

Exploration of the functional conservation across major phylogenetic boundaries to define the ancestry of genetic functions will also be important. Within this framework, the study of biological innovations, such as the origin of new genes or their neo- or sub-functionalization, will be of particular relevance to legume biologists, considering the restricted occurrence of nitrogen-fixing symbiosis and its proven functional link to the much older (approximately 400 million years ago) and certainly more ubiquitous association of plants with mycorrhizal fungi [19,20].

The availability of legume genomes opens an unusually valuable treasure chest, where efficient sequestration of nitrogen, phosphate and carbon has been hiding within the dynamic structure of gene networks. Unwinding this biological wealth might provide an important insight on how to preserve nonrenewable resources and the health of the environment at the same time as fulfilling the increasing need for sustainable biomass production.

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#### Techniques & Applications

## Boosting tandem affinity purification of plant protein complexes

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**Protein-interaction mapping based on the tandem affinity purification (TAP) approach has been successfully established for several systems, such as yeast and mammalian cells. However, relatively few protein complex purifications have been reported for plants. Here, we highlight solutions for the pitfalls and propose a major breakthrough in the quest for a better TAP tag in plants.**

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#### The rise of TAP

Over the past 20 years, a wide variety of methods have been developed to explore protein interactions. Co-immunoprecipitation or yeast two-hybrid were often the method of choice, but the emergence of powerful, ultrasensitive high-throughput mass spectrometry (MS), together with the availability of comprehensive protein sequence repertoires, has favored the development of methods relying on *in situ* affinity purification of protein complexes.

Especially, the tandem affinity purification (TAP) approach, based on the expression of a bait protein fused to a double affinity tag (the TAP tag), has proven to be of great value. The classical TAP tag consists of two immunoglobulin G (IgG)-binding domains of protein A from *Staphylococcus aureus*, a specific protease cleavage site for elution by addition of the tobacco (*Nicotiana tabacum*) etch virus (TEV) protease and a calmodulin-binding peptide (CBP). Purification steps were optimized for highest recovery while maintaining protein complex integrity. TAP of protein complexes was first demonstrated in yeast (*Saccharomyces cerevisiae*) [1] and was soon applied in a wide variety of organisms, giving rise to high-quality and comprehensive protein-interaction networks [2,3]. Nowadays, databases are filled with protein-interaction data from TAP experiments, but in the plant research field, the TAP approach considerably lags behind. Here, we review and discuss the use of TAP in plants and provide solutions to problems associated with the technology.

### TAP in plants: a brief overview

Until now, only a limited number of purifications from plant material through TAP have been reported, and most have been performed with the traditional yeast tag or with a plant-adapted version, called the improved TAP tag (TAPi) [4]. The latter contains the same modules as the traditional yeast TAP tag but with an optimized codon sequence for plants and an intron for higher gene expression and without cryptic nuclear localization signals. Both the traditional and the TAPi tags were used with success for complex purifications from *Arabidopsis thaliana* [4–10] and rice (*Oryza sativa*) [11,12]. A protein complex from *Arabidopsis* was also purified with an alternative TAP tag (TAPa), in which the CBP domain was replaced by a 9xMyc and 6xHis sequence, preventing the non-specific purification of endogenous calmodulin-binding proteins and allowing purification of cation-dependent enzyme complexes because no EGTA-containing buffers are required [13]. Furthermore, the TEV cleavage site was replaced by the more-specific and low-temperature-active human rhinovirus 3C protease cleavage site. Although this tag has often been used as an epitope tag for protein gel blotting [14] and in co-immunoprecipitation [14,15] or chromatin immunoprecipitation experiments [15], only a single protein complex has been characterized with the TAPa tag [13]. In conclusion, in plants, the traditional yeast TAP and the TAPi tags perform best so far.

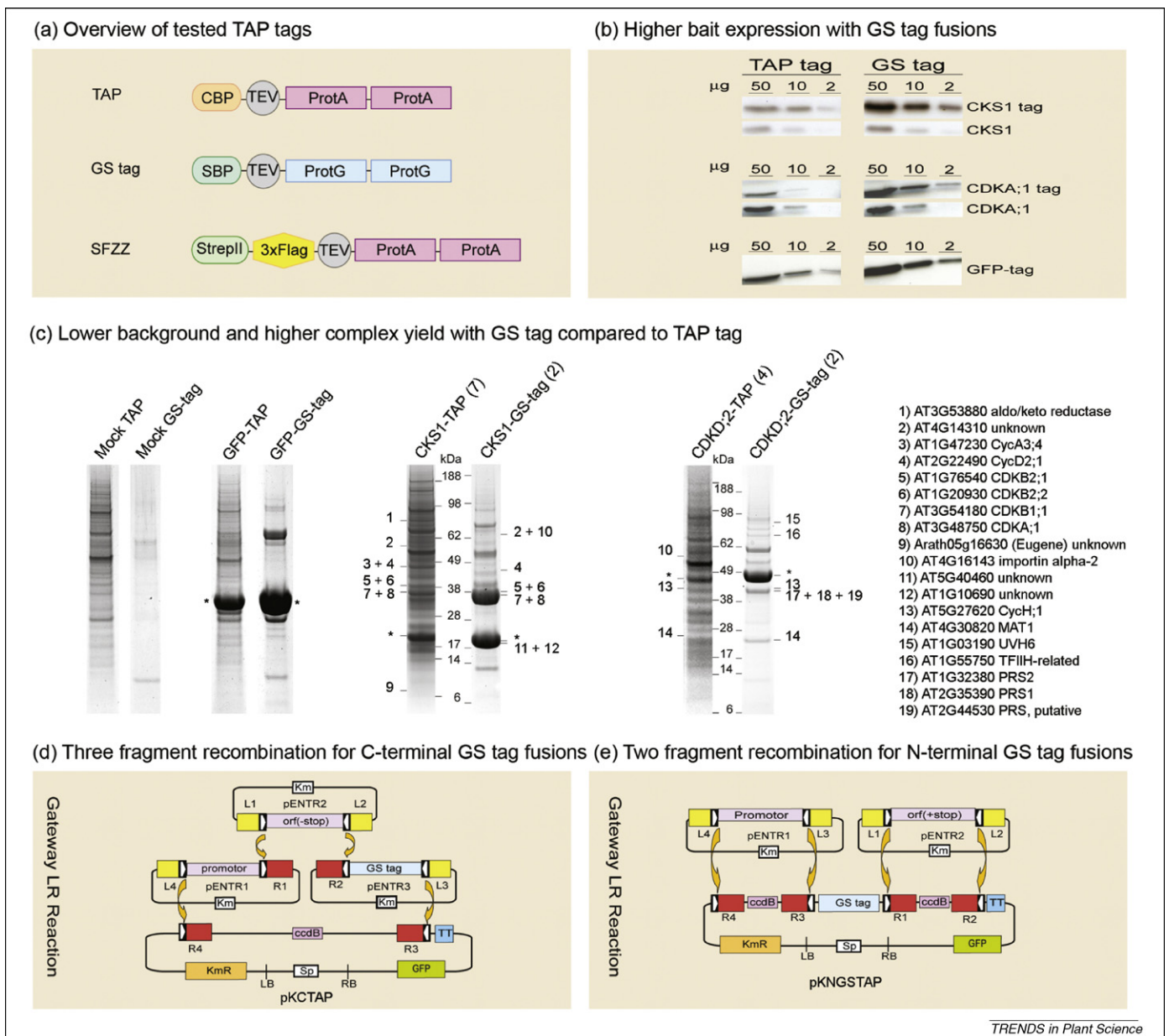
### TAP in plants: pitfalls and solutions

Approximately ten years after the proof of concept in yeast, the few TAP data from plants demonstrate that problems are associated with the method. Indeed, unlike in yeast, efficient homologous recombination is not feasible in higher plants. So, the endogenous protein and the tagged counterpart will compete for complex assembly. To overcome this pitfall, different strategies can be followed. The TAP-tagged protein can be introduced into a mutant background, where the endogenous protein is suppressed by RNA interference [16] or is eliminated by transferred DNA (T-DNA) insertion [13]. These complementation

approaches determine the functionality of the tagged protein and increase the success rate of the purification because more interactors are available for complex assembly with the tagged protein. A more generic approach for increasing competition is overexpression of the tagged bait, a strategy used in all successful TAP reports in plants so far. Another problem is false negative interactors, especially when low-abundant complexes are studied. Because proteins are present in a high dynamic range, varying from only 10–100 copies to more than  $10^7$  copies per cell, and because they cannot be amplified like polynucleotides by PCR, the success rate of TAP depends on the amount of protein complexes purified and the MS sensitivity. One possibility for circumventing the problem of false negatives is the combination of multiple TAP eluates from parallel purifications [13]. Alternatively, the amount of protein extract can be increased before purification. When studying basic cell biological processes, plant cell suspension cultures have a major advantage compared to whole plants because they are fast growing and provide an unlimited supply of synchronizable biological material. Moreover, the PSB-D culture used previously [5] has a ploidy level of 8C, meaning that more proteins are available for complex assembly. The suspension culture is ideal for investigating the cell cycle [5], but it can also be used to isolate complexes involved in other fundamental processes, such as primary metabolism, gene expression or cell-wall synthesis.

### The quest for a better TAP tag

Despite the valuable strategies described above, it was clear that a major leap forward would only be possible through further optimization of complex purification. Therefore, we evaluated different TAP tags for plant cells (Figure 1a). In line with the TAPa tag [13], we replaced the CBP part in the traditional TAP tag with linear peptide epitopes to reduce background. Although the final TAP eluates obtained with this SFZZ tag (Figure 1a) looked often much ‘cleaner’ on gel, the purified amount of complexes was systematically low and only a few interacting proteins could be sequenced (see supplementary Table S1). Probably the TAPa tag also has to deal with this low complex yield because six different TAP eluates had to be pooled to identify the COP9 signalosome complex [13]. Despite a layer of background proteins sticking to the calmodulin resin, in our laboratory, the best results with respect to complex yield were, until recently, always obtained with the traditional or the TAPi tags [4]. Background proteins sticking to the resins and other false positives from non-specific binding to complexes after protein extract preparation can be determined by mock and exogenous protein purifications using, for example, green fluorescent protein (GFP) or  $\beta$ -glucuronidase. This list of proteins is then systematically subtracted from the original prey list. To get rid of bait-specific false positives absent in the control TAP list, it is also valuable to repeat purifications and to give more confidence to interactions that were confirmed in multiple experiments [5] or that achieved the best protein identification scores. Assigning confidence scores to interactions by integrating interaction data with other data sources is also rewarding, a method



**Figure 1.** Evaluation of alternative TAP tags in *Arabidopsis* cell suspension culture. **(a)** Overview of tested TAP tags. Abbreviations: 3xFlag, three copies of Flag tag; ProtA, immunoglobulin G (IgG)-binding domain of protein A; ProtG, IgG-binding domain of protein G; SBP, streptavidin-binding peptide; StrepII, StrepII tag. **(b)** Higher bait expression with GS tag fusions: 50  $\mu$ g, 10  $\mu$ g and 2  $\mu$ g of total protein extracts of cultures expressing CKS1-tag, CDKA;1-tag or GFP-tag were separated via SDS-PAGE. Both TAP and GS tag fusions were analyzed via immunoblotting with antibodies against bait proteins. For CKS1 and CDKA;1, endogenous protein levels are also shown. **(c)** Comparison of final eluates with the traditional TAP and GS tag protocols: final eluates were precipitated, separated on 4–12% NuPAGE gradient gels and visualized with Coomassie G. Background levels were analyzed with mock and GFP tag purifications. Proof of concept was demonstrated for CKS1 (At2g27960) and CDKD;2 (At1g66750). Preys identified via MALDI-TOF/TOF and confirmed in multiple experiments are indicated (see [supplementary Table S2](#)). The number of purifications used to determine the confirmed interactors is shown in parentheses. Asterisks indicate bait proteins. **(d)** Schematic representation of the cloning strategy for GS fusions to the C terminus or **(e)** to the N terminus of the bait protein. Abbreviations: ccdB, toxic killer gene for negative selection; KmR, neomycin phosphotransferase II gene for selection of transformed plant cells; LB, left border for T-DNA insertion; RB, right border for T-DNA insertion; Sp, streptomycin and spectinomycin resistance gene; TT, cauliflower mosaic virus 35S transcription terminator.

often applied in prediction of protein–protein interactions [17,18].

### A new TAP tag for plants: the GS tag

In our continuous search for an ideal TAP tag for plants, we recently evaluated the GS tag [19], which combines two IgG-binding domains of protein G with a streptavidin-binding peptide, separated by two TEV cleavage sites. This tag, developed to study mammalian protein complexes, has been reported to give a tenfold increase in

bait recovery compared to the traditional TAP tag. We adapted the GS protocol for plant cells (see [online supplementary Box S1](#)) and tested background levels by comparing two mock and two GFP purifications with the traditional TAP tag ([Figure 1c](#)). Background levels, counted as the average number of proteins identified in two experimental repeats, dropped from 62 to 8 and from 87 to 11 proteins for mock and GFP purifications, respectively, making MS analysis much less labor intensive and the identification of genuine protein interactions easier,

especially with low-abundant complexes. An additional benefit of the GS tag is the higher cellular concentration levels of the bait protein (Figure 1b) and the concomitantly higher complex incorporation and yield, as shown by the stronger A-type cyclin-dependent kinase (CDKA;1) band in the CDK subunit 1 (CKS1) GS-tag purification (Figure 1c). As a final proof of the GS tag superiority, we present results obtained with two cell-cycle baits, CKS1 and the D-type CDK-activating kinase CDKD;2 (Figure 1c). Only the experimentally confirmed interactors are represented and compared with those obtained with the traditional TAP tag [5] (see also online supplementary Table S2). For CKS1, most of the interactors confirmed previously in seven purifications with the traditional TAP tag were found with the GS protocol with only two purifications. In addition, some new interesting interactions could be detected with the GS tag only. The known partners of CDKD;2, the H-type cyclin (CycH;1) and CDK-activating kinase assembly factor 'ménage à trois' 1 (MAT1), previously discovered in four purifications with the traditional TAP tag, were identified in two purifications with the GS protocol. Moreover, using the GS tag we demonstrated that, as in rice [11], the *Arabidopsis* CDKD;2 is part of the transcription factor IIIH (TFIIH) complex, because ultraviolet hypersensitive 6 (UVH6) and a TFIIH-complex-related transcription factor co-purified. Furthermore, CDKD;2 might link regulation of cell division with nucleotide biosynthesis because of co-purification of three phosphoribosyl diphosphate synthetases (PRSs).

### Conclusion and perspectives

We have shown that the GS tag outperforms the traditional TAP tag in plant cells, both concerning specificity and complex yield. Recently, we replaced the TEV protease cleavage sites in the GS tag with the rhinovirus 3C cleavage site for improved protein complex stability during purification. Combined with the latest and most sensitive MS technology, this tag should bring protein complex analysis in plants to its full bloom. Cloning with these tags is compatible with the Gateway system [20] (Figure 1d,e), and vectors for C- or N-terminal cloning are available at <http://www.psb.ugent.be/gateway/>.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.tplants.2008.08.002](https://doi.org/10.1016/j.tplants.2008.08.002).

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