternative promoters or tags.

Cell suspension cultures versus *Arabidopsis* seedlings

For fast generation of an unlimited supply of biomass, we initially developed a TAP protocol for *Arabidopsis* cell suspension cultures, derived from the ecotype Landsberg erecta. After cocultivation with *Agrobacteria* containing the TAP expression vector, transformed cells are directly selected in liquid culture containing the appropriate selection agents, omitting the time-consuming step of clonal selection of transformed calli. Each transformed culture represents a mixture of many independent transformation events, eliminating deleterious effects when the transfer DNA (T-DNA) cassette is expressed too highly or too weakly, depending on the number of T-DNA insertions or the place of integration in the genome. Consequently, reproducible bait accumulation levels are obtained, often in the range of the endogenous protein, reflecting high levels of post-translational regulation of the bait proteins[18]. Because of the high ploidy level of these cultures (9*n*), the overall gene dosage is higher than *in planta*. This often leads to higher protein levels for the endogenous bait and its interactors in cell cultures. Therefore bait fusions are preferentially expressed from the *35S* promoter, because the fusion protein has to compete with its endogenous counterpart during complex assembly, and because important functional elements, such as enhancers, are often missed in endogenous promoter entry clones[18]. Although these cultures seem artificial, the use of such systems is more the rule than the exception in, e.g., mammalian protein complex studies. TAP on *Arabidopsis* cell suspension cultures (**Box 1**) has proven to be an excellent hypothesis-generating tool. So far, our platform covered a wealth of basic cellular processes, such as cell cycle regulation[18,19,23,24,26,39–43], hormone signaling[21,44–49] and intracellular trafficking[22,49–51]. We explored different cellular compartments, including technically more challenging compartments, such as mitochondria[42] and membranes[51,52]. When analyzing complexes residing in or

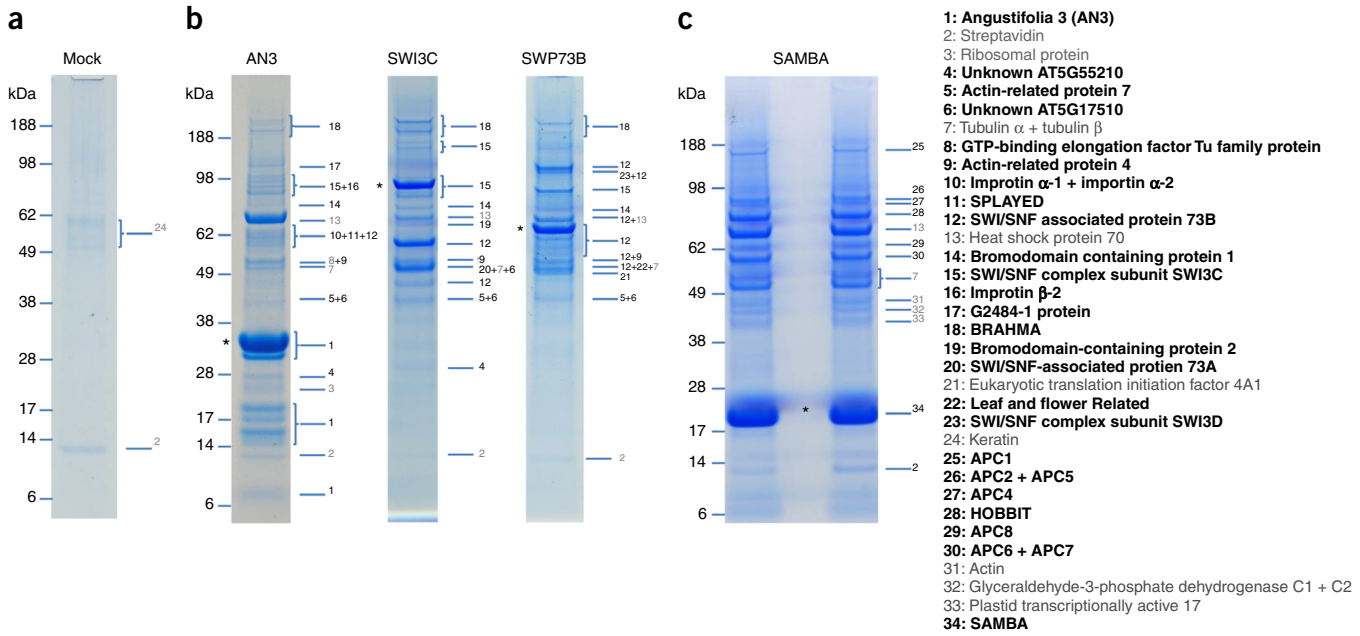

Figure 3 | SDS-PAGE analysis of TAP eluates, showing the high specificity and reproducibility of the GS-TAP protocol. (**a**,**b**) Proteins in GS-TAP eluates obtained from wild-type cell cultures (mock) (**a**), or cell cultures expressing GS fusions to AN3 (AT5G28640), SWI3C (AT1G21700), SWP73B (AT5G14170) (**b**) or SAMBA (AT1G32310) (**c**), were separated by high-performance SDS-PAGE at neutral pH (NuPAGE) and stained with Coomassie G. In **c**, TAP eluates from a technical repeat are shown using SAMBA as bait, demonstrating the high reproducibility of the TAP protocol. Proteins identified by MS and visually as protein bands are listed. Bait proteins are indicated with an asterisk. True interactors are indicated in bold, and nonspecific proteins are indicated in gray. APC, anaphase-promoting complex subunit.

attached to membranes, we implement the nonionic detergent digitonin during protein extraction and TAP. Dynamic alterations of the protein complex composition can efficiently be mapped through simple treatment of the culture with a specific agent and comparison with mock treatments. For this, a wide variety of chemicals can be applied to the cultures, such as phytohormones[21,48] or synchronization drugs[53]. Growing the cultures under conditions inducing differentiation to tracheary elements and secondary wall deposition even enabled the characterization of membrane-bound protein complexes involved in the lignin biosynthesis pathway[52]. We mostly work with the dark-growing PSB-D (dark) culture, but when analyzing light-regulated processes TAP can be performed as efficiently in the light-growing PSB-L (light) culture. This is nicely demonstrated by the characterization of the UV-B photoreceptor complex involved in the initiation of photomorphogenic responses[37]. In addition, these cultures can be used for chromatin immunoprecipitation to map gene regulatory networks[54].

Nevertheless, not all cellular pathways are active in these suspension cells, leading to false-negative results when certain biological processes are investigated. In particular, analysis of the proteins functioning in a certain plant developmental context is problematic owing to the proliferative nature of cell cultures and their lack of differentiation. To broaden the applicability of our TAP platform, we extended our method to *Arabidopsis* seedlings growing in liquid culture. Ideally, the transgene is expressed from the endogenous promoter and transformed in the corresponding mutant background to prevent competition with the endogenous bait protein during complex assembly. Complementation of the mutant phenotype immediately renders information on the functionality of the fusion protein. When the functional endogenous promoter and corresponding mutant are not available, we express transgenes from the constitutive *35S* promoter. In some cases, final bait accumulation levels with the *35S* promoter might be artificially high, leading to high levels of uncomplexed bait interfering with the detection of low abundant interactors during MS, or leading to aberrant protein interactions[55]. In that case, transgenic lines with the *35S* promoter are selected, which show more moderate expression levels, e.g., when the T-DNA is inserted in a less transcriptionally active chromatin region. For fast biomass generation, we provide guidelines for efficient selection of transformed plants and seed upscaling, with an optimized protocol for growing seedlings in liquid culture. Furthermore, the total protein input and TAP purification protocol were adapted for TAP on seedlings, where we start from 50 g of seedlings. However, when studying dynamics of low-abundance protein complexes, acting for example in a specific growth zone during leaf development, it is more feasible to use maize leaves[56], because it is very technically demanding to dissect the different growth zones of a developing organ in *Arabidopsis* and to obtain enough biomaterial for differential TAP. Therefore, we are currently transferring our platform to economically important crop species such as maize and rice.

Protein identification by ultrasensitive MS using the TAIRplus database

An important step after TAP is the unambiguous identification of all proteins present in the final eluate. The emergence of ultrasensitive MS and the availability of well-annotated plant genome sequences significantly increased the efficiency of this

TABLE 1 | Optimization of the TAP strategy.

		MALDI-TOF/TOF				LTQ Orbitrap Velos	
		GS^{TEV} (200 mg)		GS^{rhino} (200 mg)		GS^{rhino} (25 mg)	
Gene_ID	Protein name	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
AT1G32310	SAMBA	x	x	x	x	x	x
AT2G20000	CDC27b, HOBBIT	x	x	x	x	x	x
AT2G39090	APC7	x	x	x	x	x	x
AT3G48150	APC8, CDC23	x	x	x	x	x	x
AT5G05560	APC1	x	x	x	x	x	x
AT1G06590	APC5			x	x	x	x
AT1G78770	APC6, CDC16			x	x	x	x
AT2G04660	APC2			x	x	x	x
AT3G57860	UVI4-like/OSD1			x	x	x	x
AT4G21530	APC4		x	x	x	x	x
AT3G16320	CDC27a			x	x	x	x
AT2G18290	APC10					x	x
AT4G22910	CCS52A1					x	x
AT4G11920	CCS52A2					x	x
AT5G13840	CCS52B					x	x
AT4G33270	CDC20-1					x	
AT4G33260	CDC20-2					x	x
AT3G05870	APC11					x	
AT2G42260	UVI4			x			x
AT1G29200	Unknown protein	x					
AT5G60540	Pyridoxine biosynthesis 2	x					

Replacement of the GS^{TEV} tag by the GS^{rhino} tag, and implementation of ultrasensitive MS allowed downscaling of the TAP protocol, leading to more relevant interactions. TAPs were performed using SAMBA as bait in cell suspension cultures. The amount of total protein extract used as input is shown between brackets. Two experiments (Exp) are shown per condition. Nonspecific proteins were subtracted.

identification. We integrated the linear trap quadrupole (LTQ) Orbitrap Velos mass spectrometer for more accurate and sensitive protein identification.

In our approach, eluted proteins are submitted to a short (7 min) gel electrophoresis run on a gradient gel. The main objective of this step is not to separate the proteins but to remove any sample contaminants, such as detergents, that are not compatible with MS. After protein staining, the whole protein-containing zone from the top to the front is processed as one sample, and proteins are in-gel-digested with trypsin. Next, the complex mixture of peptides is separated by a nano-LC system in-line connected to an LTQ Orbitrap Velos mass spectrometer.

From each MS scan, the ten most abundant peaks are selected for fragmentation and MS/MS acquisition in the ion trap. Peak lists are generated, and the Mascot search engine is used for MS-based protein homology identification against the TAIRplus database. This database contains all 35,386 protein sequences present in the TAIR10 database[30]. However, to increase the number of peptide-spectrum matches (PSMs) and to minimize false positive identifications, we extended the TAIR10 protein database with sequences of all types of non-TAIR possible contaminants in TAP or in proteomics experiments in general. These contaminants include the cRAP protein sequences, a list of proteins commonly found in proteomics experiments, which are present either by accident or by unavoidable contamination of protein samples (The Global Proteome Machine, http://www.thegpm.org/crap/). In addition, commonly used tag sequences and typical TAP contaminants, such as sequences derived from the resins or the proteases used, were added. The TAIRplus database contains in total 35,839 sequence entries, and it is accessible at http://www.psb.ugent.be/tapdata (see Equipment). Proteins identified by at least two rank-1 peptides with a PSM confidence of >99%, of which at least one is unique to the protein, are retained. To estimate the false discovery rate (FDR) at the peptide level, a separate search is performed against a decoy database consisting of the reversed TAIRplus sequences. The number of positive identifications obtained from the decoy database is used to estimate the number of false positive PSMs with TAIRplus, assuming that there is an equal probability for incorrect PSMs in both TAIRplus and TAIRplus reversed[57]. The estimated FDR, calculated from a large data set of 543 TAP experiments and 503,629 PSMs for TAIRplus, is 1.4%. Implementation of ultrasensitive MS combined with the use of the GS^{rhino} tag enabled downscaling of the total protein input derived from cell suspension cultures from 200 mg to 25 mg, as compared with MS through tandem matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF). This was nicely demonstrated with SAMBA as bait (**Table 1**). The downscaling opens perspectives to apply chemical agents to the culture, such as, treatment with the cell proliferation synchronization agent aphidicolin. These improvements also allowed us to extend our TAP platform to *Arabidopsis* seedlings.

Determination of nonspecific proteins and data analysis

Despite the high specificity of the double AP procedure, many of the identified proteins after TAP are nonspecific interactors.

TABLE 2 | Overview of available vectors for TAP cloning.

Strategy	Entry vectors			Destination vectors
C-terminal fusions with *35S*, or endogenous promoter	Promoter	Bait	TAG	pKCTAP (with *nptII* and *GFP* marker)
	pEN-L4-2-R1 (with *35S* promoter)	pEN-L1-bait-L2 (without stop codon)	pEN-R2-GS_rhino-L3	pHCTAP (with *hpt* and *RFP* marker)
				pK7m34GW (*nptII*)
	pEN-L4-P_end-R1			pH7m34GW (*hpt*)
				pB7m34GW * (*bar*)
N-terminal fusions with *35S* or endogenous promoter	Promoter	TAG	Bait	pKCTAP (with *nptII* and *GFP* marker)
	pEN-L4-2-R1 (with *35S* promoter)	pEN-L1-NGS_rhino-L2	pEN-R2-bait-L3 (with stop codon)	pHCTAP (with *hpt* and *RFP* marker)
				pK7m34GW (*nptII*)
	pEN-L4-P_end-R1			pH7m34GW (*hpt*)
				pB7m34GW (*bar*)
N-terminal fusions with *35S*, and freedom of selection marker	Tag	Bait		pK7m24GW2 (*35S* on vector backbone; *nptII*)
	pEN-L4-NGS_rhino_R1	pEN-L1-bait-L2 (with stop codon)		pH7m24GW2 (*35S* on vector backbone; *hpt*)
				pB7m24GW2 (*35S* on vector backbone; *bar*)
N-terminal fusions with *35S*, and kanamycin selection	Promoter	Bait		pKNGS_rhino (*tag* on vector backbone; *nptII*)
	pEN-L4-2-L3 (with *35S* promoter)	pEN-L1-bait-L2 (with stop codon)		

More information on the composition of the used vectors and the cloning strategy can be found in **Figure 4**. All vectors can be ordered at http://gateway.psb.ugent.be/ . *When using the *bar* gene in cell culture, the phosphinothricin derivative bialaphos is preferably used.

These proteins are mainly high-abundance and promiscuous proteins present in almost all TAP eluates. Nonspecific background proteins are mostly determined by negative control experiments. In the past, we used mock wild-type protein extracts or protein extracts derived from cultures or seedlings expressing tagged heterologous bait proteins, such as GFP or β-glucuronidase, to define a background list.

Over time, we created an oversized background list (J.V.L., D.E. and G.D.J., unpublished data), comprising all negative control experiments that we ever performed, ranging from the original TAP tag giving a lot of background to the semipure TAP samples obtained with GS^{rhino}. This list filtered out most background proteins, but because of its size, i.e., ~1,800 proteins, the risk of losing bona fide interactors was increasing. In contrast, if we used a more restricted background list established with a set of 25 negative control experiments using our GS^{rhino} protocol on seedlings or cell cultures (J.V.L., D.E. and G.D.J., unpublished data), we observed that many proteins that were reoccurring in GS^{rhino}-TAP experiments with numerous unrelated baits were not present in this restricted background list, and thus they were escaping the background filtering. Therefore, we decided to take advantage of the huge amount of GS-TAP eluates analyzed on the LTQ Orbitrap Velos, and we built a list of nonspecific proteins based on the occurrence of proteins in 543 TAP experiments using 115 different bait proteins (**Fig. 5a**).

To reduce any bias produced by baits functioning in a similar biological process or possessing a similar molecular function, baits were classified into 62 groups: baits were grouped together if they were present in the same protein complex—based on protein complex knowledge, either from in-house data or from the literature—if they belonged to the same gene family[58] or if they were active in the same biological process according to their gene ontology annotation[59]. A stringent threshold was arbitrarily set in such a way that proteins present in three or more different

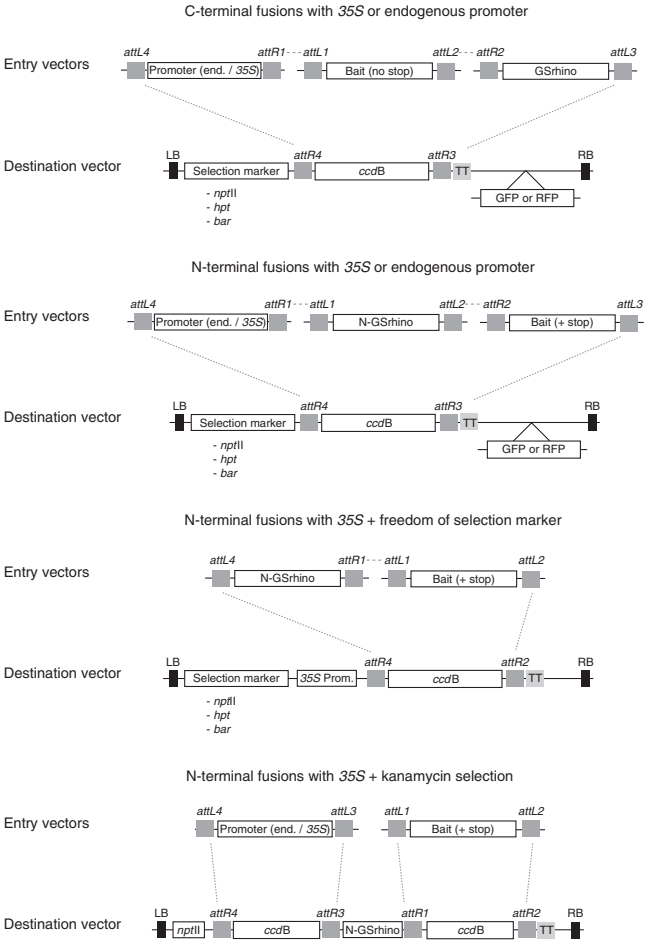

Figure 4 | Overview of the different multisite Gateway cloning strategies for fusion of the GS^{rhino} tag to the bait protein. More information on the exact names of the vectors can be found in **Table 2**. *att*, Gateway recombination site; *ccd*B, negative selection marker gene for gateway cloning; TT, transcription terminator; *npt*II, neomycin phosphotransferase II gene for Kanamycin selection; *hpt*, hygromycin phosphotransferase gene for hygromycin selection; *bar*, phosphinothricin acetyl transferase gene for selection with phosphinothricin (plants) or Bialaphos (cell culture).

bait groups were considered nonspecific. As such, a new list of 760 nonspecific proteins was generated (**Supplementary Table 2**). This is a more elegant and efficient way of filtering nonspecific proteins, with a decreased risk of losing bona fide interactors. More than 95% of all proteins identified in the 25 negative GS^{rhino} control experiments are present in our new list of nonspecific proteins, demonstrating its performance. In contrast, 18% of the list could not be found in our old extended background list of ~1,800 proteins, built merely with negative control experiments, further proving the efficacy of our new list.

The list contains mostly ubiquitously expressed proteins, as shown by an expression breadth analysis on the corresponding genes (**Fig. 5b**). They mainly include highly expressed housekeeping proteins such as ribosomal proteins, actins, tubulins, heat-shock proteins, elongation factors and metabolic enzymes. An additional set of sticky background proteins is present at a much lower concentration, and it is identified in some experiments, while escaping the detection limit in others. Although some of the nonspecific proteins might represent true interactors (for example, heat-shock proteins that assist in bait protein folding and complex assembly), often they are less relevant for further investigation and are ignored.

Semiquantitative analysis, by comparing the relative protein abundance across the large data set of 543 different TAP experiments, allowed us to retain genuine interactors present in the list of nonspecific proteins. For this, we first calculated normalized spectral abundance factors (NSAFs)[60], on the basis of the total number of spectra identifying each protein, normalized by the length of the protein and the total number of identified spectra in the sample. Next, nonspecific proteins were grouped into subsets, according to the number of bait groups in which they were identified. After plotting the average NSAF of nonspecific proteins per subset, it was clear that the most frequent nonspecific binders (n = 51–62) have the highest relative protein abundance (**Supplementary Fig. 1**), representing the most abundant background proteins, and that the average NSAF decreases proportional to the frequency of occurrence to a minimal value of 0.4%. However, for nonspecific proteins identified in only three or four bait groups, an increase was observed. This reflected the presence of a substantial amount of true interactors in these subsets, a consequence of the stringent filtering that was applied. In practice, for low-abundance nonspecific binders, identified in 3–10 different bait groups, the observed minimal average NSAF of 0.4% was used to calculate ΔNSAFs for nonspecific proteins in individual

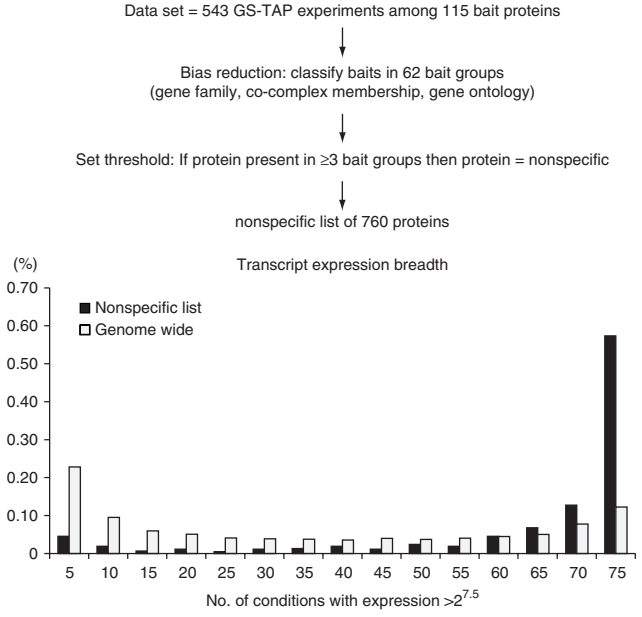

Figure 5 | Schemes for the determination of nonspecific proteins and transcript expression breadth of the nonspecific gene list. To compare the expression breadth for the list of nonspecific genes, the number of experiments in which a gene was expressed above background (expression value >$2^{7.5}$) was determined using an unbiased expression compendium. Starting from the CORNET microarray compendium 2 (TAIR10), covering 111 experiments[66], similarities between all experiments were computed using the Pearson correlation coefficient (PCC), and 75 unique experiments were retained (PCC < 0.95). Subsequently, for each gene, the number of experiments in which the expression was above background was counted. Genes lacking expression in any of the 75 experiments were discarded.

TAP experiments. A threshold of ΔNSAF ≥ 1% enabled the identification of possible bona fide interactors that would otherwise have been designated as nonspecific (**Supplementary Fig. 2**).

Finally, reproducibility is a very important indicator for the reliability of an interaction. Therefore, we always perform TAP experiments in duplicate. For TAP on cell culture (**Box 1**), we prepare one total protein extract, which is then divided over two TAP purifications performed in parallel as a technical repeat. Because of the highly reproducible TAP expression levels obtained in cell culture and because the transformed cell cultures represent a mixture of different transformed cells[18], it is not necessary to perform a biological repeat. For TAP on seedlings, however, we generate biomass coming from two independent transgenic lines, allowing biological TAP repeats. Validation of an interaction in such a repeat further adds confidence to the interaction, whereas interactions only found once should be handled with more care. Nevertheless, as demonstrated with the cell cycle interactome[24], interactions only found once might be true interactions, representing more transient or weak interactors that are worth selecting for confirmation through another PPI technique. Moreover, additional validation can be obtained when common interactions are observed between data obtained with N- and C-terminal TAP fusions, or in reverse TAP experiments using the preys as bait. Once a final interactor list is obtained, networks can be visualized using software such as Cytoscape[61], and additional information can be projected onto the network. As such, a better understanding of complex networks can be obtained through integration of transcript expression levels, tissue specificity or subcellular localization, *cis*-regulatory elements, protein motifs and protein domains.

MATERIALS

REAGENTS

- β-glycerophosphate (Sigma-Aldrich, cat. no. G9422)
- Acetic acid (CH_3CO_2H, glacial, 100%; Merck, cat. no. 100063)
- Acetone (CH_3COCH_3; Biosolve, cat. no. 010305)
- Acetonitrile (CH_3CN, 99.9%; Acros Organics, cat. no. 32675-0010)
- Agar, plant tissue culture (Lab M, cat. no. MC029)
- Benzonase nuclease (Novagen, cat. no. 70664-3)
- Bialaphos glufosinate analog (Goldbio, cat. no. B0178)
- C18 Reprosil-HD beads, 5 µm (Dr. Maisch, cat. no. r15.b9h)
- Coomassie brilliant blue G (Sigma-Aldrich, cat. no. B0770)
- Desthiobiotin (Sigma-Aldrich, cat. no. D-1411)
- Disinfectol (Chem-Lab NV, cat. no. CL00.0112)
- DTT (Sigma-Aldrich, cat. no. D0632)
- E-64 protease inhibitor (Sigma-Aldrich, cat. no. E3132)
- EDTA (Duchefa, cat. no. E0511)
- EDTA-free Ultra complete protease inhibitor cocktail tablets (Roche, cat. no. 06538282001)
- Ethylene glycol, anhydrous, 99.8% (Sigma-Aldrich, cat. no. 293237)
- Formic acid, 98–100%, standard laboratory reagent (SLR), extra pure (Fisher Scientific, cat. no. F/1850/PB08)
- HCl, 37% (wt/vol; Merck, cat. no. 100317)
- HPLC-grade water (Acros Organics, cat. no. 26830)
- Hygromycin (Duchefa, cat. no. H0192)
- IgG-Sepharose FF (GE Healthcare, cat. no. 17-0969-01)
- Iodoacetamide (GE Healthcare, cat. no. RPN6302)
- Kanamycin (Duchefa, cat. no. K0126)
- Liquid nitrogen
- $MgCl_2$ (Merck, cat. no. 105833)
- 2-(N-Morpholino)ethanesulfonic acid (MES; Duchefa, cat. no. M1503)
- Milli-Q ultrapurified water (from a Millipore system)
- Murashige and Skoog medium (Duchefa, cat. no. M0221)
- Myo-inositol (Sigma-Aldrich, cat. no. I5125)
- Na_3VO_4 (Sigma-Aldrich, cat. no. S6508)
- NaCl (VWR International, cat. no. 27810295)
- NaClO, 12% Cl (Roth, cat. no. 9062)
- NaF (Merck, cat. no. 106449)
- NH_4HCO_3 (Sigma-Aldrich, cat. no. A6141)
- NP-40 (USB, cat. no. 19628)
- NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, cat. no. NP0007)
- NuPAGE sample reducing agent (Invitrogen, cat. no. NP0004)
- Phosphinothricin/glufosinate (Sigma-Aldrich, cat. no. 45520)
- PMSF (Sigma-Aldrich, cat. no. P7626)
- *p*-Nitrophenyl phosphate (Sigma-Aldrich, cat. no. 71768)
- Peroxidase anti-peroxidase soluble complex antibody (Sigma-Aldrich, cat. no. P1291)
- Rhinovirus 3C protease (GE Healthcare, cat. no. 27-0843-01)
- Sterile water
- Streptavidin-Sepharose (GE Healthcare, cat. no. 17-5113-01)
- Sucrose (VWR Prolabo Chemicals, cat. no. 27483)
- Trichloroacetic acid, 6.1 N (Sigma-Aldrich, cat. no. T0699)
- Trifluoroacetic acid (Fisher Scientific, cat. no. T/3258/PB05)
- Tris (Biosolve, cat. no. 20092391)
- Trypsin gold, mass spectrometry grade (Promega, cat. no. V5280)
- Tween 20 (Duchefa, cat. no. P1362)

EQUIPMENT

- NuPAGE gradient gel, 4–12% (wt/vol) (Life Technologies, cat. no. NP0321)
- Tube, 5 ml (Becton Dickinson and Company, cat. no. 352063)
- Tube, 50 ml (e.g., Corning, cat. no. 430829 or Greiner, cat. no. 227261)
- Centrifuge tubes (Nalgene, cat. no. 525-2244)
- Cooled-down centrifuge for 1.5- and 2.0-ml tubes (e.g., Eppendorf, 5424R)
- Cooled-down centrifuge for 50-ml tubes (e.g., Eppendorf, 5810R)
- Filter tips (Greiner, 1,000 µl, cat. no. 740288; 200 µl, cat. no. 739288; 100 µl, cat. no. 772288; 10 µl, cat. no. 771288)
- Gel loading tips (Costar, cat. no. 4853)
- Glass plate
- Filter disc, grade 3 hw (Sartorius, cat. no. FT-3-303-055)
- Hand blender (e.g., Braun MR500)
- Ionizing air blower (e.g., Aerostat XC; Simco, cat. no. 4002667)
- LTQ Orbitrap Velos (Thermo Fisher Scientific) or equivalent high-performance mass analyzer. The LTQ Orbitrap Velos combines the high mass accuracy and ultra-high resolution of the Orbitrap mass analyzer, with the increased sensitivity and improved cycle time of the LTQ Velos. Specifications: mass range *m/z* 50–2,000, *m/z* 200–4,000; resolution 60,000 at *m/z* 400 at a scan rate of 1 Hz; mass accuracy <3 p.p.m. with external calibration, <1 p.p.m. with internal calibration; dynamic range >5,000 within a single scan
- Miracloth mesh, 22–25 µm (Calbiochem, cat. no. 475855)
- Mobicol column 'classic' (ImTec, cat. no. M1002)
- Mobicol column, 0.35-µm bottom filter (ImTec, cat. no. M513515)
- Nano LC system, UltiMate 3000 RSLC nano (Dionex)
- Nitrile gloves
- Orbital shaker (e.g., Bellco Biotechnology 7744-10220)
- Parafilm (Bemis flexible packaging, cat. no. PM-996)
- Peristaltic pump P-1 (GE Healthcare, cat. no. 18-1110-91)
- Poly-Prep chromatography column (Bio-Rad, cat. no. 731-1550)
- Protein LoBind tube (Eppendorf, 1.5 ml, cat. no. 022431081, 2.0 ml, cat. no. 022431102)
- Safe-Lock tubes, 1.5 ml (Eppendorf, cat. no. 0030.120.086)
- Scalpels (e.g., Swann-Morton, ref. 0103 and 0308)
- SeeBlue Plus2 pre-stained standard (Life Technologies, cat. no. LC5925)
- Sintered glass filter, 47 mm (Whatman, cat. no. 1960-004)
- Sieve mesh (Retsch, cat. no. 60131000400)
- Sonicator (e.g., Branson 2210)
- SpeedVac (e.g., Hetovac VR-1, Heto Drywinner, Edwards RV3 pump)
- Stainless steel wine cooler

• Superspeed centrifuge (e.g., Sorvall centrifuge RC 5 plus with SS34 rotor)
• Tube rotator (VWR International, cat. no. 444-0500)
• Vacuum manifold (Grace, cat. no. 210351)

Software
• Mascot search engine (version 2.4.1, MatrixScience, http://www.matrixscience.com/)
• Mascot Daemon interface (Matrix Science, http://www.matrixscience.com/)
• Web browser
• MS Excel 2010 (Microsoft)

Databases
• TAIR10 (ftp://ftp.Arabidopsis.org/home/tair/Proteins/TAIR10_protein_lists/)
• TAIRplus (http://www.psb.ugent.be/tapdata)
• TAIRplus reversed (http://www.psb.ugent.be/tapdata)
• cRAP protein sequences (The Global Proteome Machine, http://www.thegpm.org/crap/)
• List of nonspecific proteins (**Supplementary Table 2**)

REAGENT SETUP

1/2 MS medium Dissolve 2.15 g of Murashige and Skoog medium, 10 g of sucrose, 0.5 g of MES and 0.1 g of myo-inositol in 1 liter of Milli-Q water. Set the pH to 5.7 with KOH and sterilize it by autoclaving. The final concentrations are as follows: 2.15 grams per liter Murashige and Skoog, 10 grams per liter sucrose, 0.5 grams per liter MES and 0.1 grams per liter myo-inositol, pH 5.7. After cooling the medium to ~60 °C, add the appropriate filter-sterilized antibiotic to reach the following final concentrations: 25 µg/ml kanamycin or 20 µg/ml hygromycin or 15 µg/ml phosphinothricin; 1/2 MS medium can be stored for up to 3 months at room temperature (20–25 °C) or at 4 °C in the dark if antibiotics are added.

1/2 MS plates Add 8 g of plant tissue agar to 1 liter of 1/2 MS medium (pH 5.7) and sterilize it by autoclaving. Add the appropriate antibiotics as described for liquid 1/2 MS medium; 1/2 MS plates can be stored at 4 °C in the dark for up to 3 months.

Sterilization solution (fresh) Add 11.6 ml of 12% (wt/vol) NaClO and 150 µl of Tween 20 to 18.4 ml of sterile H_2O. The final concentrations are as follows: 4.6% (wt/vol) NaClO and 0.5% (vol/vol) Tween 20.

Extraction buffer (fresh) Mix 2.5 ml of 1 M Tris-HCl buffer (pH 7.6), 1.5 ml of 1 M $MgCl_2$, 15 ml of 1 M NaCl, 0.5 ml of 20% (vol/vol) NP-40, 100 µl of 0.1 M Na_3VO_4, 200 µl of 0.5 M NaF, 1 ml of 0.1 M PMSF, 20 µl of 5 mM E64 and 5 ml of ethylene glycol, and then add Milli-Q water to a final volume of 100 ml. Dissolve 0.557 g of *p*-nitrophenyl phosphate, 1.296 g of β-glycerophosphate and two tablets of protease inhibitor cocktail in the buffer on ice. The final concentrations are as follows: 25 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 150 mM NaCl, 15 mM *p*-nitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1% (vol/vol) NP-40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 µM E64, EDTA-free Ultra complete tablet (1/10 ml) and 5% (vol/vol) ethylene glycol.

Wash buffer (fresh) Mix 1 ml of 1 M Tris-HCl (pH 7.6), 15 ml of 1 M NaCl, 0.5 ml of 20% (vol/vol) NP-40, 100 µl of 0.5 M EDTA, 1 ml of 0.1 M PMSF, 20 µl of 5 mM E64, 5 ml of ethylene glycol and add Milli-Q water to a final volume of 100 ml. Keep the buffer on ice until use. The final concentrations are as follows: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% (vol/vol) NP-40, 0.5 mM EDTA, 1 µM E64, 1 mM PMSF and 5% (vol/vol) ethylene glycol.

Streptavidin elution buffer (fresh) Combine 0.1 ml of 1 M Tris-HCl (pH 7.6), 1.5 ml of 1 M NaCl, 50 µl of 20% (vol/vol) NP-40, 10 µl of 0.5 M EDTA, 2 µl of 5 mM E64, 0.1 ml of 100 mM PMSF and 0.5 ml of ethylene glycol, and add Milli-Q water to a final volume of 10 ml. Dissolve 42.8 mg of desthiobiotin in the buffer and keep it on ice. The final concentrations are as follows: 10 mM Tris/HCl (pH 7.6), 150 mM NaCl, 0.1% (vol/vol) NP-40, 0.5 mM EDTA, 1 µM E64, 1 mM PMSF, 5% (vol/vol) ethylene glycol and 20 mM desthiobiotin.

HCl/acetone solution Add 411 µl of 37% (wt/vol) HCl to 100 ml of acetone, and store it at −20 °C until use. The final concentration is 50 mM HCl in acetone. The solution can be stored for up to 1 year at −20 °C.

Reducing buffer (fresh) Dissolve 200 mg of NH_4HCO_3 and 50 mg of DTT in 50 ml of HPLC water. The final concentrations are as follows: 6.66 mM DTT and 50 mM NH_4HCO_3.

Alkylating buffer (fresh) Dissolve 200 mg of NH_4HCO_3 and 500 mg of iodoacetamide in 50 ml of HPLC water. The final concentrations are as follows: 55 mM iodoacetamide and 50 mM NH_4HCO_3.

Trypsin digest buffer (fresh) Dissolve 100 µg of trypsin in 100 µl of 50 mM acetic acid. Make a replacement buffer by adding 100 mg of NH_4HCO_3 and 2.5 ml of acetonitrile to 22.5 ml of HPLC water. Add 790 µl of replacement buffer to 10 µl of trypsin dissolved in 50 mM acetic acid. The final concentrations are as follows: 12.5 µg of trypsin per ml in 50 mM NH_4HCO_3 and 10% (vol/vol) acetonitrile.

Solvent A (loading) Solvent A contains 0.1% (vol/vol) trifluoroacetic acid and 2% (vol/vol) acetonitrile in HPLC-grade water. Solvent A can be stored at room temperature for 1 week.

Solvent A′ (gradient) Solvent A′ is 0.1% (vol/vol) formic acid in HPLC-grade water. Solvent A′ can be stored at room temperature for 1 week.

Solvent B′ (gradient) Solvent B′ is 0.1% (vol/vol) formic acid and 80% (vol/vol) acetonitrile in HPLC-grade water. Solvent B′ can be stored at room temperature for 1 week.

***Agrobacteria* harboring TAP expression vectors** Clone the desired GS^{rhino} expression vector (**Table 2**) according to a standard multisite Gateway LR reaction and transform it into *Agrobacterium tumefaciens* strain C58C1 RifR pMP90 by electroporation, as previously described[17].

***Arabidopsis thaliana* plants** Either wild-type or mutant plants can be used for tranformation of the TAP expression vector. In general, we use ecotype Col-0; however, other ecotypes might be considered as well.

PROCEDURE

Biomass production ● TIMING ~7.5 months

1| *Preparation of T_1 and T_2 seeds (Steps 1–8)*. Transform five wild-type or mutant *Arabidopsis thaliana* Col-0 ecotype plants through a standard floral dip method[62] with *Agrobacteria* containing the TAP GS^{rhino} expression vector (see Reagent Setup). ▲ **CRITICAL STEP** Preferably, transform the corresponding mutant (e.g., homozygous T-DNA insertion in gene encoding the endogenous bait protein) if available, because complementation of the mutant phenotype can generate information on the functionality of the TAP fusion protein. In addition, this will prevent competition between the TAP fusion protein and the corresponding endogenous protein during protein complex assembly, thus increasing the success rate.

2| Collect seeds from five independently floral-dipped plants using a sieve mesh, and then collect seeds from each plant in 1.5-ml Safe-Lock tubes.

3| Sterilize the seeds according to standard methods[62,63], such as vapor-phase or liquid bleach seed sterilization. ■ **PAUSE POINT** Seeds can be stored for at least 1 year in a dry atmosphere at room temperature.

4| Put circa 400–500 seeds from each parent line onto 1/2 MS plates supplemented with the appropriate selective agent.

5| Place plates for 2–3 d at 4 °C for stratification in the dark.

6| Transfer the plates to the growth chamber in a normal 16-h light/8-h dark cycle at 21 °C for 2–3 weeks.

7| Select approximately ten resistant T_1 plants from each parent line and transfer them to soil. At this stage, genomic DNA can be isolated to confirm insertion of the T-DNA TAP expression cassette by a standard PCR procedure.

8| Collect T_2 seeds using a sieve mesh, and sterilize them using a standard method.
■ **PAUSE POINT** Seeds can be stored for at least 1 year in a dry atmosphere at room temperature.

9| *Expression analysis of the TAP fusion protein (Steps 9–12)*. From each line, put ~6 mg (100–200 seeds) of sterilized seeds (see Step 3) in 30 ml of selective 1/2 MS medium in a 100-ml Erlenmeyer flask.

10| Grow the seedlings on a rotating platform (80–90 r.p.m.) in a growth chamber at 21 °C under a normal 16-h light/8-h dark cycle.

11| After 6 d, harvest seedlings on a sintered glass filter, freeze them in liquid nitrogen and store them at –80 °C.
The seedlings can be stored for at least 1 year.

12| Prepare total protein extract by homogenization of ~100 mg of seedlings with mortar and pestle in liquid nitrogen or with a bead mill, followed by standard freeze-thaw protein solubilization in extraction buffer and clearance of the extract by a two-step centrifugation in a microcentrifuge at maximum speed. Analyze 30 µg of total protein extract by SDS-PAGE and western blotting according to standard methods[17] using peroxidase anti-peroxidase soluble complex antibody (1/2,500) for the detection of TAP fusion proteins.
? TROUBLESHOOTING

13| *T_3 seed upscaling (Steps 13–16)*. Select two lines with positive TAP fusion expression for T_3 seed upscaling.

14| Put circa 100 sterile seeds from each selected line onto selective 1/2 MS plates, and grow the plants as described in Steps 5 and 6.

15| Select circa 50–resistant T_2 plants from both selected lines, and transfer them to soil.

16| Collect and pool all T_3 seeds. For one TAP experiment, circa 2.5 g of T_3 seed is required.
■ **PAUSE POINT** Seeds can be stored for at least 1 year in dry atmosphere at room temperature until further use.

17| *Sterilize 20 batches of 125-mg seeds (Steps 17–23)*. Put 125 mg of seeds in a 50-ml sterile tube (×20).

18| Wash the seeds for 2 min with 30 ml of 70% (vol/vol) Disinfectol and mix gently.

19| Spin down the seeds (1 min at 3,220*g* at room temperature) and decant the supernatant.

20| Wash the seeds for 2 min with 30 ml of 90% (vol/vol) Disinfectol, mix gently, and then perform Step 19.

21| Wash the seeds for 10 min in 30 ml of sterilization solution, vortex briefly, and then perform Step 19.

22| Wash the seeds 4–6 times with 30 ml of sterile water until the supernatant is clear. Briefly spin down in between and decant the supernatant. The final pH of the supernatant should be ≤6.

23| Transfer 20 batches of 125 mg of seeds to 5 ml of sterile water, and add the mixture to a 2-liter Erlenmeyer flask containing 600 ml of 1/2 MS medium.

24| *Growing the T_3 plants (Steps 24 and 25)*. Grow the seedlings on a rotating platform (80–90 r.p.m.) in a growth chamber at 21 °C under a normal 16-h light/8-h dark cycle.

25| Harvest the seedlings after 6 d; harvest each flask separately on a sintered glass filter through a filter disc. Gently manipulate the seedlings until most of the liquid is removed. Isolate ~100 mg of seedlings to confirm TAP fusion expression analysis (see Step 12). Freeze the remaining seedlings in liquid nitrogen and store them at –80 °C.
? TROUBLESHOOTING
■ **PAUSE POINT** Seedlings can be stored for at least 1 year at –80 °C.

Protein extraction ● TIMING ~2 h
▲ **CRITICAL** To minimize protein degradation during protein extraction and TAP, always precool reagents and equipment and perform all steps at 4 °C. To decrease contamination with nonspecific proteins, such as keratin, which might interfere with protein identification of low-abundance co-purified proteins, always work in a clean dust-free environment and wear protective nitrile gloves and a lab coat. An ionizing air blower can be used for charge neutralization over the workbench to prevent the attraction of dust.

26| Grind 50 g of harvested plant material in liquid nitrogen with a kitchen blender for 10 min in a stainless steel wine cooler and allow the remaining liquid nitrogen to evaporate.

27| Add 100 ml of ice-cold extraction buffer.

28| Add 100 µl of Benzonase (2,500 units) to a final concentration of 0.1% (vol/vol), and mix it for an additional 2 min.
▲ **CRITICAL STEP** Benzonase is a nuclease targeting both RNA and DNA. Through Benzonase treatment, we minimize the loss of chromatin complexes during centrifugation, which is important when analyzing transcription factor– or chromatin-related complexes. In addition, indirect DNA-mediated interactions are prevented, lowering the false-positive rate[7,64].

29| Transfer the mixture to four precooled centrifugation tubes and incubate them for 30 min at 4 °C on a tube rotator.

30| Centrifuge the tubes at 36,900*g* for 20 min at 4 °C, carefully isolate the supernatants and repeat the centrifugation.

31| Filter the supernatants through a double layer of Miracloth mesh.

32| Pool the protein extract in a 100-ml flask and determine the protein concentration via a standard Bradford method[17]. Keep 100 µl of protein extract aside for western blot analysis to confirm the expression and stability of the TAP fusion protein during extraction. Add 25 µl of 5× SDS sample buffer[17], heat the mixture for or 10 min at 95 °C and store it at –20 °C until it is used for western blot analysis.

IgG- and streptavidin-based AP ● TIMING ~5 h
33| *IgG-based AP (Steps 27–33)*. Transfer 250 µl of IgG-Sepharose beads onto a Poly-Prep chromatography column and wash the column with 2.5 ml of extraction buffer.

34| Remove the bottom of a 50-ml tube with a Stanley knife and pull the Poly-Prep column through the tube. Seal the junction between the column and the tube with Parafilm.
▲ **CRITICAL STEP** This step allows loading of a bigger volume onto the IgG beads on the column.

35| Load the total protein extract stepwise on the assembled IgG-Sepharose Poly-Prep column (**Supplementary Fig. 3**), and then draw the extract through the column with a peristaltic pump at a flow rate of 1 ml/min.

36| Remove the 50-ml tube and mount the IgG-Sepharose Poly-Prep column on a two-way valve in a vacuum manifold system. Wash it with 37.5 ml of wash buffer by applying vacuum.
▲ **CRITICAL STEP** Be careful not to drain the column dry, and be sure to close the two-way valve in time.

37| Carefully transfer IgG beads to a 1.5-ml protein LoBind tube using a 1-ml blue pipette with a 3–4-mm tip cutoff, and add 1 ml of wash buffer and 100 U rhinovirus 3C protease.

38| Incubate the mixture for 1 h at 4 °C on a tube rotator. After 30 min, apply an additional boost of 100 U of protease for efficient elution.

39| Transfer the mixture onto a Mobicol column and collect the eluate in a 2-ml protein LoBind tube. Wash the beads on a Mobicol column two times with 500 µl of wash buffer, and add the wash to the eluate.

40| *Streptavidin-based AP (Steps 34–37)*. Equilibrate 100 µl of streptavidin-Sepharose beads three times with 1 ml of wash buffer in a 5-ml tube.

41| Incubate the pooled eluate (2 ml) on the equilibrated streptavidin beads for 1 h at 4 °C on a tube rotator in the 5-ml tube.

42| Transfer the mixture to a Poly-Prep chromatography column and wash it with 10 ml of wash buffer on a vacuum manifold system.

43| Apply 1 ml of streptavidin elution buffer to the Poly-Prep column, incubate it for 5 min and slowly collect the eluate by gravity in a 1.5-ml protein LoBind tube.

Protein concentration ● TIMING overnight incubation + 2 h

44| Add 333 µl of trichloroacetic acid per 1 ml of streptavidin eluate to obtain a final concentration of 25% (vol/vol), and mix it well by inverting the tube.

45| Incubate the mixture overnight on ice to precipitate the proteins.

46| Centrifuge the precipitated proteins down at 20,800*g* in a microcentrifuge for 15 min at 4 °C.

47| Carefully remove the supernatant and wash the pellet twice with 500 µl of ice-cold HCl/acetone solution. Centrifuge in between as in Step 46, and remove the supernatant.

48| Air-dry the pellet for 1 h on ice and store the eluate at –80 °C.

▲ **CRITICAL STEP** Keep the tube open to dry the pellet, but cover the ice box with plastic wrap to eliminate contamination of the sample with dust.

■ **PAUSE POINT** The protein pellet can be stored at –80 °C for at least a couple of weeks.

Protein separation and staining ● TIMING ~2 d

49| Dissolve the proteins present in the pellet (Step 48) in 30 µl of 1× NuPAGE sample buffer supplemented with 1× NuPAGE sample reducing agent.

50| Heat the protein sample for 10 min at 70 °C and load it on a precast 4–12% (wt/vol) gradient NuPAGE Bis-Tris gel, as previously described[17].

▲ **CRITICAL STEP** When more than one sample is being analyzed, keep one empty lane in between two sample lanes to avoid cross-contamination of samples. Apply in the first lane a standard protein molecular weight (MW) marker; for example, load 2.5 µl of SeeBlue Plus2 pre-stained standard.

51| Run the gel at 200 V for 7 min.

52| Visualize the proteins by Coomassie brilliant blue G-250 staining, as previously described[17].

53| In parallel, evaluate bait protein extraction and stability by analyzing 30–50 µg of total protein extract from the input sample (see Step 32) by standard SDS-PAGE, western blotting and detection with peroxidase anti-peroxidase soluble complex antibody (see Step 12).

In-gel protein digestion ● TIMING ~2 d

▲ **CRITICAL** Be aware of keratin or dust contamination, wear nitrile gloves and a lab coat at all times, and work in a clean environment. Use keratin-free material and filter tips. Freshly prepare all solutions just before use.

54| Destain the gel twice for 1 h in 25 ml of HPLC-grade water, and shake it gently on an orbital shaker.

55| Take a gel image for documentation.

56| Remove the water solution; incubate the gel for 40 min in 25 ml of reducing buffer while shaking it gently on an orbital shaker.

57| Remove the reducing buffer and incubate the gel for 30 min in 25 ml of alkylating buffer in the dark, and shake it gently on an orbital shaker.

58| Remove the alkylating buffer and wash the gel for 30 min in 25 ml of HPLC-grade water, and shake it gently on an orbital shaker.

59| Transfer the gel to a glass plate and cut out a broad protein zone per lane with a fresh scalpel.
▲ **CRITICAL STEP** From the gel lane, cut the gel zone containing the whole protein sample, by using the protein MW marker as a reference, starting from the height of the lowest MW marker until ~1 mm underneath the well. Cut the gel zone into gel plugs (Step 60) no smaller than 1 mm^2. Take care not to crush the gel plugs while removing the solutions in Steps 62–70; therefore, use a fine gel-loading tip placed on a 1-ml filter tip.

60| Slice the gel zone into 16–20 gel plugs with a scalpel and transfer them to a 1.5-ml protein LoBind tube filled with 600 µl of HPLC-grade water.

61| Wash the gel plugs twice with 600 µl of HPLC-grade water.

62| Remove the water solution, dehydrate the gel plugs in 600 µl of 95% (vol/vol) acetonitrile and shake them for 10 min.

63| Remove the acetonitrile solution, rehydrate the gel plugs in 600 µl of HPLC-grade water and shake them for 10 min.

64| Remove the water solution and repeat dehydration as described in Step 62.

65| Remove the acetonitrile solution and transfer the tube to ice. Add 90 µl of trypsin digest buffer.
▲ **CRITICAL STEP** Check whether all plugs are covered after the rehydration in Step 65; if not, add some extra trypsin digest buffer before incubating at 37 °C.

66| Incubate the mixture for 30 min at 4 °C to allow the gel plugs to rehydrate. Afterward, incubate the mixture for 3.5 h at 37 °C to digest the proteins.
■ **PAUSE POINT** After digestion, the sample can be stored overnight at 4 °C.

67| Sonicate the sample for 5 min in a sonication bath.

68| Transfer the solution covering the gel plugs to a fresh 1.5-ml protein LoBind tube and set it aside; this sample contains the peptides.

69| Add 300 µl of 95% (vol/vol) acetonitrile to the gel plugs and shake them for 10 min. Gel plugs should be completely dehydrated and have a white appearance.

70| Transfer the acetonitrile solution from the gel plugs to the tube with the peptides from Step 68; this is the trypsin digest.

71| Use a needle to punch a hole in the cap of the 1.5-ml tube (containing the trypsin digest; Step 70), and place it with the cap closed in a SpeedVac. Completely dry the trypsin digest (~2–3 h). Afterward, cover the cap with Parafilm to close the hole.
■ **PAUSE POINT** Dried trypsin digests can be stored for several days (or even weeks) at –20 °C.

LC-MS/MS analysis on LTQ Orbitrap Velos ● TIMING ~0.5 d

72| Setup the nano LC system in-line connected to the LTQ Orbitrap Velos, with a trapping column of 100 µm internal diameter × 20 mm (length), 5 µm C18 Reprosil-HD beads and a reverse-phase separating column of 75 µm internal diameter × 150 mm, 5 µm C18 Reprosil-HD beads.

73| Solubilize the peptides in 15 µl of loading solvent A and load 5 µl on the trapping column.

74| After washing and back-flushing from the trapping column, separate the peptides on the separating column with a gradient of x% solvent A′/100-x% solvent B″ from 2% to 50% during 30 min, at a flow rate of 300 nl/min, followed by a washing step reaching 100% solvent B′.

75| Operate the LTQ Orbitrap Velos in data-dependent mode to automatically switch between MS and MS/MS acquisition for the ten most abundant peaks in a given MS spectrum. Acquire full-scan MS spectra in the Orbitrap at a target value of 1E6 with a resolution of 60,000. Isolate the ten most intense ions for fragmentation in the linear ion trap, with a dynamic exclusion of 20 s. Set the target value for filling the ion trap to 1E4 ion counts.

Data analysis and filtering ● TIMING ~0.5 d

76| Create Mascot generic files with Mascot Distiller. Allow grouping of spectra with a maximum intermediate retention time of 30 s and a maximum intermediate scan count of 5 if possible. Group with a 0.005-Da precursor tolerance. Use no de-isotoping and a relative signal-to-noise limit of 2. Generate a peak list only when the MS/MS spectrum contains more than 10 peaks.

77| Use the Mascot Daemon interface to search the peak lists with the Mascot search engine against the TAIRplus database. Set the parameters as outlined in the following table:

Parameter	Value
Variable modification	Oxidation (M)
	Methyl (DE)
Fixed modification	Carbamidomethyl (C)
Enzyme	Trypsin/P
Maximum missed cleavages	1
Charge	2+ and 3+
Peptide tolerance	10 p.p.m.
# ^{13}C	1
MS/MS tolerance	0.5 Da
Instrument	ESI-TRAP

M, methionine; DE, aspartic acid, glutamic acid; C, cysteine; P, proline (trypsin also cuts when a cleavage site is followed by proline residue).

78| In the Mascot results URL, create Mascot select summary (protein hits) with the following settings: significance threshold $P < 0.01$, maximum number of hits AUTO, MudPIT scoring, ion score or expect cutoff <0.01, require bold red.

79| Export search results as a .csv file, including same-set proteins, protein coverage, protein length, protein PI, peptide start, end, homology threshold and identity threshold.

80| Open the results .csv file in Excel and filter for rank 1 peptides. Retain only identifications from *Arabidopsis* (TAIR10) with at least two matched high-confidence rank 1 peptides, of which at least one is unique to the protein. Create a protein identification list in which same-set proteins are included in a separate column.

81| Cross-check the protein identification list against the list of nonspecific proteins (**Supplementary Table 2**), and retain only specific binders in the final list.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
12	No or very low bait expression	The TAP fusion protein is unstable The endogenous promoter lacks some important functional elements	Fuse the TAP tag to the other protein end (N-terminus versus C-terminus) Use the constitutive *35S* promoter or clone a larger promoter fragment
		The endogenous promoter is not active in 6-d-old seedlings	Use the constitutive *35S* promoter or harvest biomass (Step 25) from a more relevant developmental stage
25	The liquid medium appears contaminated with bacteria or fungi	Insufficient seed sterilization	Discard biomass harvested from contaminated Erlenmeyer flasks
81	No bait or interactors identified	Bait expression was too low	See the troubleshooting guidance for Step 12, 'No or very low bait expression'
		The protein complex is not active in 6-d-old seedlings	Generate biomass from a more relevant developmental stage or under more relevant conditions
		The endogenous bait protein interferes with the protein complex assembly of the tagged bait High contamination with keratin, interfering with the identification of low-abundance interactors	Transform the bait fusion construct in the corresponding mutant or overexpress the bait fusion Always work in a clean, dust-free environment, and wear nitrile gloves and a lab coat at all times
		The bait fusion protein or unstable interactors were degraded during protein extract preparation	Evaluate bait fusion protein degradation by SDS/western blotting. Always keep all samples and buffers cold. Add additional protease or proteasome inhibitors
		Inefficient IgG elution due to steric hindrance of 3C rhinovirus cleavage site	Evaluate by analyzing the IgG beads after elution for the presence of uncleaved bait protein. Fuse the TAP tag to the other protein terminus
	Bait protein or interactors from other TAP experiments are identified	Samples are cross-contaminated during sample preparation or identifications are the consequence of column-memory effects	Be very careful when preparing samples. Perform blank runs in between experiments, or use a tandem LC configuration

● TIMING

Biomass production: ~7.5 months

Steps 1–8, preparation of T_1 and T_2 seeds: 4 months
Steps 9–12, expression analysis of T_2 seeds: 8 d
Steps 13–16, T_3 seed upscaling: 3 months
Steps 17–23, T_3 seed sterilization: 0.5 d
Steps 24 and 25, growing the T_3 plants: 6 d

Tandem affinity purification: 1 d

Steps 26–32, protein extraction: 2 h
Steps 33–39, IgG-based AP: 3.5 h
Steps 40–43, streptavidin-based AP: 1.5 h

Sample preparation and LC-MS/MS analysis: ~5 d

Steps 44–48, protein concentration: overnight incubation + 2 h
Steps 49–53, protein separation and staining: 2 d
Steps 54–71, in-gel protein digestion: 2 d
Steps 72–75, LC-MS/MS analysis: ~0.5 d
Steps 76–81, data analysis and filtering: ~0.5 d
Box 1, Tandem affinity purification: 1 d

ANTICIPATED RESULTS

Since the previous publication[17] of our detailed TAP protocols on *Arabidopsis* cell cultures in 2011, many improvements have been made to our platform (**Table 1**). Replacement of the TEV protease cleavage sites by rhinovirus 3C sites allows elution at 4 °C instead of 16 °C, identifying more relevant interactors. Implementation of ultrasensitive MS increased the sensitivity, which means that less total protein input is required (25 mg instead of 200 mg) to detect more interactors. For TAP on cell suspension cultures, we now collect ~15–20 g of cells from 1 liter of 3-d-old culture. From 2.5 g of these cells, total protein extract is derived at a concentration of ~15–20 mg/ml.

To explore protein complexes functioning in a developmental context, we provide protocols for fast biomass generation and further adapted our TAP protocol. During seedling biomass production, we collect ~7.5 g of 6-d-old seedlings per Erlenmeyer flask. Typically, using 50 g of seedlings, we obtain 100–120 ml of protein extract with a concentration of ~4–5 mg/ml, giving 400–600 mg as total protein input. The difference in protein concentration between seedlings and cell suspension cultures reflects the high protein content of the suspension cells.

When we performed this protocol using SAMBA, a plant-specific APC regulator, as bait in *Arabidopsis* seedlings, we identified 37 proteins by MS. After data filtering against our list of nonspecific proteins, the bait protein SAMBA and 11 specific co-purifying proteins remained in the list[25]. These represent all core subunits of the APC, except APC11, which is more difficult to detect by MS probably because of its small size (10.1 kDa). In addition, the APC activator CCS52A2 was co-purified. It was further shown by Y2H that SAMBA binds to the APC through direct interaction with APC3b. Mutant analyses demonstrated that SAMBA is involved early in plant development through the regulation of A-type cyclin stability[25].

In another work[29], this time using AN3, an important regulator of leaf development[65], as bait in *Arabidopsis* seedlings, 76 proteins were identified significantly by MS. After subtraction of nonspecific proteins, 14 proteins were retained that were also found with TAP in cell cultures using AN3 or other members of the complex as bait[29]. Among these proteins are several plant homologs of SWI/SNF chromatin remodeling complex subunits. Moreover, through semiquantitative analyses using normalized spectral counts (NSAF), two additional proteins were retained from the nonspecific interactors and considered as genuine (**Supplementary Fig. 2**). These represent two actin-related proteins (ARP4 and ARP7), known core subunits of SWI/SNF chromatin remodeling complexes[29]. In conclusion, these data indicate that AN3 associates with chromatin remodelers to regulate transcription during leaf development, acting as a key player at the switch between cell proliferation and cell differentiation in a developing leaf.

In our hands, when using this TAP approach on cell cultures, we identify an average of 5.6 specific interactors in common per bait protein used, in a duplicate TAP experiment. The success rate of identifying at least one confirmed interactor per bait protein used, found in both duplicate TAP experiments, is 65%.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS J.V.L., D.E. and G.D.J. designed the research. B.C., N.D.W., G.P., E.V.D.S., L.V. and J.V.L. performed experiments. D.E., J.V.L., K.V., L.M. and G.D.J. analyzed the data. K.G. and E.W. provided protocols for LC-MS/MS analysis. M.D., A.V., K.V., K.G., L.M. and E.W. commented on the manuscript. J.V.L., D.E. and G.D.J. wrote the manuscript.

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