MOLECULAR AND BIOCHEMICAL ANALYSIS OF TRICHOME PATTERNING IN *ARABIDOPSIS THALIANA*

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BURCU DARTAN

Aus

İSTANBUL

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Berichterstatter:	Prof. Dr. Martin Hülskamp
	Prof. Dr. Ute Höcker
Prüfungsvorsitzender:	Prof. Dr. Siegfried Roth
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ZUSAMMENFASSUNG

Für die nicht zufällige Verteilung von Trichomen in der Blattepidermis von *Arabidopsis thaliana* ist ein *de novo* Musterbildungsmechanismus verantwortlich. Dabei bestimmt das Zusammenspiel von MYB-, bHLH- und WD40 Proteinen, ob eine epidermale Zelle Trichom wird oder nicht. Diese Proteine regulieren die Expression der Gene, die für die Initiation von Trichomen erforderlich sind. Zur Untersuchung der Trichommusterbildung wurden genetische Analysen von Trichominitiationsmutanten in *Arabidopsis* Blättern, sowie Hefe-Zwei-Hybrid Studien mit den Trichommusterbildungsproteinen durchgeführt. Außerdem lieferte der Vergleich mit ähnlichen Mechanismen wie der Wurzelhaarmusterbildung mehr Einblicke. Bis heute sind biochemische Informationen über die spezifischen, intrinsischen Eigenschaften der Trichommusterbildungsproteine und ihre direkten Protein-Protein-, sowie Protein-DNA Wechselwirkungen immer noch rar. In dieser Arbeit wurde die bakterielle Expression und Reinigung der Proteine GL1, GL3, EGL3, TTG1 und TRY, die als die Schlüsselkomponenten des MYB-bHLH-WD40-Komplexes dienen, das erste Mal erfolgreich ausgeführt. GST-Pull-Down Experimente mit den hier aufgereinigten Proteinen zeigen direkte und neue Interaktionen zwischen den Komponenten des MYB-bHLH-WD40-Komplexes. Des weiteren wurde TTG2, ein vor kurzem entdeckter Regulator der Trichomentwicklung, bakteriell erfolgreich exprimiert, gereinigt und für die Analyse von *in vitro* Protein-Interaktionen via GST-Pull-Down und Affinitätsreinigungs-experimente benutzt. Ebenso wurden spezifische Antikörper gegen GL1, EGL3, TTG1, TTG2 und TRY Proteine produziert, gereinigt und für die Detektion des jeweiligen Proteins aus *Arabidopsis* Pflanzenextrakten getestet. Diese gereinigten Proteine ebnen den Weg für, biochemische Experimente, die die Untersuchung von Protein-Protein- und Protein-DNA-Interaktionen, Proteinbeweglichkeiten und posttranslationale Proteinmodifikationen zum Ziel haben, um offene Fragen zum Verständnis der Trichommusterbildung zu beantworten.

ABSTRACT

In the *Arabidopsis* leaf epidermis, a *de novo* patterning mechanism is responsible for the non-random distribution of trichomes. Whether an epidermal cell becomes a trichome or not, is determined by the interplay of MYB-bHLH-WD40 proteins. These proteins are regulating the expression of genes required for initiation of trichomes. The trichome pattern formation in *Arabidopsis* leaves has been investigated by genetic analyses of trichome initiation mutants and yeast two-hybrid interactions among these trichome patterning proteins. Additionally, the comparison to similar mechanisms, such as root hair patterning has revealed further insights. However, biochemical information about specific intrinsic properties of the trichome patterning proteins and their direct protein-protein and DNA-protein interactions is still rare. In this study, bacterial expression and purification of the GL1, GL3, EGL3, TTG1 and TRY proteins, which serve as the key components of the MYB-bHLH-WD40 complex, is successfully performed for the first time. GST pull-down experiments conducted with these purified proteins reveal direct and novel interactions among the members of the MYB-bHLH-WD40 complex. Moreover, TTG2, a recently identified regulator of trichome development, is bacterially expressed, purified and analysed for its *in vitro* protein interactions via GST pull-down and affinity purification experiments. Specific antibodies against GL1, EGL3, TTG1, TTG2 and TRY proteins are also produced, purified and tested for detection of these proteins from *Arabidopsis* plant extracts. These pure proteins and antibodies pave the way to future biochemical experiments investigating protein-protein as well as protein-DNA interactions, protein mobility and posttranslational modifications, with the aim to answer open questions for understanding of the trichome patterning.

PUBLICATIONS

Digiuni, S., Schellmann, S., Geier, F., Greese, B., Pesch, M., Wester, K., Dartan, B., Mach, V., Srinivas, B. P., Timmer, J., Fleck, C. & Hülskamp, M. A competitive complex formation mechanism underlies trichome patterning on *Arabidopsis* leaves. *Molecular Systems Biology* **4**, 217 (2008).

For this publication, I have conducted the GST pull-down experiments showing the interactions between TRY and GL3, as well as EGL3 and GL1 proteins.

ABBREVIATIONS AND GENE NAMES

µ	micro
µg	microgram
µL	micro liter
^{0}C	Degrees Celsius
35S	35s promotor from Cauliflower Mosaic Virus
AtPLD	*Arabidopsis thaliana PHOSPHOLIPASE D*
bHLH	Basic- Helix- Loop- Helix
Bp	base pair
BSA	Bovine Serum Albumin
CaMV	Cauliflower Mosaic Virus
cDNA	complementary DNA
CDS	Coding Sequence
ChIP	Chromatin Immunoprecipitation
Col	Columbia
CPC	*CAPRICE*
CPL3	*CAPRICE-LIKE3*
Da	Dalton
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EGL3	*ENHANCER OF GLABRA3*
EMSA	Electrophoretic Mobility Shift Assay
et al.	*et alii* (Lat.) and others
ETC1	*ENHANCER OF TRIPTYCHON and CAPRICE1*
ETC2	*ENHANCER OF TRIPTYCHON and CAPRICE2*
ETC3	*ENHANCER OF TRIPTYCHON and CAPRICE3*
GFP	Green Fluorescent Protein
GL1	*GLABRA1*
GL2	*GLABRA 2*
GL3	*GLABRA3*
GSH	Glutathione
GST	Glutathione-S-Transferase
HA	Hemagglutinin epitope
HD	Homeodomain
HD-Zip	Homeodomain Zipper
His	Histidine
HRP	Horseradish Peroxidase

IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria Bertani
L*er*	Landsberg *erecta*
LR	Gateway LR Recombination
MBP	Maltose-Binding Protein
MCS	Multiple Cloning Site
Min	Minute
mL	milliliter
mM	milimolar
mRNA	messengerRNA
Ni-NTA	Nickel-Nitrilotriacetic Acid.
OD	Optical Density
P.pastoris	*Pichia pastoris*
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
pGL2	Promoter of GL2 gene
PROSEARCH	Search Prosite Database for Patterns in a Protein Sequence
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic Acid
Rpm	revolutions per minute
RT	Room Temperature
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulfate
SDSC	Sand Diego Supercomputer Center
Strep	Strep-Tactin®.
TAIR	The Arabidopsis Information Resource
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TCL1	*TRICHOMELESS1*
TRY	*TRIPTYCHON*
TTG1	*TRANSPARENT TESTA GLABRA1*
TTG2	*TRANSPARENT TESTA GLABRA2*
WER	*WEREWOLF*
YFP	Yellow-Fluorescent Protein

All gene- and mutant names are written in italics. Wildtype-genes are written in capital letters. Proteins are written in non-italic capital letters.

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1. INTRODUCTION

1.1. *ARABIDOPSIS* TRICHOMES

In nature, from zebra skin to seashell, peacock to mushroom, patterns can be observed in almost all organisms. Pattern is described as "a particular way in which something is done, organized or happens" [1]. How pattern formation occurs in nature has motivated many scientists including plant biologists to find explanations for these mechanisms. Establishment of a proper pattern is probably one of the key steps in developmental processes of organisms.

The need for plant scientists to find a model organism in order to dissect plant development was fulfilled by a weed from a mustard family - *Arabidopsis thaliana*. *Arabidopsis* was chosen to understand the molecular organization of a plant due to its convenience for reproduction, growth, the easy accessibility to create genetic modifications, and availability of its genome sequence [2].

Larkin *et al.* have performed studies on *Arabidopsis* leaf epidermis. Their observation of hair cells on *Arabidopsis* leaves, known as trichomes, has shown that the initiation of trichomes is much less frequent than expected by a chance event. Trichomes are never found as nests; in order to initiate trichome development on leaf epidermis, establishment of a minimum distance between two developing trichomes is required. The proper establishment of trichome pattern is a non-random event and it is not achieved by cell lineage [3]. This implies the presence of a cross-talk between the cells accomplishing the pattern.

Main functions of trichomes are resistance to herbivores; extreme temperature fluctuations and UV light [4]. The most revealing studies related to trichome research aim to understand how the initiation and development of specialized cells from initially equivalent cells is achieved. Factors making trichomes ideal models for such studies include accessibility of these cells, the presence of several mutants specifically affecting trichome development in the plant and the viability of plants lacking trichomes [5]. Therefore, the formation of trichomes is widely studied and serves as a basis for the analysis of cellular development of *Arabidopsis*.

Mature trichome cells of the *Arabidopsis* leaves are comparatively large, polarised cells, which have 3 branches and a large nucleus at their first branching

points. The observation of wild-type trichome development as well as trichomes of mutant plants has shown that the first step in trichome differentiation is the commitment of an epidermal cell which then expands and starts endoreduplication. The unicellular trichome cells undergo endoreduplication cycles, which allow for more rapid growth and extension [6, 7]. A stalk then arises perpendicularly to the leaf plane which then expands and a second branch forms. The expansion continues until the third branch has formed and a papillae surface develops at the surface of the mature trichome [8]. The development of a trichome from an epidermal cell is depicted in Figure 1.

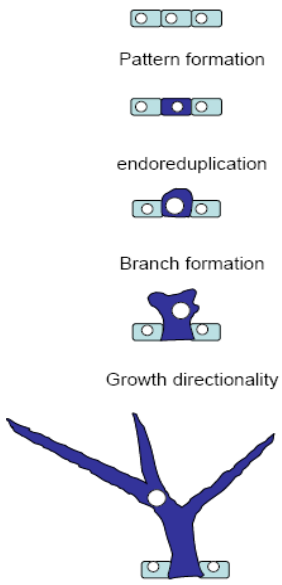

Figure 1 *Trichome development* [9]

1.2. PATTERN FORMATION IN ORGANISMS (TURING AND MEINHARDT & GIERER MODELS)

The *de novo* formation of a stable pattern in biological systems was first delineated by Turing's equations which were then modelled by Meinhardt and Gierer (Activator-Inhibitor Model) [10, 11]. This mathematical model postulates the formation of an activator which enhances the production of itself by an autocatalytic loop. The increased activator concentration in one cell accelerates the production of inhibitor which moves into the neighbouring cell where it suppresses the activator (Lateral Inhibition Mechanism). The observation of different trichome initiation mutants suggested that this model could give ideas about how the patterning in

trichomes is established [7]. In the case of trichome formation, the activator in the selected cell continues to produce more activator molecules that overcome equilibrium between the activator and inhibitor concentrations. The statistical fluctuations between the selected and neighbouring cells lead to the formation of a stable pattern and to the initiation of the trichome cell fate. The comparison of the mechanisms for trichome and root hair patterning in *Arabidopsis* has revealed that similar mechanisms are involved in both events [12].

1.3. DIFFERENT GENE FAMILIES IN TRICHOME FORMATION

Over the recent years, the observation of trichome initiation in different mutant plants has shown that some gene mutations cause decrease in trichome density and number. On the other hand, mutations in some other genes increase the number of trichome forming cells or formation of trichome nests in *Arabidopsis.* Therefore, the genes responsible for the trichome cell fate are divided into two categories. The members of the first category are the activators of trichome initiation due to the fact that trichome formation is decreased when these genes are mutated. The second category is composed of the genes whose mutations cause increase in trichome forming cells namely the inhibitors of trichome initiation. Several of these genes have been cloned and analysed by genetic analysis.

1.3.1. ACTIVATORS OF TRICHOME INITIATION

The mutations of some genes either as single or in combination with other mutations result in a decrease of trichome formation. In this sense, these genes are referred to as the activators of the trichome cell-fate. These genes include *TRANSPARENT TESTA GLABRA1 (TTG1)* [13], *GLABRA1 (GL1)* [14, 15], *GLABRA3 (GL3)* [16, 17], *ENHANCER OF GLABRA3 (EGL3)* [18], *GLABRA2 (GL2)* [19] and *TRANSPARENT TESTA GLABRA2 (TTG2)* [20]. The cloning of these genes has revealed that they encode proteins containing tryptophane-aspartic acid (WD40), myeloblastosis (MYB), basic Helix-Loop-Helix (bHLH), Homeodomain-Zipper (HD-Zip) or tryptophane-arginine-lysine-tyrosine (WRKY) domains.

1.3.1.1. WD40 REPEAT PROTEINS

One of the first mutant screens of *Arabidopsis* resulted in the identification of *ttg1* mutant plants. The *ttg1* mutant plants show lack of trichomes on the surfaces of leaves and at the stem base, transparent seed coat and absence of seed coat

mucilage [13]. The cloning of *TTG1* revealed that this locus encodes a WD40 repeat protein [21]. WD40 is known to be the protein-binding motif, enabling protein-protein interactions for several different cellular processes. The structure of WD40 repeats has been shown to be a beta-propeller [22]. Although TTG1 structure is currently unknown, it is likely that the WD40 repeats may mediate protein-protein interactions for trichome initiation.

1.3.1.2. MYB PROTEINS

The second gene which resulted in glabrous plants (i.e., plants lacking trichomes) when mutated was the *GL1* gene. *gl1* mutant plants show no trichomes on leaf surfaces [14, 15, 23, and 24]. The cloning of the *GL1* revealed that this locus encodes a two repeat (R2R3) MYB protein [23].

Two homologous genes for *GL1* have been cloned and they have been shown to play a role in epidermal patterning. The first gene is called *WEREWOLF (WER)*. Its mutation causes increased root hair formation; *WER* overexpression reduces the number of root hair cells produced [25, 26]. Overexpression of the second homologous gene -*At MYB23*- shows phenotypes similar to the *GL1* overexpression phenotype which is recognized by a decrease in number of trichomes along the middle part of the leaf and production of ectopic trichomes on cotyledons and hypocotyls [27, 28].

In 1982, the first gene containing MYB domain was discovered and named *c-MYB*. It encodes an oncogene from avian retroviruses, causing acute leukaemia [29]. Since then, several MYB domains containing proteins have been characterised both from animals and plants. MYB domains are recognized by the presence of highly basic amino acids and conserved tryptophan residues that form a helix-turn-helix structure made of 53 amino acids [30, 31]. MYB domains consist of imperfect repeats referred to as R1, R2 and R3 repeats. One study reports that R2R3 repeat MYB proteins in *Arabidopsis* genome constitute the largest family of *MYB* genes in plants [32].

1.3.1.3. BASIC HELIX LOOP HELIX (bHLH) PROTEINS

Another gene which is involved in trichome initiation is *GL3*, encoding a bHLH protein. An alignment of consensus sequences using the HLH motif designates the presence of 133 genes containing bHLH domains in *Arabidopsis*. These proteins have a wide range of functions from plant metabolism to development [33]. bHLH proteins are identified by their conserved structural domain, which is

composed of two amphipathic α-helices, a loop and a basic region. The basic region has been shown to be important for DNA binding whereas the HLH domain is needed for homo and/or heterodimerization [34]. *gl3* mutant plants, which lack the bHLH region of the GL3 protein, have reduced numbers of trichomes; whereas overexpression of GL3 results in more trichome formation than observed in wild-type plants. Yeast two-hybrid and genetic analyses demonstrate that GL3 interacts with GL1 and TTG1, and that for the GL1-GL3 complex to completely function, the TTG1 protein is required [16].

A homologous gene for *GL3* is *EGL3*. It has also been cloned and it has been shown that *EGL3* functions in epidermal cell fate and differentiation as well as other *TTG1*-dependent pathways. Although the *egl3* mutant plants do not have differences in their trichome numbers or densities compared to the trichomes on wild-type leaves, the double mutants of *gl3egl3* have glabrous leaves [18]. The comparison of phenotypes of these two bHLH mutants is shown in Figure 2.

GL3 and EGL3 can interact with R2R3 repeat MYB domain proteins GL1, WER and AtMYB23, as well as the R3MYB proteins and TTG1 in yeast two-hybrid experiments [16, 17, 18, 28, 35 and 36]. It has been shown that the MYB domain, which is the region important for DNA binding, also plays a role in interaction with bHLH proteins [37].

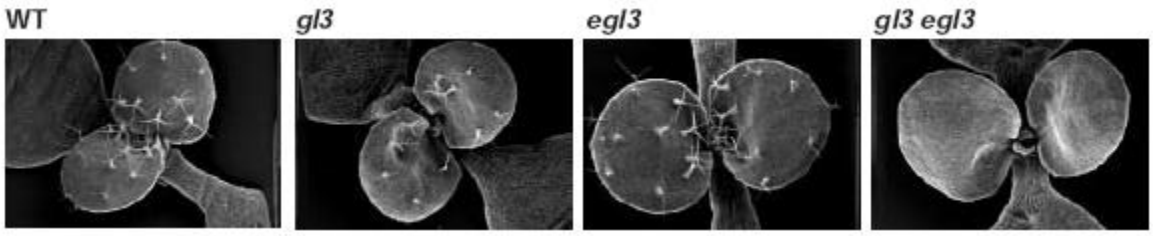

Figure 2 *Redundancy of bHLH proteins* [38]

1.3.1.4. HD-ZIP PROTEINS

It has been found that the *GL2* encodes a HD-Zip protein. Mutant plants lacking the *GL2* show reduced trichome density, ectopic root hair formation as well as aborted trichome formations with the shape of spikes [39, 40].

Initially, *GL2* was thought to be involved in trichome morphogenesis but not in trichome initiation. However, reduced trichome density in *gl2* mutant plants, the lack of trichomes on the *gl2gl3* plants' leaves and a dose-dependent increase in

trichome numbers and their frequencies in *pGL2::GL2* plants suggests a possible role for GL2 protein also in trichome initiation pathway [19, 40].

The homeodomain (HD) is a 60 amino acid conserved domain encoded by the homeobox DNA present in all eukaryotic organisms. The HD forms a DNA-binding helical structure. The structure and position of the HD has been shown to determine the variety of developmental processes in which the HD-containing transcription factors function [41].

The GL2 protein contains both a highly basic domain that may stipulate DNA specific contact and a strongly acidic domain that might mediate transcriptional activation complex protein-protein interactions [40].

1.3.1.5. WRKY PROTEINS

Although it has not been shown to have a role in trichome initiation, a WRKY transcription factor *TTG2* has been elucidated to have a similar role like *GL2* in trichome development. Mutant plants lacking the functional *TTG2* show similar trichome phenotypes like the *gl2* mutant plants. It has been suggested that the *TTG2,* together with *GL2,* acts downstream of trichome initiation and promotes the outgrowth of trichomes on the leaf epidermis [20]. The expression patterns of *TTG2* and *ttg2* phenotypes indicate an affiliated role for TTG2 with MYB-bHLH-TTG1 complexes in the regulation of their common target genes [42].

WRKY domain containing transcription factors are plant specific proteins that have the conserved N-terminus domain and Zinc-finger like motif. This domain is named after the presence of the WRKYGQK amino acid sequences serving as the DNA binding motif. The transcription factors containing this conserved domain have been shown to be involved in plant defence as well as metabolic processes and trichome development mechanisms [43, 44].

1.3.2. INHIBITORS OF TRICHOME INITIATION

Mutations in some genes results in formation of more trichomes or trichome nests. In addition, the overexpression of these genes shows a decrease in number of trichomes or even glabrous leaves in some cases. These genes are therefore named as inhibitors of trichome initiation. Surprisingly, all the inhibitor genes cloned so far contain only the MYB conserved domain. The difference between the activator and inhibitor MYB proteins is that the former are R2R3 repeat MYB

proteins, whereas the latter contain only the R3 repeat, lacking the activation domain [45, 46, and 47]

A mutation in the gene coding for the TRIPTYCHON (TRY) protein causes initiation of more than one trichome from an initiation site which would normally produce only one trichome cell [7]. Nevertheless, a study by Schnittger *et al.* has shown that overexpression of the trichome initiation activators in combination with removal of the inhibitor *TRY* is not sufficient for transformation of all epidermal cells into trichomes [48]. This indicates the presence of other inhibitory factors of trichome initiation.

The mutation of the *CAPRICE (CPC)* gene, which is a homologue of *TRY,* results in reduced number of root hairs and increased trichome density, whereas overexpression of this gene leads to increased number of root hairs and absence of leaf, stem and sepal trichomes. It has been reported that *CPC* also encodes a R3 MYB domain protein [45, 47].

The overexpression of the *TRY* and *CPC* in a wild-type background leads to glabrous leaves whereas the mutation of *TRY* together with *CPC* results in increased numbers of trichomes and trichome clusters. This shows that *TRY* and *CPC* are inhibitors of trichome initiation. The analysis of *try* single and the *trycpc* double mutants and their similar expression patterns suggest that these two inhibitory genes are involved in the Lateral Inhibition Mechanism in trichome and root hair patterning in *Arabidopsis* [47].

In addition to *TRY* and *CPC*, four other *R3 MYB* genes have been cloned from *Arabidopsis*. The mutant and overexpression phenotypes together with the identification of common interaction partners denote that all these six genes are functional homologues and they have redundant roles in trichome and root hair patterning [35, 49, 50, and 51].

These results show that an important protein family for controlling trichome and root hair patterning in *Arabidopsis* is composed of single and double repeat MYB proteins. The reason for presence of several redundant MYB proteins is still not known. They have been suggested to have overlapping roles in plant development as well as functions in different organs [26, 52]. When the functions of MYB proteins from other organisms are also considered, it is seen that this conserved domain has roles in a vast variety of cell proliferation and differentiation events [53, 54, 55, 56, and 57]. However, how this difference in roles of these proteins containing conserved domains is exactly ensured is still under investigation. One possibility is

the different regulation of these proteins [24, 28, and 50]. Another feature of these similar proteins could be the differences in their tertiary structures enabling different protein and/or DNA interactions. Although much is known about animal MYB domain protein properties and their structure, only one MYB protein from plants has been crystallized. This is a MYB protein from *Antirrhinum*, involved in establishment of floral symmetry [58, 59]. Nevertheless, none of the structures of MYB proteins from *Arabidopsis* have been currently solved.

1.4. TRICHOME PATTERNING MECHANISM

The patterning in *Arabidopsis* epidermis is achieved by the translation of developmental cues into interplay of readout genes to establish the developmental cell fate of the plant [7]. Should the Meinhardt & Gierer Model be used to explain the mechanism of patterning, the requirements of the model have to be fitting to the actual observations in nature. The presence of the same types of genes and similar interactions both in the root and the shoot of *Arabidopsis* puts forward the similar mechanisms for trichome initiation and root hair cell patterning [36]. However, there are some differences between patterning mechanisms in these organs. The first difference is that positional cues specify the formation of root hairs only over the cleft between two underlying cortex cells. In the cells that have a single underlying cortex cell, activator complex is formed by interaction of R2R3 MYB-bHLH-WD40. This launches the transcription of GL2 to trigger the non-root hair cell fate. In this cell, the R3 MYB protein concentration is also increased. These small R3 MYB proteins then move into the neighbouring cell where they form the inactive complex, which cannot produce GL2 to gain the non-root hair fate so they can develop as root hairs [12, 26, 38, and 60].

The first assumption of the Meinhardt & Gierer Model is the autocatalytic loop activating the production of activators in the pattern forming cells. This feature of the model is still not elucidated for patterning mechanisms.

The second assumption is the activation of inhibitors by the activators. The same expression pattern of *GL1*, *TRY*, *GL3* and *EGL3* in wild-type and different mutant backgrounds support the transcriptional activation of the *TRY* by *GL1* and *GL3* [18, 24, 36, 47, 61, and 62]. The only proof of the direct interaction of activators with the proposed target DNAs were shown by Electrophoretic Mobility Shift Assays (EMSA) and yeast one-hybrid [63, 64, and 65]. These studies shed light on the interaction of MYB proteins with their target DNA sequences important for

epidermal patterning. However, EMSA experiments have only been conducted for WER and CPC. The ability of other transcription factors described in the patterning pathway to bind directly *in vitro* to other DNA sequences have not been shown yet. In addition to the EMSAs, Chromatin Immunoprecipitation (ChIP) experiments have also been conducted to show that GL3 and TTG1 can bind to the promoters of *GL2, CPC, TRY* and *ETC1*. These results are consistent with the activation of inhibitors by the activators stated in the Meinhardt & Gierer Model [66]. These assays were performed by fusion tags due to the absence of the specific antibodies against patterning proteins.

Another postulate of this model is the Lateral Inhibition which requires the cell autonomous behaviour of the activator and mobility of the inhibitor. The increased concentration of the activator in one cell causes the increase of this cell's potential to become a trichome cell. On the other hand, this cell starts producing also more inhibitor which moves to the neighbouring cell where it inhibits the trichome initiation by inhibiting the activator function in this cell [11]. One of the important experiments in trichome development has shown the counteraction of *GL1* by *TRY*. Trichome formation can be initiated not only from the epidermal but also from the subepidermal cell layers and trichomes can be initiated on organs otherwise would be devoid of trichomes when *GL1* is overexpressed in a *try* background [61, 67]. The second evidence for this postulate came with the comparison of expression and protein localization of *CPC* [46, 68]. Apart from movement of the CPC in root, micro-projectile bombardment experiments also provide evidence for TRY and CPC movement in *Arabidopsis* cotyledons and leaf epidermal cells. On the other hand, in the same assay, GL1 and GL3 were shown to be cell autonomous [62]. Moreover, leaf sector experiments show that GL1 acts locally [7]. These data support movement of inhibitors which is predicted by the Meinhardt & Gierer Model. However, the actual mechanism of movement of these proteins is still not known and only speculated to be through plasmodesmata [46, 62, and 68]. Whether this mechanism for movement holds true is a hypothesis that still has to be tested by other tools such as microinjection.

The inhibitor, which has moved from the trichome initial to the neighbouring cells, presumably prevents the formation of the activator complex as well as the production of the inhibitor complex in these cells. Yeast three-hybrid experiments have shown that the inhibitor TRY can compete with GL1 for binding to GL3, thus forming an inhibitory complex, which prevents the activation of downstream genes for trichome formation. This inhibitory complex forms in the epidermal cells which have received TRY from their neighbouring trichome initiating

cells [17]. The competition of CPC with WER for binding to GL3 and EGL3 has also been shown by yeast three-hybrid experiments [69]. Nevertheless, the competition of these proteins for binding to GL3 has not been shown by any other assay. It would be of great importance to compare the binding affinities of GL1/WER and TRY/CPC to GL3.

Genetic and yeast two-hybrid analyses show that GL3, as well as its homologue EGL3, interacts with GL1 and TTG1, and that TTG1 is required for GL1-GL3/EGL3 complex function [16,18]. Similar interactions are also observed in yeast two-hybrid experiments of WER/MYB23 with GL3/EGL3 [37]. The formation of this MYB-bHLH-WD complex is possibly realized also in the *Arabidopsis* leaf epidermis to initiate the trichome cell fate. The cell, which perceives the difference between activator and inhibitor concentrations, processes this information to go into the determined cell fate. The formation of the activator complex in leaf and root epidermis is likely to trigger the formation of the trichome and the non-hair cell fate by activating the *GL2* that is necessary for the determination of the epidermal pattern [12, 38, 60, and 69]. However, it is still a question if these interactions are occurring in a direct manner or if some intermediates that intervene the interaction of these individual proteins are existing.

In summary; trichome initiation is comparable to root hair cell formation in *Arabidopsis*, as both are mediated by the formation of the activator complex composed of R2R3 MYB proteins - bHLH proteins - WD40 protein in the trichome or non-root hair initials. The formation of the activator complex initiates the transcription of *GL2* and the trichome cell fate or the non-root hair cell fate. The activator complex also activates other R3 MYB transcription factors which then move to the neighbouring cells where they compete with the R2R3 MYB proteins for binding to the bHLH proteins and form the inactive complex. As a consequence, trichome or non-root hair cell fate cannot be triggered in these cells. The comparison of these two mechanisms is illustrated in Figure 3.

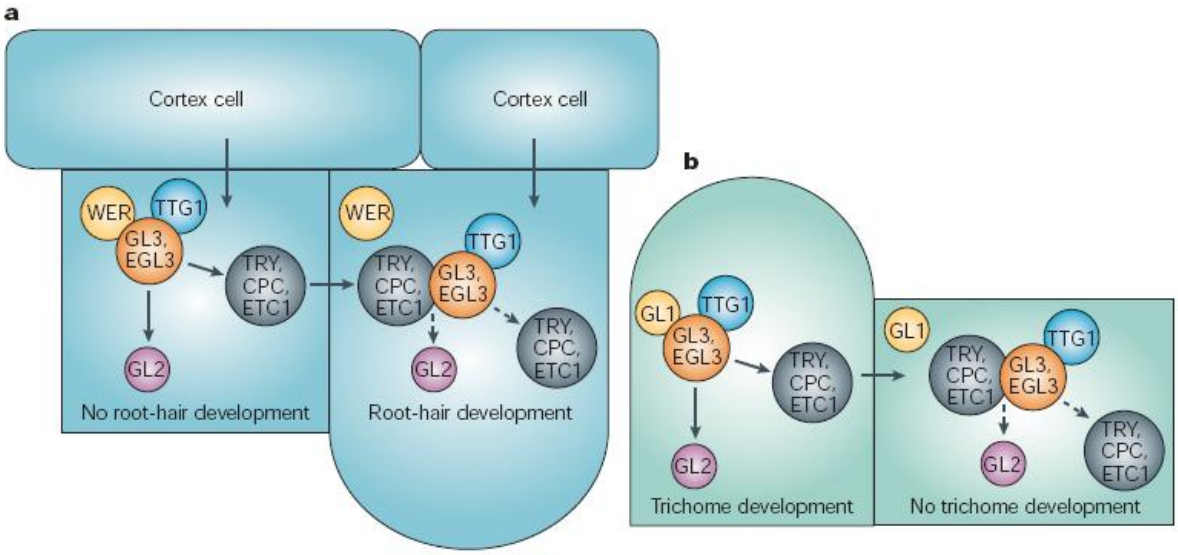

Figure 3 *Patterning mechanism in* Arabidopsis *epidermis* [38]
Different organs have similar machinery
a) Patterning in root epidermis
b) Patterning in leaf epidermis

1.5. OTHER MECHANISMS INVOLVED IN TRICHOME FORMATION

Bouyer *et al.* have recently shown that a substrate-depletion mechanism is also playing a role in trichome initiation, in addition to the activator-inhibitor model. TTG1 has been shown to be depleted from neighbouring cells to the trichome initials in *Arabidopsis* leaves. The same study also indicates the mobility of TTG1, most probably via the plasmodesmata. Due to the lack of depletion of the TTG1-YFP protein in *gl3* plants, increased expression of *GL3* in trichomes and the interactions between GL3 and TTG1, GL3 seems to be the factor that traps the TTG1 protein in the incipient trichome cells [70].

1.6. AIM OF THE PROJECT

Trichome pattern formation in *Arabidopsis* has been analysed in the light of the Meinhardt & Gierer Model in recent years. However, currently the vast majority of reports giving insights about trichome pattern formation in *Arabidopsis* are based on the models depending on genetic, yeast two/three-hybrid analyses and the comparison of the molecular data obtained from the root hair patterning system. There are still limitations existing as to how observations made in nature could be

explained by these models. One of the most important limitations is the absence of proteins in hand except WER and CPC. This fact handicaps the performance of experiments enlightening how the direct protein-protein, protein-DNA interactions are established, how the structural and biochemical properties of similar proteins can define their different roles in trichome patterning.

This project attempts to analyse and characterise the patterning proteins biochemically. It aims to express, purify and further characterise the proteins involved in *Arabidopsis* trichome initiation machinery. Purified proteins allow for experiments to be carried out that further help to understand the patterning mechanism at a biochemical level. Moreover, until now, the lack of antibodies for the patterning proteins made it possible to work only with gene fusion tags which may not always reflect the real biological situations. Consequently, this study endeavours to obtain pure antibodies against patterning proteins. These antibodies may in the future be used to conduct experiments in requirement for the native proteins. Additionally, it is also this project's aim to test *in vitro* the interactions which had been genetically shown previously. It is of interest to test the functionality of proteins *in vitro* and also to see whether these interactions are occurring in a direct manner. In summary, this work contemplates biochemical data to explain the features of the patterning mechanism, which are so far not discovered.

2. RESULTS

The prerequisite to biochemically study a mechanism is to have the proteins in hand. Therefore, the proteins which are thought to be involved in trichome patterning were tried to be expressed using different expression systems. Since it is better to express eukaryotic proteins in eukaryotic systems, initially the expression of GL1, GL3, EGL3, TTG1, TRY and CPC proteins in two different eukaryotic expression systems were tried. However, it was not possible to express these proteins using neither the *S.cerevisiae* nor the *P.pastoris* expression systems (Data not shown). Another eukaryotic expression system, *Arabidopsis* suspension cultures, was also tried. This also did not yield production of the proteins that were intended in this expression system (Data not shown). After these trials, the only choice left was to use different bacterial expression systems as an alternative. The proteins to be expressed were intended to be used in different purposes. Hence; the expression of different proteins either using no tag or with different fusion tags was attempted in this study.

2.1. PREDICTED PI AND MOLECULAR WEIGHTS OF PATTERNING PROTEINS

In order to provide appropriate experimental conditions for proteins, one should find a proper buffer in which proteins are soluble, stable and can exert their biochemical activities. One of the important features of the buffer chosen is the pH value. Therefore, at first the predicted pI values of different proteins from The *Arabidopsis* Information Resource (TAIR) Database were analysed (Table 1).

Table 1 *pI values and molecular weights of proteins identified in TAIR database* [71a].

Protein Name	pI value	Molecular Weight (Da)
GL1	7.18	26339
GL3	5.93	70538
EGL3	4.81	66619
TTG1	4.49	37892
TRY	9.99	13005
CPC	10.21	11385
TTG2	9.39	47141

2.2. CHARACTERISATION OF DIFFERENT PROTEINS IN DIFFERENT BUFFER CONDITIONS

As explained in Introduction, proteins acting in the patterning pathway contain different classes of conserved domains. Furthermore, as can be seen in Table 1, all trichome patterning proteins have different computed pI values, which indicate that these proteins might have different behaviours under the same conditions. Since a combination of these proteins would be used in this work (such as in *in vitro* pull-down assays), it would be important to determine the common preferred buffer conditions that a combination of proteins of interest could function properly.

Therefore, initially a test purification step for all the proteins that were used in this study was done as a His- tag fusion via Nickel-Nitrilotriacetic Acid (Ni-NTA) resin. After purification of each protein individually, the purified proteins were dialysed against several different buffers. The behaviours of proteins for different buffering reagents were analysed and the optimum buffer to work with was determined. After dialysis, the protein samples were first centrifuged and then analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). These treatments were applied to His-GL, His-GL3, His-EGL3, His-TTG1 and His-TRY proteins- the key players of trichome patterning. SDS-PAGE analyses have shown that the proteins were precipitating when they were in MES buffer, whereas HEPES buffer was shown to destabilize the proteins. When an SDS-PAGE was done for HEPES buffered proteins that were left for overnight at 4^0C, it was observed that

proteins started showing smears instead of clear bands. As an example, His-GL1 protein is shown in different buffers after overnight storage at 4^0C in Figure 4. On the other hand, proteins in buffers of Phosphate, Tris or Carbonate could still be detected by Coomassie staining after SDS-PAGE, even after several weeks of storage at 4^0C. This showed that the optimum buffer to work with is a Tris or Phosphate based buffer. pH values of the different Tris buffers were tested for pH 7, pH 7.5, pH 8 and pH 8.5. The best Tris buffer condition was found to be pH 7.5 and pH 8, due to the reason that pH 7 value is very close to the pI value of the GL1 protein. Carbonate buffer was not used for the experiments due to its high pH value (pH > 9). The buffers that were used for different proteins are explained in the Materials & Methods section in detail.

Table 2 *Different buffering reagents that were tested and corresponding protein behaviour.*

Buffering Reagent	Protein
HEPES pH 7.5	Proteins were not stable
MES pH6.2	Proteins were not soluble
Phosphate pH 7.4	Proteins were both stable and soluble
Carbonate pH 9	Proteins were both stable and soluble
Tris	Proteins were both stable and soluble

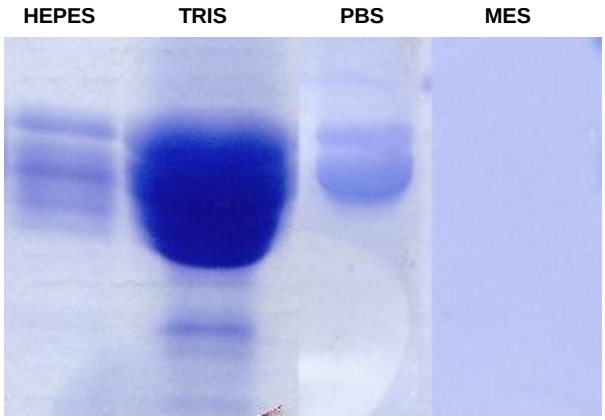

Figure 4 *SDS-PAGE of the His-GL1 in different buffers.*

2.3. THE STRUCTURAL DIFFERENCES OF THE MYB PROTEINS INVOLVED IN TRICHOME PATTERNING

The MYB proteins involved in trichome patterning exert different functions for activation or inhibition of trichome patterning despite containing conserved domains [14, 15, 23, 45, 47, and 48]. How this is achieved by these proteins containing similar sequences is still not known. One of the reasons could be the differences in their tertiary structures. Therefore, it would be of great importance to compare the structures of the MYB proteins acting as activators and inhibitors for trichome patterning. For this reason GL1 and TRY were chosen as representatives, since they have been shown to be the key players in activation and inhibition machineries respectively [14, 15, 23, and 48].

Among plant MYB proteins, the only one with a solved structure is a MYB domain protein from *Antirrhinum* [58]. However, the alignment of protein sequences of the *Antirrhinum* MYB protein together with GL1 or TRY did not yield any results that would help conduct homology modelling. When an alignment for closest homologue with a known structure in the Protein Databank (PDB) [71b] is conducted, the result yields to a MYB protein from chicken, which is still far away. Also the database search by Predict Protein Database [71c] did not yield any results for GL1 and TRY proteins based on the known structures in the databases of 3D-Jigsaw [71d] and Swiss-Model [71e], and PDB. This hindered the Homology Modelling or 3D Structure Alignment for GL1 and TRY protein structures. Therefore, it would be important to crystallize the GL1 and TRY in order to solve their three-dimensional structures to enlighten the differences in their activities. In order to do this, GL1 and TRY had to be obtained as extremely pure, highly concentrated and devoid of any tags, which may interfere with their tertiary structures. This required the expression and purification of GL1 and TRY.

2.3.1. EXPRESSION OF PROTEINS WITHOUT TAG

The CDS's of GL1, GL3, TTG1, TRY and CPC proteins – the key players in trichome initiation representing one protein from each domain class- were cloned into the pET3a vectors. GL1, TRY and CPC proteins were successfully expressed using the inducible T7 promoter for high levels of protein expression in bacteria. Expression was achieved with a final concentration of 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction for 3 hours. GL3 and TTG1 proteins could not be expressed with the usage of this vector. Expressions were analysed by taking

an aliquot from cell culture after induction, running these aliquots in SDS-PAGE and staining of the gels with Coomassie staining (Figure 5).

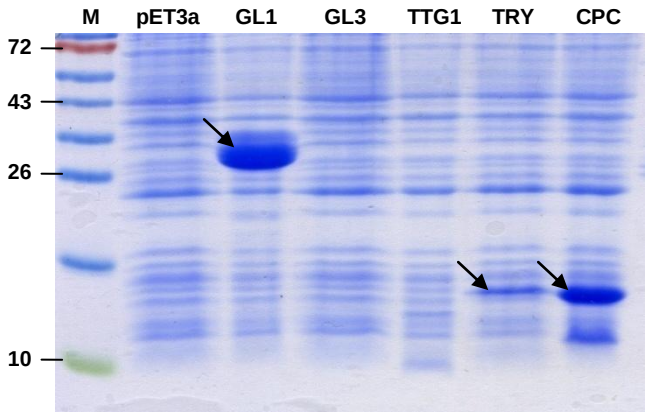

Figure 5 *SDS-PAGE of the cell extracts after induction containing the pET3a vector constructs. M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the left side Arrows indicate the expressed proteins.*

2.3.2. PURIFICATION OF GL1 AND TRY EXPRESSED VIA THE pET3a VECTOR

GL1 and TRY proteins which were expressed using the pET3a vector were purified via gel filtration chromatography since this vector does not contain any sequences for fusion tags. Two rounds of gel filtration chromatography were performed in order to get a highly pure protein. During gel filtration, a bigger pore sized column for first and a smaller pore sized column for the subsequent purification were used. All of the elution fractions containing the protein of interest after the first gel filtration chromatography were combined due to the fact that first round of purification did not result in a single band of protein in SDS-PAGE (Data not shown). After two rounds of gel filtration chromatography, GL1 was still not observed as a single band on SDS-PAGE (Figure 6). On the other hand, by the same method, TRY protein was nicely obtained in a soluble form although some contaminants were present in SDS-PAGE even after the second gel filtration step (Figure 7). In order to get rid of these contaminants, cation exchange chromatography was performed. After the cation exchange, the TRY protein was nicely pure and solubilised. After this step, the concentration of the protein was not high enough to proceed to crystallization (Figure 8). Therefore, the elution fractions of 44 to 49 were combined and the protein was concentrated by the use of Amicons.

However, the concentration of the protein resulted in formation of aggregates, which is a major drawback for crystallization of proteins [72]. The aggregation of these proteins could not be prevented by changing to other buffers since the initial tests already showed that TRY protein is not stable in a HEPES and not soluble in a MES buffer. Therefore purification of TRY was pursued by another method.

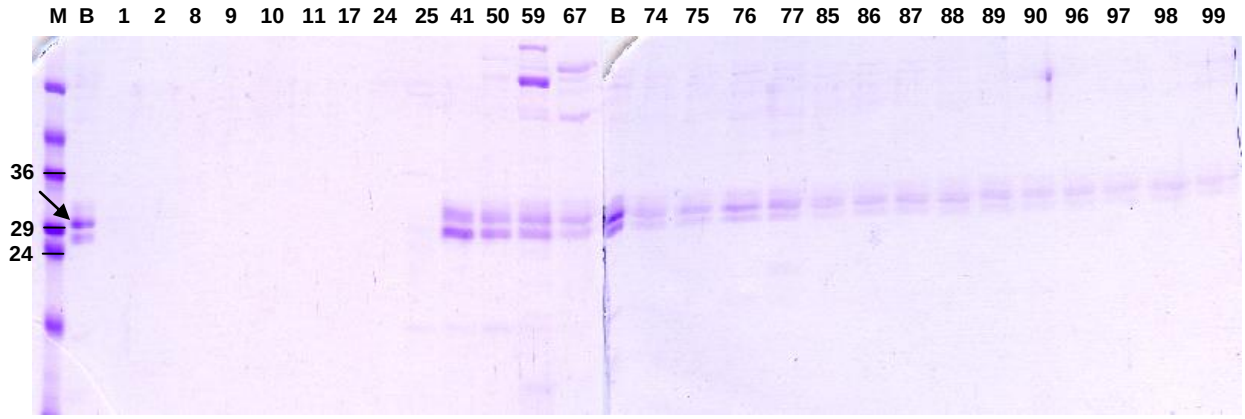

Figure 6 *SDS-PAGE after 2nd purification of GL1 by gel filtration chromatography.*
B: The sample before loading to the second gel filtration column.
Numbers correspond to the elution fractions.
M: Unstained protein molecular weight marker, with corresponding MW values (kDa) shown on the left side
Arrow indicates the band corresponding to theoretical size of GL1

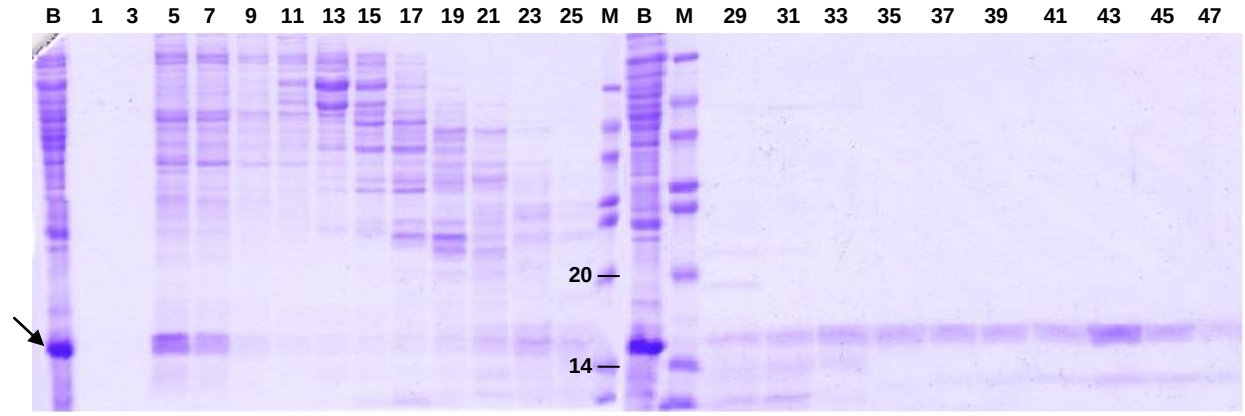

Figure 7 *SDS-PAGE after 2nd purification of TRY by gel filtration chromatography.*
B: The sample before loading to the second gel filtration column.
Numbers correspond to the elution fractions.
Elutions from number 29 to 47 correspond to the peak in the chromatogram which is calculated to be the size of 13kDa. Therefore they were combined and concentrated.
M: Unstained protein molecular weight marker, with corresponding MW values (kDa) shown in the figure.
Arrow indicates the band corresponding to theoretical size of TRY.

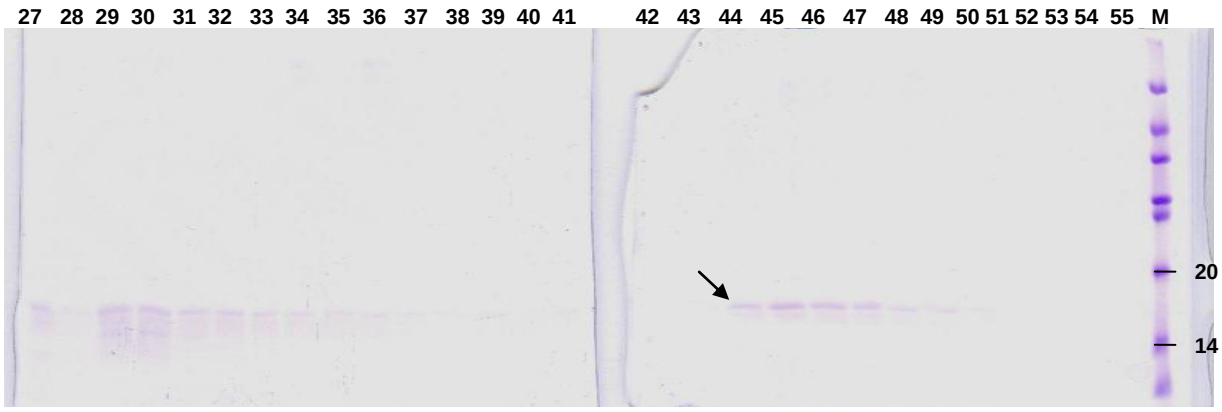

Figure 8 *SDS-PAGE after cation exchange chromatography of TRY.*
Fractions of 44-49 from the cation exchange chromatography were combined and concentrated
The numbers correspond to the elution fractions.
Arrow indicates the band corresponding to theoretical size of TRY

2.3.3. EXPRESSION OF PROTEINS WITH N-TERMINAL MBP- TAG

As mentioned above, one of the intentions of this study was to crystallize the patterning proteins. The observation that these proteins could not be obtained in a desired manner when they were expressed by a vector without a fusion tag, paved the way for the usage of a cleavable tag fused to the protein of interest. For this reason, the pMALC2 vector was chosen for expression purpose since it contains a Maltose Binding Protein (MBP) tag, which is also known to solubilise the fusion proteins [73, 74]. This vector also has a Factor Xa protease cleavage site between the tag and the coding sequence of the desired gene. Therefore, the *GL1*, *GL3*, *TTG1* and *TRY* were cloned into this vector and the expressions of these gene products were tested after IPTG induction. With this vector; GL1, TTG1 and TRY proteins were obtained in a soluble manner but GL3 expression was not observed (Figure 9).

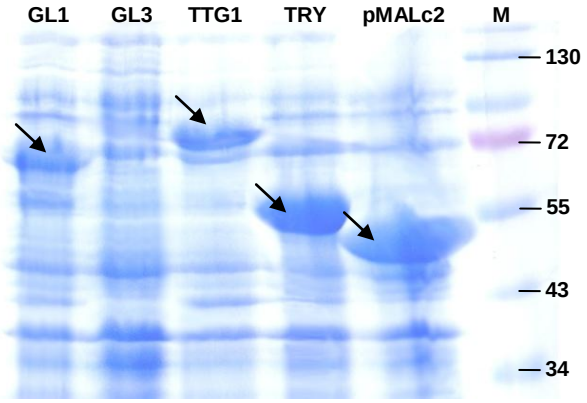

Figure 9 *SDS-PAGE of the cell extracts containing the pMALc2 constructs after induction.*
This vector contains sequences enabling N-terminal MBP- fusion of 42.5 kDa in addition to the size of the proteins. Empty vector expresses the MBP- alone.
M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.
Arrows indicate the expressed protein.

2.3.4. PURIFICATION OF MBP- TAGGED PROTEINS

The GL1, TTG1 and TRY proteins cloned into the pMALC2 vectors were successfully expressed downstream of the MBP- tag, which not only facilitated the bacterial expression of the proteins in a soluble manner but also allowed the purification via the Amylose resin [75]. These three proteins were then purified via Amylose resin successfully (Figure 10). The best purification was achieved for GL1 and TRY proteins.

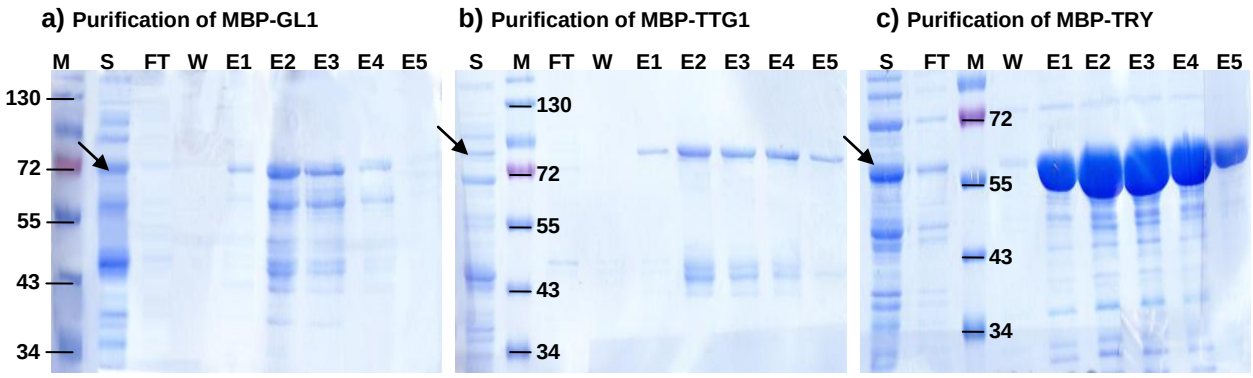

Figure 10 *SDS-PAGE showing N-terminal MBP- tagged proteins via Amylose resin.*
a) GL1, b) TTG1, c) TRY. The abbreviations refer to the following
S: The soluble bacterial cell lysate
FT: Flow through after incubation of the cell lysate with the Amylose resin
W: The combined sample from six washes
E1: First elution fraction, E2: Second elution fraction, E3: Third elution fraction
E4: Fourth elution fraction, E5: Fifth elution fraction
M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown for each figure.
Arrows indicate the MBP- tagged proteins in the cell lysates

After the purification via Amylose resin, elution fractions of 2 to 5 for tagged GL1 and TRY proteins were combined and incubated with Factor Xa protease to obtain cleavage of the tag. No Factor Xa cleavage site in the protein sequences of both GL1 and TRY were predicted to be present. However, no GL1 protein in the solution was found even after incubation for 12 hours of Factor Xa protease with the purified MBP- tagged GL1 protein (Data not shown). This could be due to the internal instability of GL1 protein under these conditions. On the other hand, the cleaved TRY after Factor Xa treatment was successfully obtained.

The cleavage reactions were performed at 15^0C in order to prevent the degradation of the proteins. As a control, same amount and concentration of the tagged protein was incubated in the same buffer without any Factor Xa protease at 15^0C. During this incubation, the TRY protein broke apart from the tag endogenously. However, the protein cleavage was still efficient after 8 hours of incubation (Figure 11).

The purified TRY, which was cleaved by the protease Factor Xa, was further polished by gel filtration chromatography. In order to ascertain that the protease does not cleave the internal protein and the starting sequences of the TRY protein are not altered, TRY protein was sequenced. The samples run on the SDS-PAGE corresponding to the TRY size were cut and used for protein sequencing. The correct cleavage of the protein from the tag was confirmed by protein sequencing using Peptide Mass Fingerprinting (Figure 12). The bars indicate the sequence that yielded from the protein sequencing blasted to the known TRY sequence from the TAIR Database. As one can see from this alignment, the protein sequence starts with the starting amino acid of TRY. In other words, no amino acid sequence from the tag has remained after the cleavage of the MBP- tag.

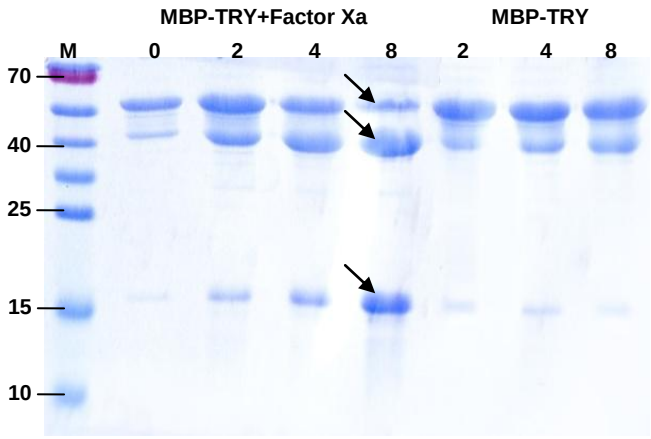

Figure 11 *SDS-PAGE showing the cleavage of MBP-TRY.*

Numbers 0, 2, 4 and 8 on the left side of the figure correspond to 0, 2, 4 and 8 hours of Factor Xa incubation of the purified MBP-TRY protein. Numbers 2, 4 and 8 on the right side correspond to 2, 4 and 8 hours of incubation of the purified MBP-TRY protein in buffer lacking the protease.

The upper bands correspond to the fusion protein, the intermediate bands correspond to the cleaved MBP tag and the lower bands correspond to the cleaved TRY.

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the left side.

Arrows indicate the theoretical sizes corresponding to the fusion protein, tag and protein.

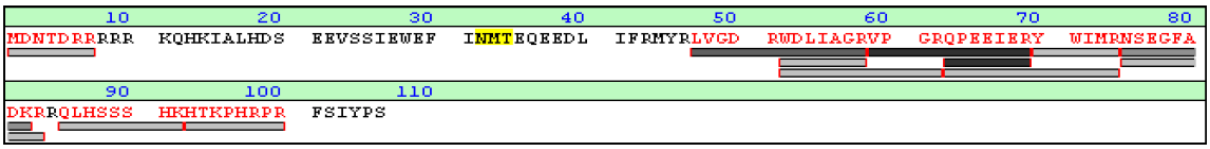

Figure 12 *Alignment of protein sequence obtained using Peptide Mass Finger printing with the known amino acid sequence of the TRY protein.*

Bars represent the protein sequence obtained from protein sequencing of TRY.

2.3.5. PURIFICATION VIA GEL FILTRATION CHROMATOGRAPHY

After cleavage of the tag by Factor Xa, initially cation exchange chromatography was performed to purify the TRY from the uncut fusion proteins and the cleaved tag. However, this did not result in pure TRY (data not shown). As an alternative, gel filtration chromatography was performed. The fusion of the TRY protein to the MBP- tag solubilised the protein. Nevertheless, the TRY protein was not obtained in a soluble manner after removal of the tag. TRY protein was always found to be eluted in the fractions which correspond to protein aggregates after gel filtration chromatography (Figure 13). This gel filtration was performed in a Tris buffer. Changing the protein to a Phosphate buffer still gave a similar result with the gel filtration (Data not shown).

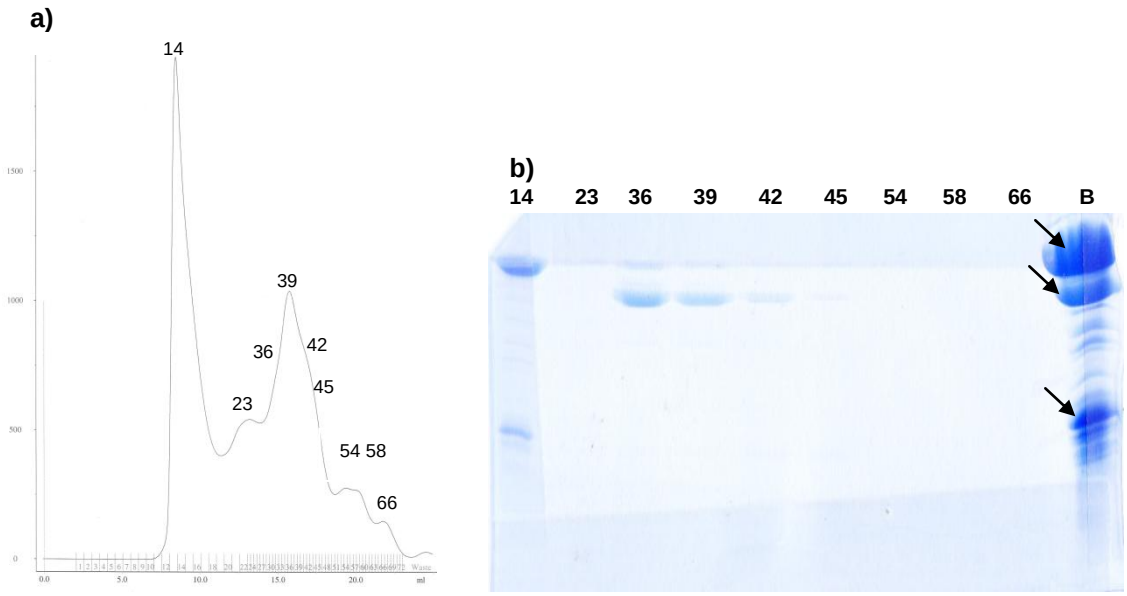

Figure 13 *Gel filtration chromatography of cleaved TRY and MBP- tag*

a) Chromatogram showing gel filtration chromatography

b) SDS-PAGE after gel filtration chromatography

Numbers correspond to the elution fractions of 14, 23, 36, 39, 42, 45, 54, 58, 66 and B is the sample loaded onto the gel filtration column.

The upper band in the gel corresponds to the uncleaved MBP-TRY protein. The intermediate band corresponds to the cleaved MBP- tag and the lower band corresponds to the cleaved TRY protein.

2.3.6. COEXPRESSION OF TRY WITH GL3 AND ITS PURIFICATION VIA GEL FILTRATION CHROMATOGRAPHY

It has been shown that coexpression of interacting partners of proteins may solubilise the individual proteins [76, 77, and 78]. Yeast two-hybrid and bimolecular fluorescence complementation (BiFC) analyses have reported that TRY protein is able to interact with GL3 protein [17, 62]. Therefore, in order to solubilise the TRY protein, MBP-TRY was coexpressed with GL3 protein containing a His- tag (Figure14). The proteins were then purified via the Amylose resin as in the case of MBP-TRY protein when expressed alone. After purification via the Amylose resin, MBP-TRY protein was observed to be eluted with GL3. This confirms that their interaction observed can occur also in bacteria. This eluate was then loaded onto gel filtration column and they were still found to be eluted together in the first fractions of the chromatography step. This observation shows that this interaction is a strong binding. However, it also indicates that the aggregation of TRY cannot be prevented even if it is together with its interacting partner and perhaps GL3 is also aggregating in a similar manner (Figure 15).

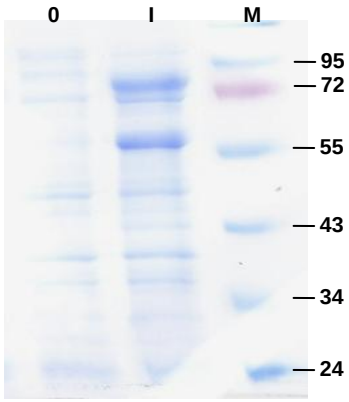

Figure 14 *SDS-PAGE showing coexpression of MBP-TRY and His-GL3*

0: cells before induction, I: cells after induction with a final concentration of 0.3 mM IPTG.

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.

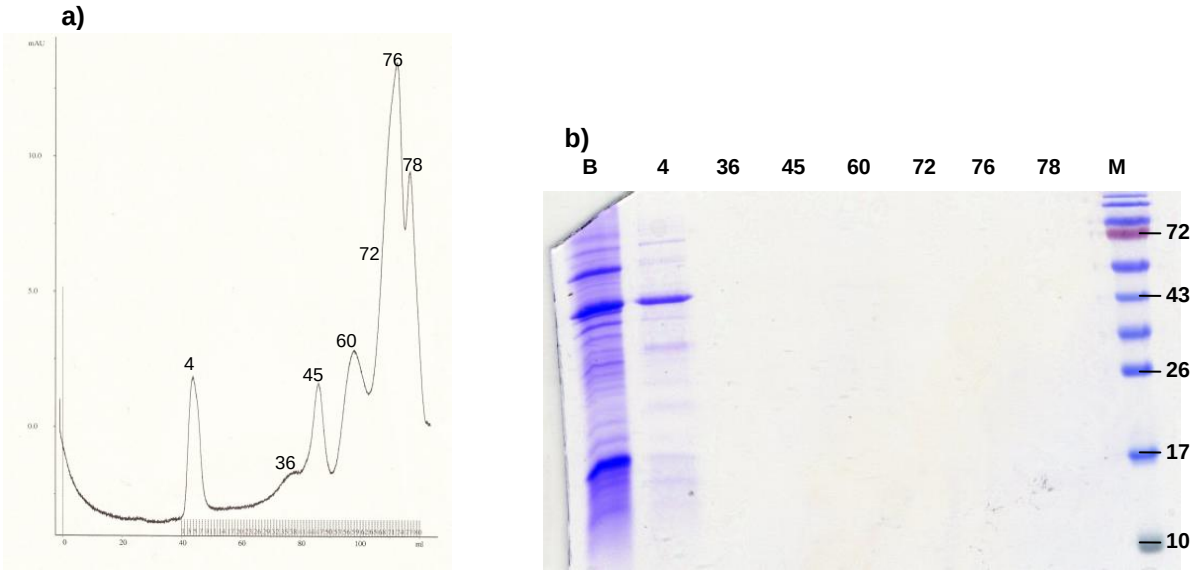

Figure 15 *Purification of the MBP-TRY and His-GL3 by gel filtration*

a) *Chromatogram showing gel purification of GL3-TRY*

b) SDS-PAGE after Gel Filtration Chromatography of the elution fractions

B: sample loaded onto the column, numbers correspond to the elution fraction numbers

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.

All of the methods mentioned above did not provide GL1 and TRY in the desired manner for crystallization of these proteins. Therefore, other methods for expression and purification of these proteins should further be performed to obtain soluble and pure GL1 and TRY.

2.4. THE DIRECT INTERACTIONS OF PROTEINS FOR TRICHOME PATTERNING

Yeast two-hybrid, genetic, BiFC and Protein complex Immunoprecipitation (Co-IP) experiments all denote the interactions among the members of the MYB-bHLH-WD40 protein complex *in planta* [17, 62, and 69]. However, the constraints of these experiments are that it is still not known whether these interactions occur in a direct manner. For this reason, it was the aim of this work to use pure proteins. As a result, the presence of these interactions would be confirmed in a direct manner *in vitro* by using the individual proteins. For the analyses of the direct interactions among patterning proteins, glutathione S-Transferase (GST) pull-down experiments using pure proteins were planned. Yet, the lack of antibodies against these proteins revealed that only the tagged proteins could be used for detection in these experiments. The CDS's were cloned into vectors containing Strep-, His- or GST- tags for high levels of expression, purification and detection of the desired proteins.

2.4.1. EXPRESSION OF PROTEINS USING A VECTOR CONTAINING C-TERMINAL HIS- FUSION TAG

The CDS's of GL1, GL3, TTG1, TRY and CPC proteins were recombined via LR into pGEX2TM-GW vector without the stop codon in the coding sequences. This provides the expression of a fusion protein with a C-terminal His- tag after IPTG induction. SDS-PAGE and Western blot analysis of the cell extracts after induction did not yield any bands corresponding to induction of the proteins (Data not shown). Therefore this system was not used for further procedures.

2.4.2. EXPRESSION OF PROTEINS WITH N-TERMINAL STREP- TAG

Since the C-terminal His- tagged proteins were not expressed and the need for another tag to do the GST pull-down experiments existed, a Strep- tag containing pASK3GW vector was used. This is a gateway compatible vector, containing a Tetracycline inducible promoter. It also allows the production of proteins with an N-terminal Strep- fusion protein. The GL1, GL3, EGL3, TTG1, TTG2 and TRY coding sequences were recombined into the vector pASK3GW. After induction, the expressed proteins except EGL3 and TTG2 could not be differentiated from the background proteins in Coomassie stained SDS-PAGEs (Figure 16 a).

However, the inductions of GL3, EGL3, TTG1 and TTG2 proteins via this vector were observed by doing a Western blot with a Strep- tag antibody (Figure 16). Due to the fact that not all of the proteins were successfully expressed, the usage of this system for further experiments was abandoned.

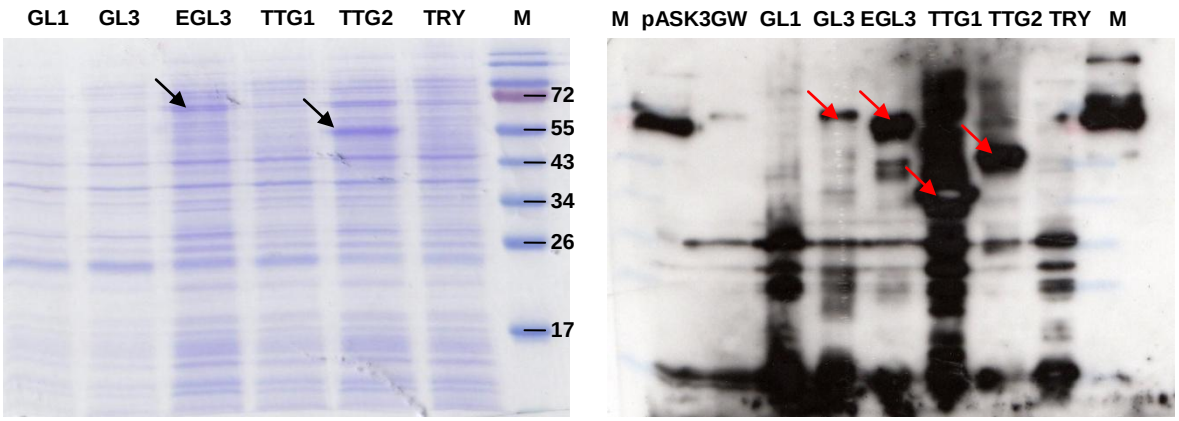

Figure 16 *SDS-PAGE analysis of the cell extracts containing the pASK3GW constructs after induction.*

a) SDS-PAGE showing the cell extracts after induction with tetracycline

b) Western blot showing the cell extracts after induction with tetracycline.

Detection was done by using anti-Strep antibody as 1^0 antibody.

This vector contains sequences enabling N-terminal Strep- fusion of 8 amino acids, in addition to the size of the proteins

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.

Arrows indicate the expressed proteins.

2.4.3. Expression of Proteins with an N-terminal His- Fusion Tag

The *GL1*, *GL3*, *EGL3*, *TTG1*, *TTG2*, *TRY*, *MYB23*, *WER*, *ETC1*, *ETC2*, *ETC3* and *CPC* were successfully expressed using the pDEST™17 vector, which contains an N-terminal His- fusion tag. In order to obtain proteins in a soluble form, expressions under different conditions were tried. Each condition refers to a combination of the following parameters: induction duration, induction temperature, final IPTG concentration and types of cells. The values for these parameters used in these trials are given in Table 3. However, none of the conditions resulted in solubilisation of the expressed proteins. Reduction of induction temperature below 20^0C resulted in no expression of the proteins. The best protein expression levels for all proteins were obtained when a final concentration of 1 mM IPTG at 37^0C for 3 hours in BL21DE3RIL (STRATAGENE) were used. Expression of each protein under these

conditions were analysed by SDS-PAGE. Western blot analysis was also done using anti-His antibody to check the integrity of the fusion tag. SDS-PAGE and Western blot analyses are shown in Figure 17. Therefore, the expression studies were proceded under these conditions in the further experiments.

Table 3 *Parameters used for soluble expression of proteins.*

Soluble expression of each protein used in this study was tested in all possible combinations of the different parameter values indicated.

Induction time (hours)	0,5	1	2	3	4	5	6
Induction temperature (^{0}C)	37	30	28	25	20	18	15
IPTG [mM]	0.1		0.3		0.5	1	
Cell types	BL21		Rosetta Gami& Rosetta Gami B		TOP10	BL21de3RIL	

Due to the reason that C-terminal His- fusion did not yield any protein expression, these N-terminal His- tagged insoluble proteins were used for further experiments. For the subsequent assays, the inclusion bodies were purified from the cytosolic fractions which were then solubilised by dialysis against the buffers needed for the GST pull-down experiments.

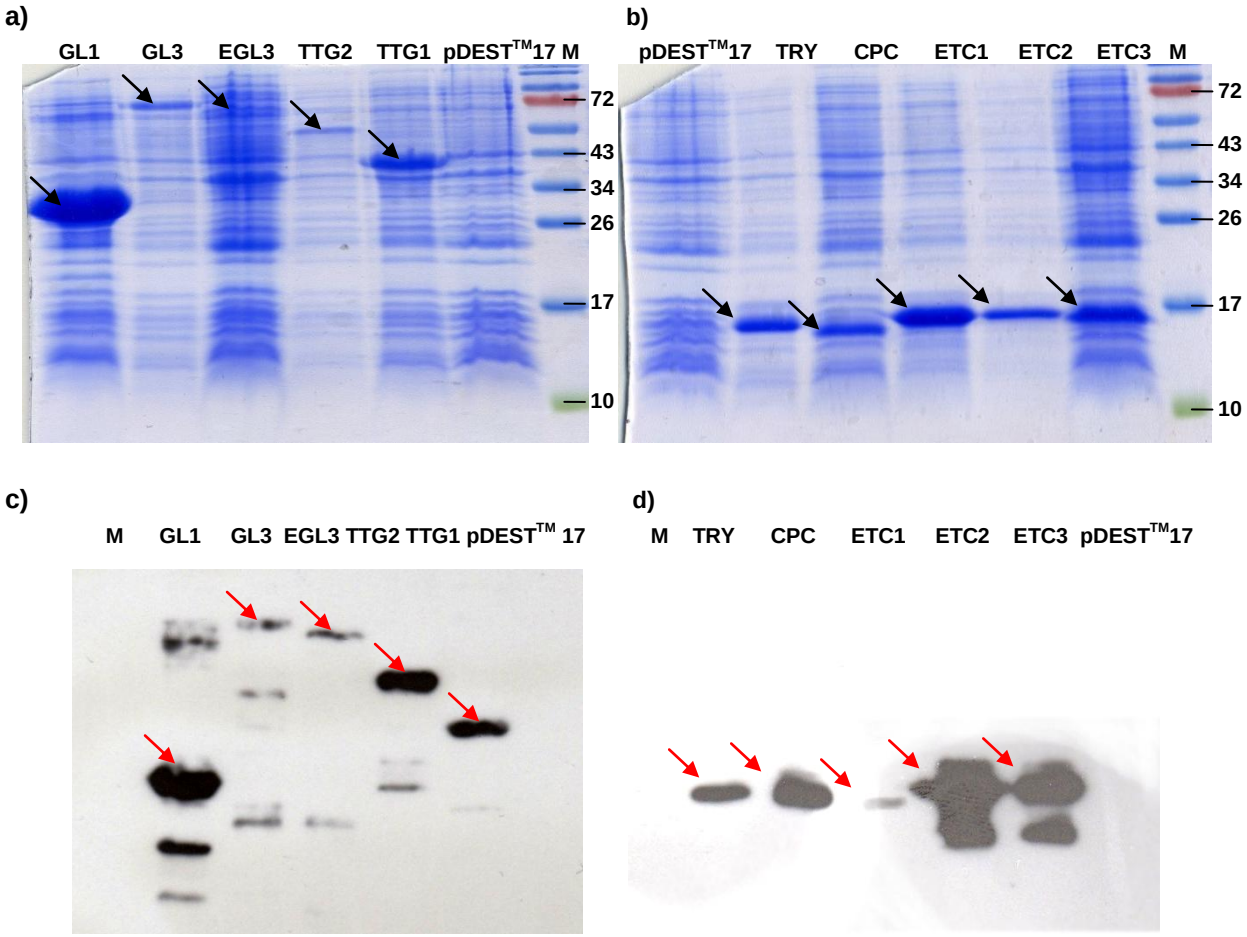

Figure 17 *SDS-PAGE (a & b) and Western blot (c & d) analyses of the cell extracts, containing the pDEST™17 constructs after induction.*

This vector contains sequences enabling N-terminal His- fusion of 2.6 kDa in addition to the size of the proteins. Empty vector expresses the His- alone

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.

Arrows indicate the expressed protein.

2.4.4. PURIFICATION OF HIS- TAGGED PROTEINS

None of the proteins were expressed when they were in fusion with the C-terminal His- tag. On the other hand, the use of N-terminal His- tag in pDEST™17 vector made it possible to express all the patterning proteins that were tested. Therefore, the purification of all the patterning proteins that were expressed together with the N-terminal His- fusion tag were accomplished through Ni-NTA resins. Proteins purified by this method were then dialysed against the buffer of interest for further experiments. Purification for each individual protein was analysed by SDS-PAGE via Coomassie staining. These results can be seen in Figure 18.

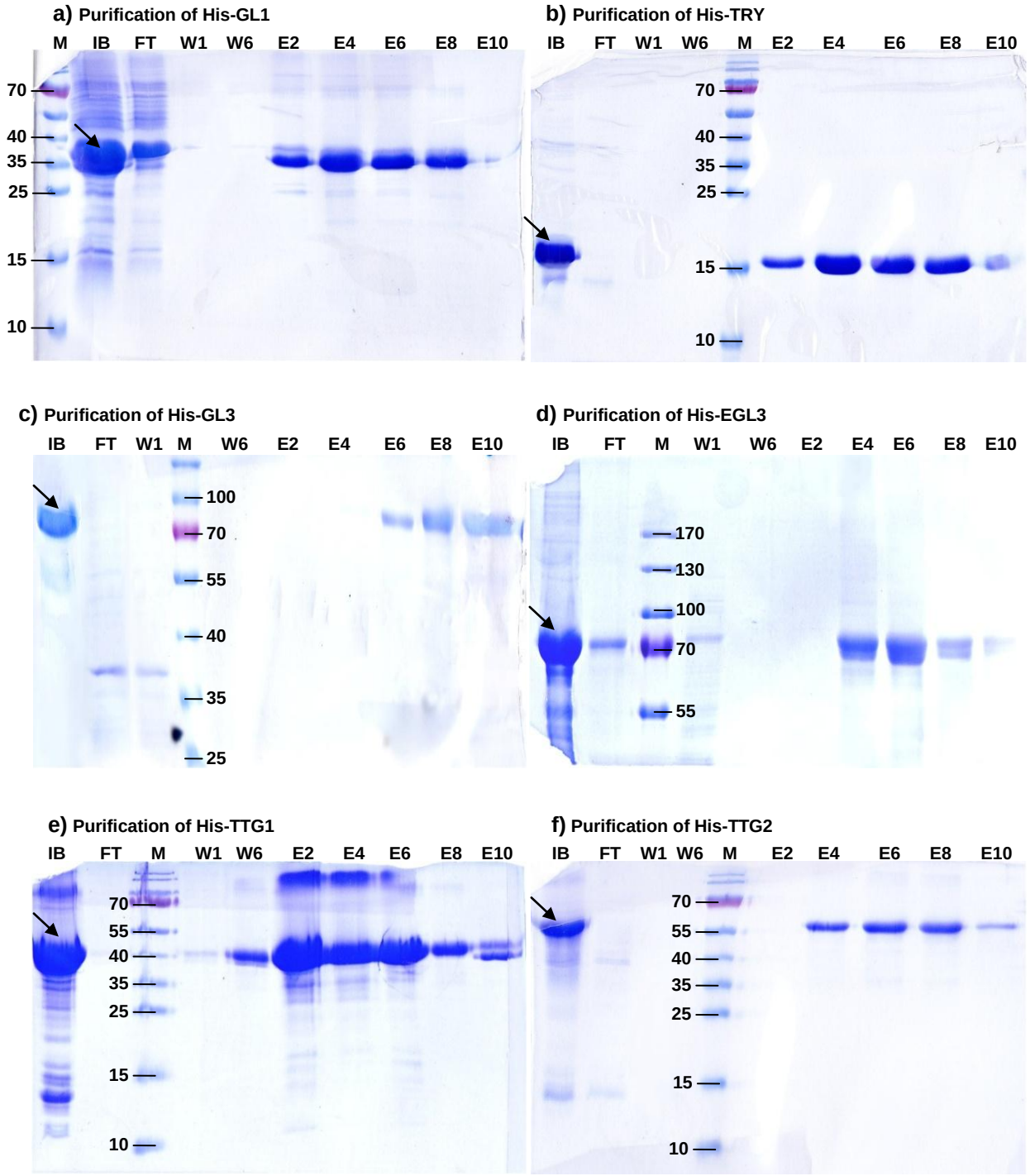

Figure 18 *SDS-PAGE showing the N-terminal His- tagged protein purifications via the Ni-NTA resin.*

The constructs used are pDEST™17 containing: a) GL1 b) TRY c) GL3 d) EGL3 e) TTG1 f) TTG2.

The abbreviations refer to the following

IB: inclusion body preparation

FT: Flow through after incubation of the inclusion body preparations with the Ni-NTA resin

W1: First washing, W6: Last (sixth) washing

E2: Second elution fraction, E4: Fourth elution fraction, E6: Sixth elution fraction, E8: Eighth elution fraction, E10: Tenth elution fraction

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown for each figure.

Arrows indicate the protein to be purified in the inclusion body preparation.

2.4.5. EXPRESSION OF PROTEINS WITH AN N-TERMINAL GST- FUSION TAG

One of the goals of this work was to confirm the protein interactions which were shown in previous studies and to analyse if they can occur in a direct manner. Therefore, GST pull-down experiments were aimed, where these interactions *in vitro* in a biochemical assay could be proven. To be used in these experiments, expression of the patterning proteins as N-terminal GST- fusions were done. Use of GST- tag was also advantageous, since GST- tag is known to solubilise proteins fused to this tag [79]. By the use of the vector pGEX2TM-GW containing an N-terminal GST- fusion tag, GL1, GL3, EGL3, TTG1, TTG2, TRY and CPC proteins were successfully expressed in Bl21DE3RIL(STRATAGENE) cells as a soluble GST- fusion. The solubilisation of these GST- fusion proteins were achieved via lyses with *N*-laurylsarcosine (sarkosyl) as suggested by Frangioni and Neel [79]. Expression of each protein under these conditions were analysed by SDS-PAGE. Western blot analysis was also done using anti-GST antibody to check the integrity of the fusion tag. SDS-PAGE and Western blot analyses are shown in Figure 19.

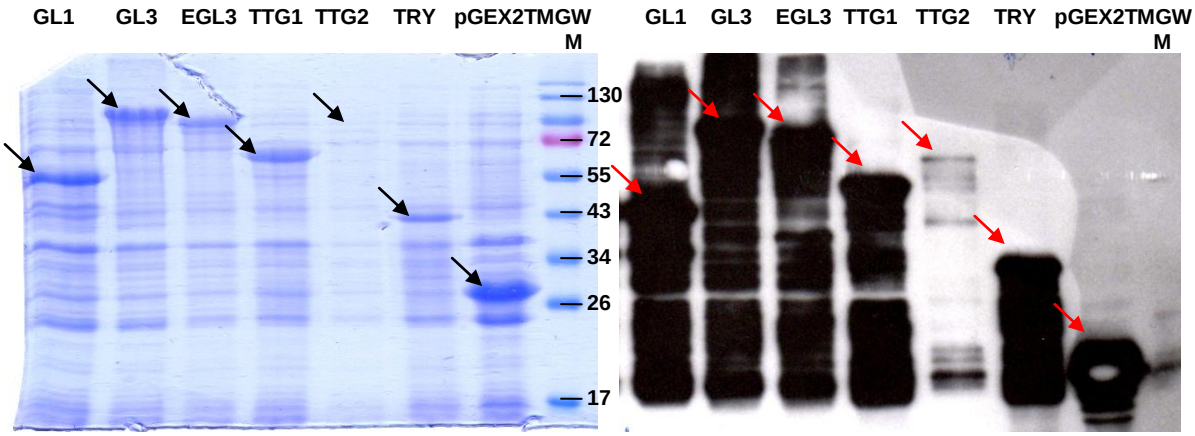

Figure 19 *SDS-PAGE and Western blot analyses of the cell extracts after induction containing the pGEX2TMGW vector constructs.*

This vector contains sequences enabling N-terminal GST- fusion of 26 kDa in addition to the size of the proteins. Empty vector expresses the GST- alone.

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.

Arrows indicate the expressed protein.

2.4.6. PURIFICATION OF GST- TAGGED PROTEINS

The GST- fusion proteins were successfully purified via the Glutathione (GSH) resin after lyses of the cells. The GST- tag enabled the successful solubility of the expressed proteins, thus no further solubilisation step was required. Figure 20 shows the SDS-PAGE analyses after purification of these proteins.

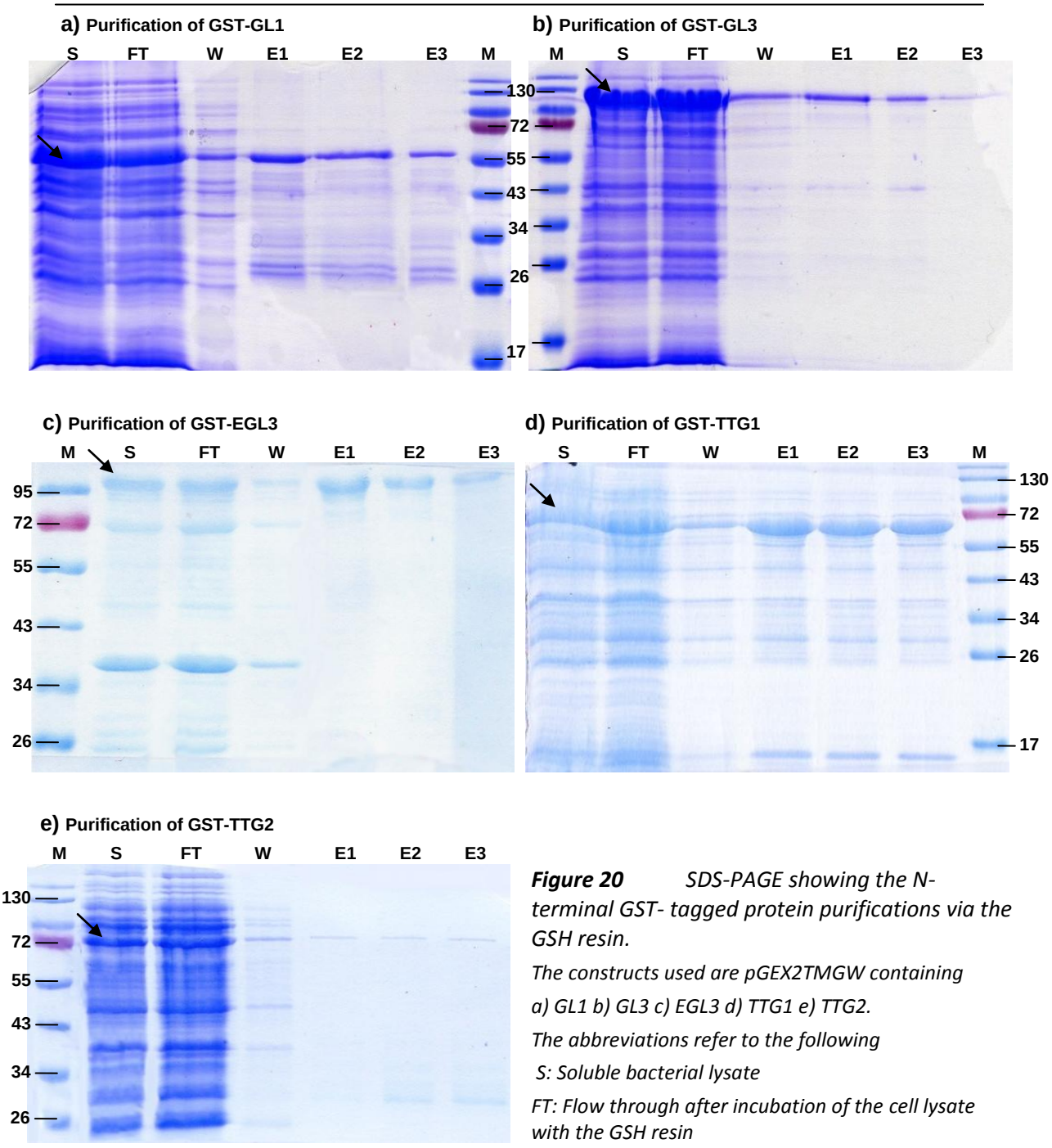

Figure 20 *SDS-PAGE showing the N-terminal GST- tagged protein purifications via the GSH resin.*

The constructs used are pGEX2TMGW containing a) GL1 b) GL3 c) EGL3 d) TTG1 e) TTG2.

The abbreviations refer to the following

S: Soluble bacterial lysate

FT: Flow through after incubation of the cell lysate with the GSH resin

W: Combined sample of six washings

E1: First elution fraction, E2: Second elution fraction, E3: Third elution fraction

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown for each figure.

Arrows indicate the protein to be purified in the cell lysate.

2.4.7. DIRECT INTERACTIONS AMONG PATTERNING PROTEINS

In order to reveal if the interactions which were shown by yeast two-hybrid analyses can also occur *in vitro*, GST pull-down experiments were attempted. For this purpose, initially GST- and MBP- tagged proteins were used since both of these proteins could be obtained from soluble fraction of the induced cells. However, the control experiment has shown that MBP can interact alone with the GST, which would interfere with the pull-down experiments (Data not shown). Thus, GST pull-downs were conducted with the GST- and His- tagged proteins. Initially, the known interactions were tested. The *in vitro* GST pull-down experiments performed have exhibited that GL1-GL3, GL1-EGL3, TRY-GL3 and TRY-EGL3 interactions can also occur *in vitro* with the purified proteins. These interactions were observed by detecting the proteins with anti-His antibodies after incubation of these two different tagged proteins in GSH resin as shown in Figure 21 a) and b). This result also indicates that these interactions are occurring in a direct manner. Apart from the known interactions, it has also been observed that GL1 can interact with the TTG1 and TRY proteins *in vitro.* Figure 21 a), b) and c) show these novel interactions, which have not been reported before in the yeast two-hybrid experiments. The formation of the predicted activator complex of GL1-GL3/EGL3-TTG1 has also been shown *in vitro* by this assay, using these three proteins together. The formation of this complex is observed by using anti-His antibody which detects the His-GL3/ His-EGL3 and His-TTG1 as shown in Figure 21 c).

During the conduction of these experiments, some problems occurred. The first one was the non-specific binding of GL1 and TRY to the corresponding resin. However, these non-specific bindings could be prevented by increasing the mild detergent Nonidet-P40 and Bovine Serum Albumin (BSA) concentrations in the pull-down buffer. Another problem was that the interactions observed when His-TRY protein was used could never be obtained with GST-TRY. This could be due to the size of the GST- tag (26 kDa) being much bigger than the TRY and hindering the interaction sites in the TRY structure.

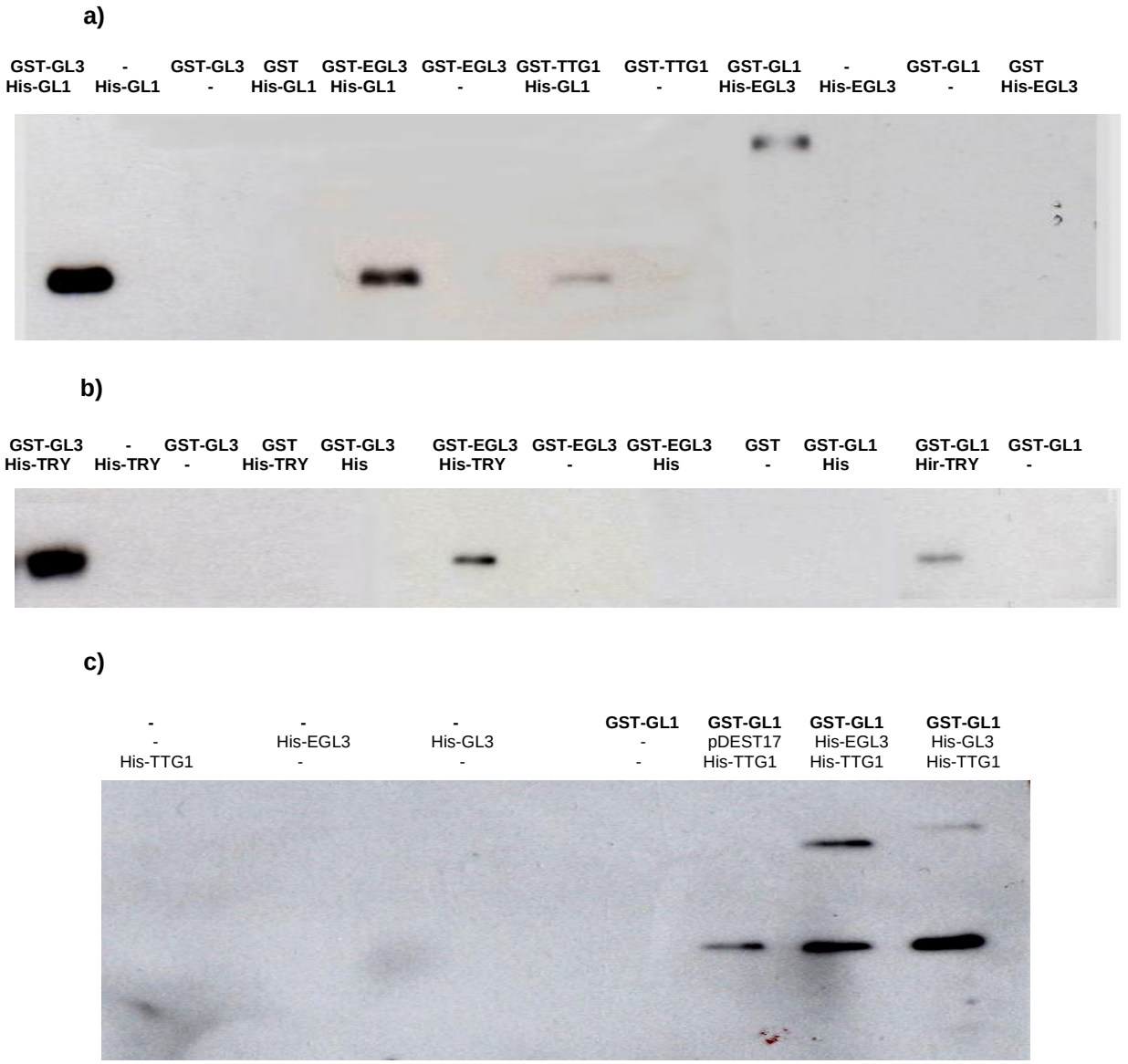

Figure 21 *Western blotting after GST pull-down experiments.*

a) Western blots of GST pull-downs showing the interactions of GL1 protein with GL3, EGL3 and TTG1 proteins

b) Western blots of GST pull-downs showing the interactions of TRY proteins with GL3, EGL3 and GL1 proteins

c) Western blots of GST pull-downs showing the interactions of TTG1, GL3, EGL3 and GL1 proteins.

In all of the GST pull-down experiments, proteins are immobilized via the GSH surface and Western blots are performed by using anti-His antibody as primary antibody.

Another interaction that was intended to be shown by GST pull-down experiments was the interaction of TTG1 with TTG2. In this assay, GST-TTG2 protein was always shown to interact with Ni-NTA resin. Another problem with the GST-TTG2 protein was its low expression levels. Therefore His-TTG2 was tried to be pulled together with GST-TTG1 via GSH resin. In this combination, His-TTG2 was also bound to GSH resin. Changing the Nonidet-P40 and BSA concentrations in this case could not prevent the non-specific binding of TTG2 to the matrix in any of the

conditions used. However, the signal after the Western blot of His-TTG2 together with GST-TTG1 was much stronger compared to the His-TTG2 bound to the resin alone. TTG1-TTG2 interaction is shown in Figure 22. This might be a confirmation to the yeast two-hybrid experiments, in which TTG1-TTG2 interaction was observed by Martina Pesch [personal communication].

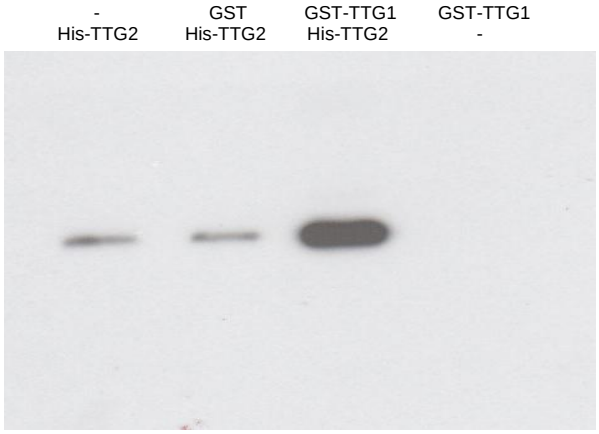

Figure 22 *Western blot after GST pull-down experiments of TTG1 and TTG2*

In the GST pull-down experiment, proteins are immobilized via the GSH surface and Western blots are performed by using anti-His antibody as the primary antibody.

2.5. COEXPRESSION AS A TOOL FOR PROTEIN-PROTEIN INTERACTIONS

During its expression, GST-TTG2 was not obtained in high concentrations compared to the other patterning proteins. Moreover, TTG2 was also expressed in inclusion bodies when fused into N-terminal His- tag. This protein was shown to interact with TTG1 in yeast two-hybrid system [Martina Pesch, personal communication].

In order to increase the stability and solubility of TTG2, it was coexpressed with its putative interacting protein TTG1, by using different overexpression vectors containing different selection genes. In this method, *TTG1* and *TTG2* were recombined into three different vectors. One of these vectors contained Kanamycin resistance (pET28 Frame C), while the other contained Ampicillin resistance (pDEST

™17 or pGEX2TMGW). After transformation of these constructs, a single colony bearing the two constructs was selected. This colony was grown for coexpression of TTG1 and TTG2. SDS-PAGE analysing the inductions of these proteins is shown in Figure 23. When the cells containing both His- tagged proteins were lysed, the expressed proteins were still found to be in the inclusion bodies. However, the coexpressed proteins were soluble when either partner was expressed in fusion with GST- tag. Cell lysates of the soluble and inclusion body fractions analysed by SDS-PAGE are shown in Figure 24.

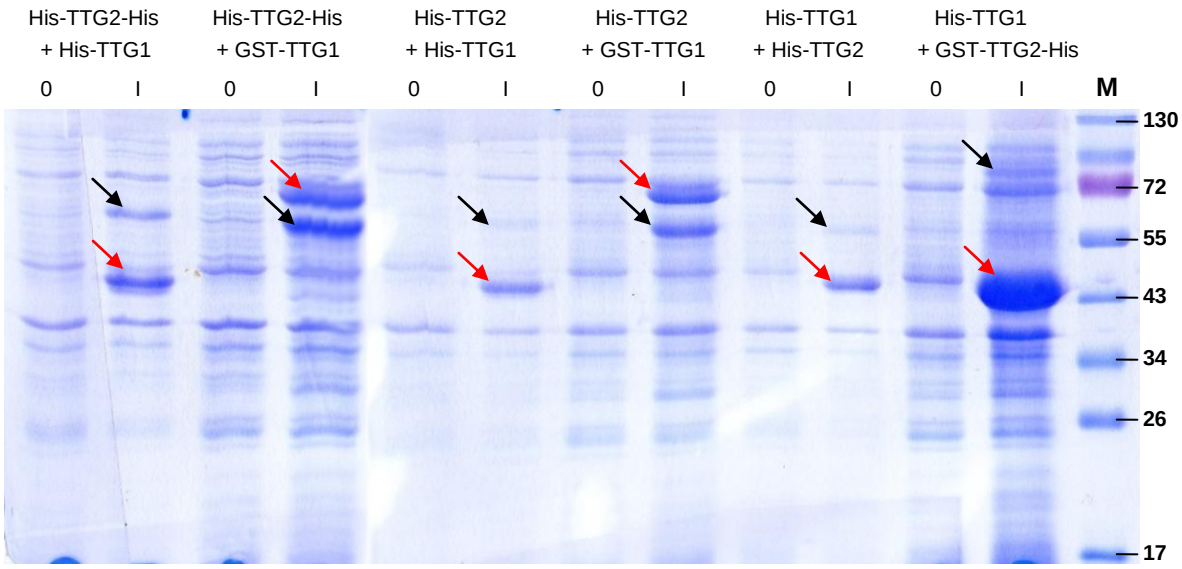

Figure 23 *SDS-PAGE of the cell extracts containing two vectors for coexpression of TTG1 and TTG2.*

The upper row of the subscription describes the proteins expressed from pET28 Frame C vector, the lower row describes the proteins expressed from pDEST™ 17 vector or pGEX2TMGW vector. The fusion tags that the protein contains are indicated as N-terminal or C-terminal fusions.

0 refers to the cell extracts before induction

I refers to the cell extracts after induction with IPTG.

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.

Black arrows correspond to the theoretical sizes of the induced proteins. Red arrows correspond to the TTG1 and black arrows correspond to the TTG2 proteins.

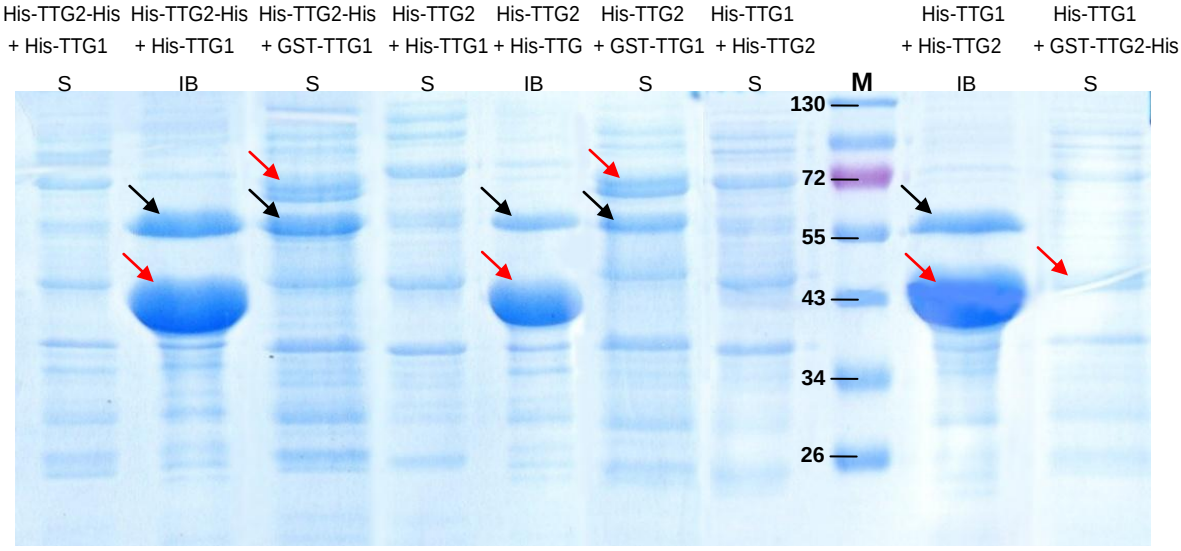

Figure 24 *SDS-PAGE of the cell lysates of TTG1 and TTG2 coexpressing cells.*

The upper row of the subscription describes the proteins expressed from pET28 Frame C vector, the lower row describes the proteins expressed from pDEST™ 17 vector or pGEX2TMGW vector. The fusion tags that the protein contains are indicated as N-terminal or C-terminal fusions.

S refers to the soluble fraction of the cell lysate

IB refers to the inclusion body preparation

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown in the figure.

Black arrows correspond to the theoretical sizes of the induced proteins. Red arrows correspond to the TTG1 and black arrows correspond to the TTG2 proteins.

In order to evaluate the interactions of TTG2 protein with other patterning proteins, initially GST pull-down experiments were performed –as explained in section 2.4.7. However, TTG2 was non-specifically binding to the corresponding resin when it was expressed either as a His- or GST- fusion protein. To show whether the TTG2 and TTG1 proteins can be coeluted after affinity purification, a single step Ni-NTA or GSH or double step Ni-NTA and GSH purification procedures were applied to these proteins. Coexpressed proteins were successively purified via Ni-NTA and GSH resin or vice versa and it has been shown that after two rounds of purification as well as single step purification via the respective tags, TTG1 and TTG2 proteins were found to be eluted only in the case of TTG1pET28frameC (N-terminal His- fusion) and TTG2 pGEX2TMGW (N-terminal GST- & C-terminal His-fusion) together. However, GL1 protein, which was not shown to interact with the TTG2 protein in yeast two-hybrid experiments [Martina Pesch, personal communication], was also eluted together with this protein after two rounds of purification. Coeluted proteins after subsequent purifications via GSH and/or Ni-NTA resins were detected in Western blots by anti-GST and/or anti-His antibodies

respectively (Figure 25). This implies that this coelution can be an artefact due to the overexpression of these proteins in the cells from a single bacterial colony. It is also known that transcription factors are sticky and that they show non-specific binding to be a big problem for this type of proteins. This could also be the case for GL1 protein, which non-specifically binds to the resins during purification. On the other hand, these could also be novel interactions as in the case of GL1-TTG1 and GL1-TRY interactions.

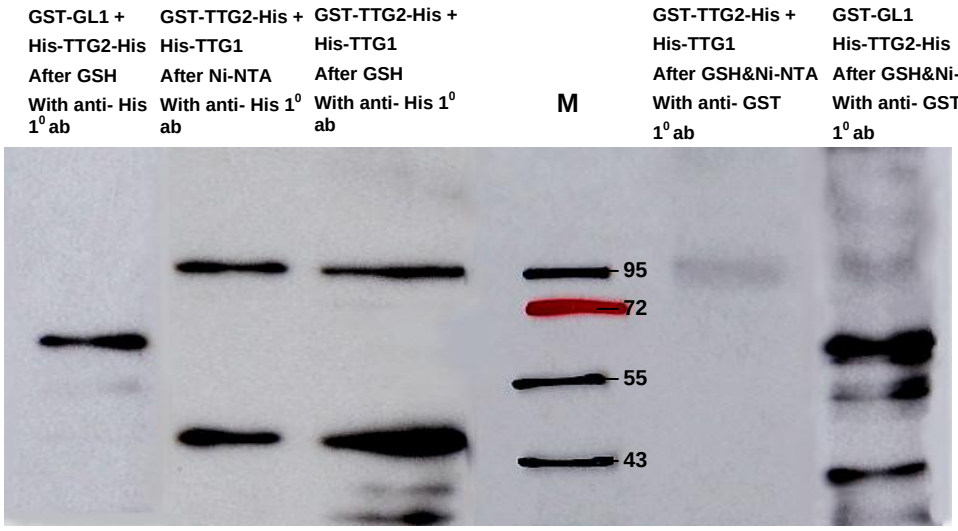

Figure 25 *Western blot after purifications of GL1-TTG2 and TTG1-TTG2 coexpressions.*

From left to right;

First lane corresponds to coelution of GST-GL1 and His-TTG2-His after one round of purification via GSH resin. Detection is done by anti-His antibody.

Second lane corresponds to coelution of GST-TTG2-His and His-TTG1 after one round of purification via Ni-NTA resin. Detection is done by anti-His antibody.

Third lane corresponds to coelution of GST-TTG2-His and His-TTG1 after one round of purification via GSH resin. Detection is done by anti-His antibody.

Fifth lane corresponds to coelution of GST-TTG2-His and His-TTG1 after subsequent GSH and Ni-NTA purifications. Detection is done by anti-GST antibody.

Sixth lane corresponds to coelution of GST-GL1 and His-TTG2-His after subsequent GSH and Ni-NTA purifications. Detection is done by anti-GST antibody.

Fourth lane is hand-made markings corresponding to the PAGE PageRuler™ Prestained protein ladder.

As already shown in this work as well as in other studies, GL3 and TRY are interacting proteins [17, 62]. *GL3* and *TRY* were cloned together into pCDFDuet™-1 and pETDuet™-1 vectors which were then coexpressed under IPTG inducible T7 promoters. Both vectors allow the expression of His- and S- tagged protein fusions. pCDFDuet™-1 vector contains Coexpression of GL3 and TRY, when they were expressed from the same vector, is shown by SDS-PAGE in Figure 26 a).

TRY was obtained in soluble form when it was expressed together with GL3 protein in pETDuet™-1. However; the growth of bacteria containing the GL3-TRY pCDFDuet™-1 construct was very slow. The expression levels of GL3 and TRY when pCDFDuet™-1 vector was used, was low and insoluble in comparison to the use of pETDuet™-1. The presences of GL3 and TRY in soluble and inclusion body fractions of the cell lysate in these experiments are shown by SDS-PAGE in Figure 26 b).

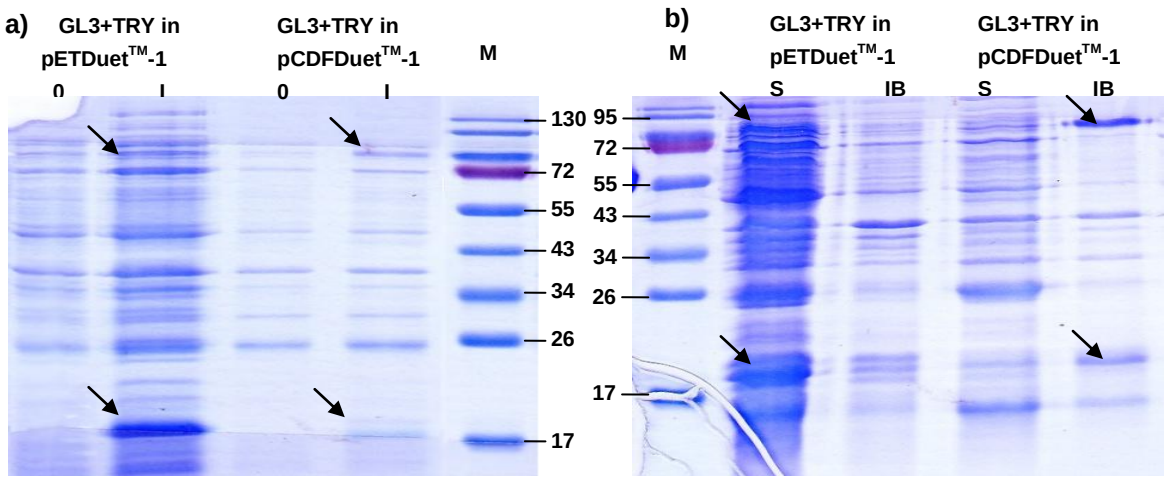

Figure 26 *SDS-PAGE showing the coexpression of GL3 with TRY*

a) *Cells grown from a single colony containing GL3 and TRY ligated to pETDuetTM-1 or pCDFDuetTM-1 vectors before and after induction.*

0 refers to cell extracts before induction; I refers to cell extracts after induction run on SDS-PAGE.

b) Cell lysates containing GL3 and TRY ligated to pETDuetTM-1 or pCDFDuetTM-1 vectors after lyses.

S refers to the soluble fraction of cell lysate, IB refers to the inclusion body fraction of cell lysate.

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown for each figure.

Arrows correspond to the theoretical sizes of the induced proteins.

In order to see if GL3, TRY and TTG1 proteins can also form a complex in bacterial cells, all three genes were aimed to be coexpressed from a single colony. In this experiment GL3-TRY pCDFDuet™-1 construct were used together with TTG1-pDEST™17 or TTG1-pGEX2TMGW. This was due to the reason that pCDFDuet™-1 vector has streptomycin/spectinomycin resistance marker whereas the latter two vectors contain ampicillin resistance marker. The results of this coexpression show that, GL3, TTG1 and TRY can be coexpressed when they are expressed in fusion to His- and S- tags, whereas the growth of bacterial cells containing His-GL3, S-TRY and GST-TTG1 were clearly reduced. Figure 27 a) shows the Coomassie staining of SDS-PAGE of the cell extracts bearing these constructs before and after induction. Coexpression of TRY protein with its interacting partner GL3 increased the solubility of this protein when other vectors were used for coexpression of these two proteins.

However, GL3, TTG1 and TRY expression from a single colony together, still yielded the production of these three proteins in the inclusion bodies. The comparison of soluble and inclusion body fractions of cells overexpressing GL3-TTG1-TRY is revealed in Figure 27 b).

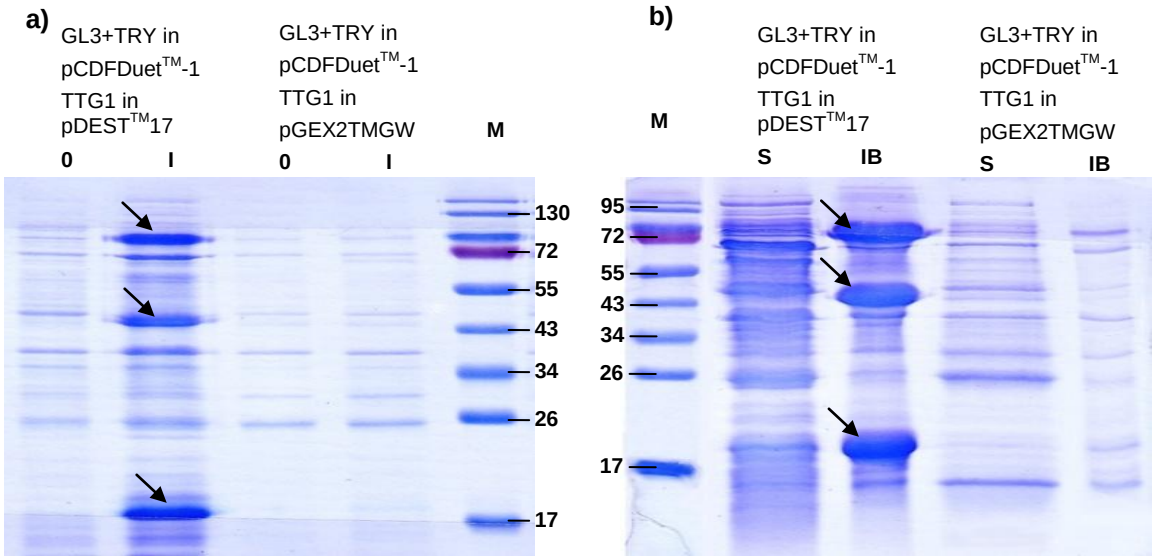

Figure 27 *SDS-PAGE showing coexpression of GL3, TTG1 and TRY proteins.*

a) Cells grown from a single colony containing GL3 and TRY ligated to pCDFDuetTM-1 and TTG1 recombined to pDEST™17 or to pGEX2TMGWvectors before and after induction.

0 refers to cells before induction; I refers to cells after induction run on SDS-PAGE.

b) Cell lysates containing GL3 and TRY ligated pCDFDuetTM-1 and TTG1 recombined to pDESTTM17 or to pGEX2TMGW vectors after lyses.

S refers to the soluble fraction of cell lysate, IB refers to the inclusion body fraction of cell lysate.

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown for each figure.

Arrows correspond to the theoretical sizes of the induced proteins.

2.6. Production of Antibodies Against Trichome Patterning Proteins

2.6.1. Purification of the Specific Antibodies

Until now, presence of none of the antibodies against GL1, GL3, EGL3, TTG1, TTG2 and TRY have been reported. Therefore, this work aimed to raise antibodies against these key players of trichome patterning. The purified His-GL1, His-EGL3, His-TTG1, His-TTG2 and His-TRY were immunised to rabbits for raising specific antibodies against these patterning proteins. Total blood serums of the animals were obtained and antibodies were purified against these His- tagged

proteins as explained in the Materials and Methods chapter. After the purification of the antibodies from the total blood serum of the rabbits, the pure antibodies were then tested against different tagged versions of the patterning proteins. Western blot analyses using the preimmunization serum as primary antibody resulted in detection of almost no signal for overexpressed proteins in bacteria. On the other hand, several bands were detected when the final total serum was used as primary antibody. The Western blot analyses of the different tagged proteins, in which the purified specific antibodies were used as primary antibodies, show that the antibody purification procedure resulted in pure and specific antibodies for the proteins GL1, TTG1, and TRY. However, several non-specific bands were observed in Western blot analyses of EGL3 and TTG2 proteins in the case of anti-EGL3 and anti-TTG2 antibodies respectively. Figures 28-34 show the Western blot analyses using these antibodies against bacterially expressed proteins containing different fusion tags. In all of the cases except MBP-TRY and Strep-EGL3, the use of fusion tag did not interfere with the antigen-antibody reactions. The Western blot analyses of the homologous proteins for GL1 or TRY revealed that the purification step provided the specific anti-GL1 and specific anti-TRY antibodies which do not cross-react with the homologues of these proteins.

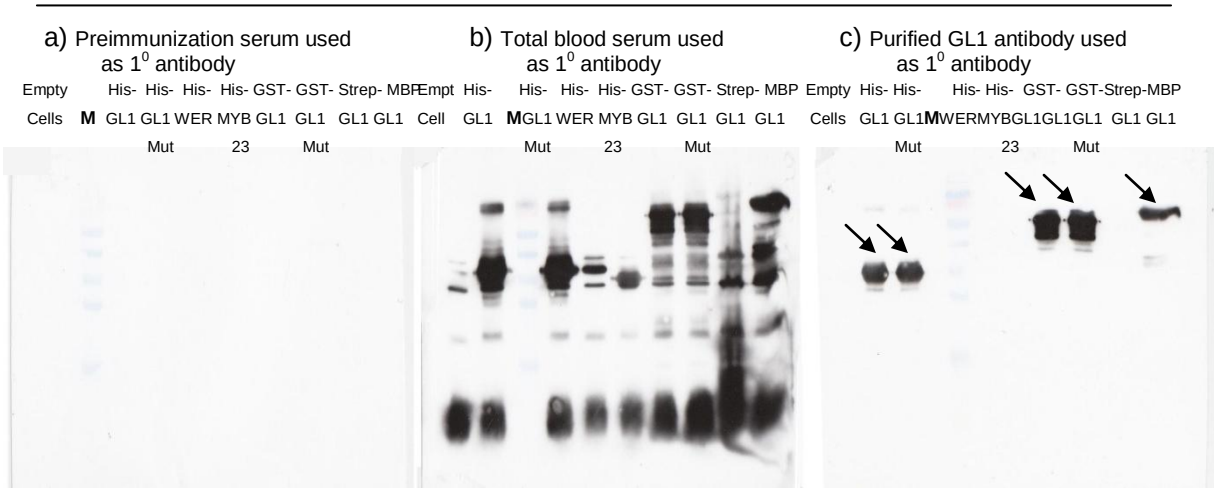

Figure 28 *Western blots of the antibodies when bacterially expressed proteins were used as samples.*

Detections were done by using preimmunization serum, total blood serum of the immunized animal or the purified antibody as primary antibodies. WER and MYB23 were used to detect the specificity of the anti-GL1 antibody, since they are homologous proteins. Empty cells refer to the cell containing the pDEST™17 vector alone after induction. The samples loaded are the cell extracts after induction pelleted from 1 mL of the culture and boiled in SDS-Gel Loading Buffer after induction. As can be seen from the last blot, purified anti-GL1 antibody does not show cross-reaction with the homologous MYB proteins WER and MYB23.

GL1mut is a single amino acid mutation containing GL1 protein, which was found not to be interacting with the GL3 protein in yeast two-hybrid interactions [Martina Pesch, personal communication].

GL1 protein containing different fusion tags were used for detection with anti-GL1 antibody to specify the interference of the tags with antigen-antibody interaction. Arrows indicate the different tagged GL1 detected by the anti-GL1 antibody specifically.

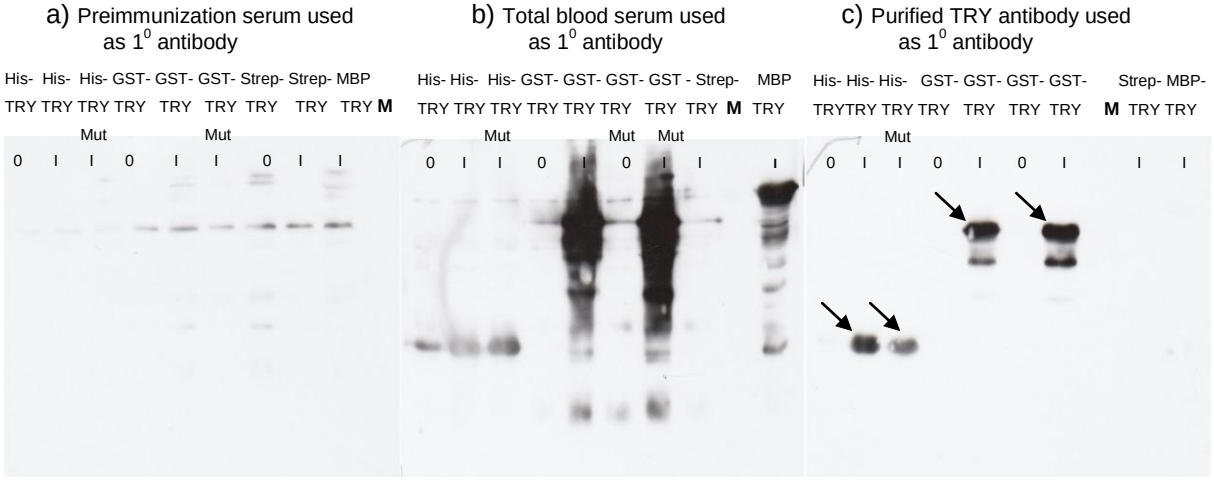

Figure 29 *Western blots of the antibodies when bacterially expressed proteins were used as samples.*

The samples loaded are the cell extracts before and after induction pelleted from 1 mL of the culture and boiled in SDS-Gel Loading Buffer after induction.

0 refers to the cells before induction. I refers to the cells after induction.

Detections were done by using preimmunization serum, total blood serum of the immunized animal or the purified antibody as primary antibodies.

TRYmut is a single amino acid mutation containing TRY protein which was found not to be interacting with the GL3 protein in yeast two-hybrid interactions [Martina Pesch, personal communication].

TRY protein containing different fusion tags were used for detection with anti-TRY antibody to specify the interference of the tags with antigen-antibody interaction. Arrows indicate the different tagged TRY detected by the anti-TRY antibody specifically.

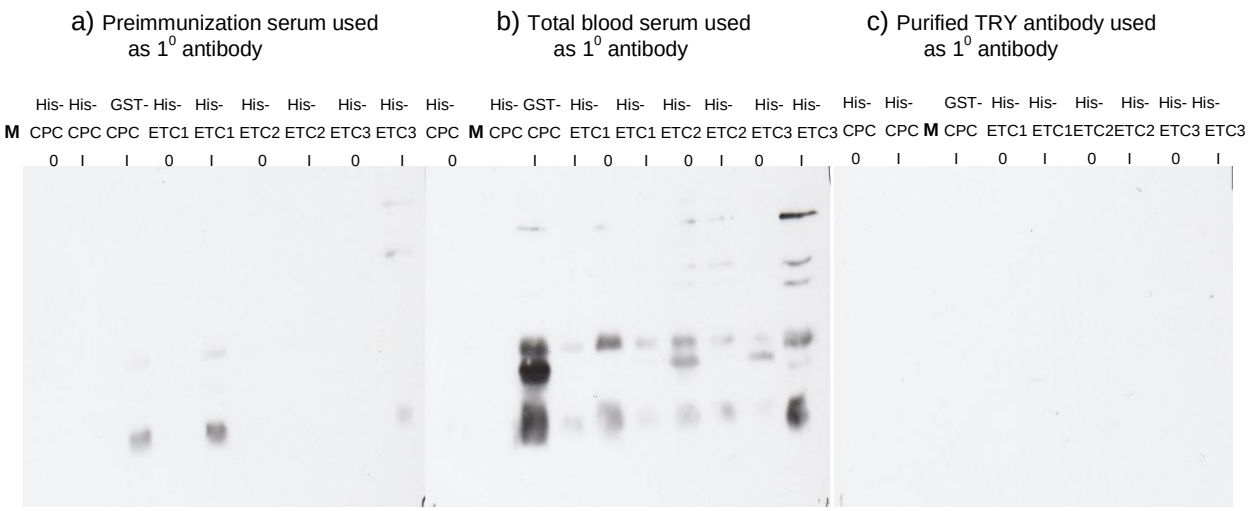

Figure 30 *Western blots of the antibodies when bacterially expressed proteins were used as samples.*

The samples loaded are the cell extracts before and after induction pelleted from 1 mL of the culture and boiled in SDS-Gel Loading Buffer after induction.

0 refers to the cells before induction; I refers to the cells after induction.

Detections were done by using preimmunization serum, total blood serum of the immunized animal or the purified antibody as primary antibodies. TRY homologous R3MYB proteins are used as samples to identify the cross-reactions with the anti His-TRY antibody. As can be seen from the last blot, anti-TRY antibody does not show cross-reaction with the homologous MYB proteins.

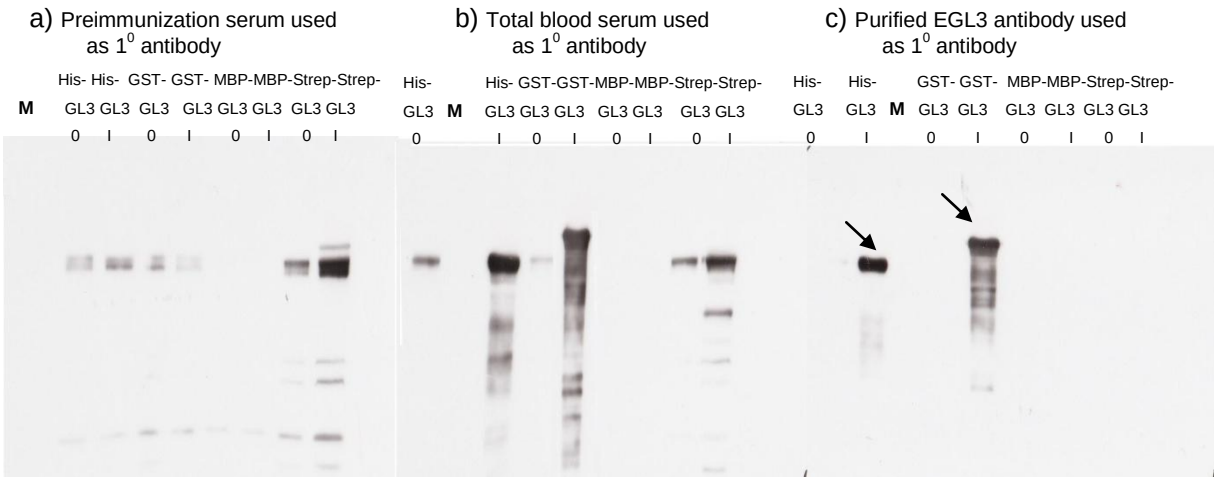

Figure 31 *Western blots of the antibodies when bacterially expressed proteins were used as samples.*

The samples loaded are the cell extracts before and after induction pelleted from 1 mL of the culture and boiled in SDS-Gel Loading Buffer after induction.

0 refers to the cells before induction, I refers to the cells after induction.

Detections were done by using preimmunization serum, total blood serum of the immunized animal or the purified antibody as primary antibodies. Different tagged GL3 proteins were used as samples to identify the cross-reactions with the anti-EGL3 antibody. As can be seen in the last blot anti-EGL3 antibody can show cross-reaction to its homologous protein GL3.

Arrows indicate the His- and GST- tagged GL3 cross-reacting with the anti-EGL3 antibody.

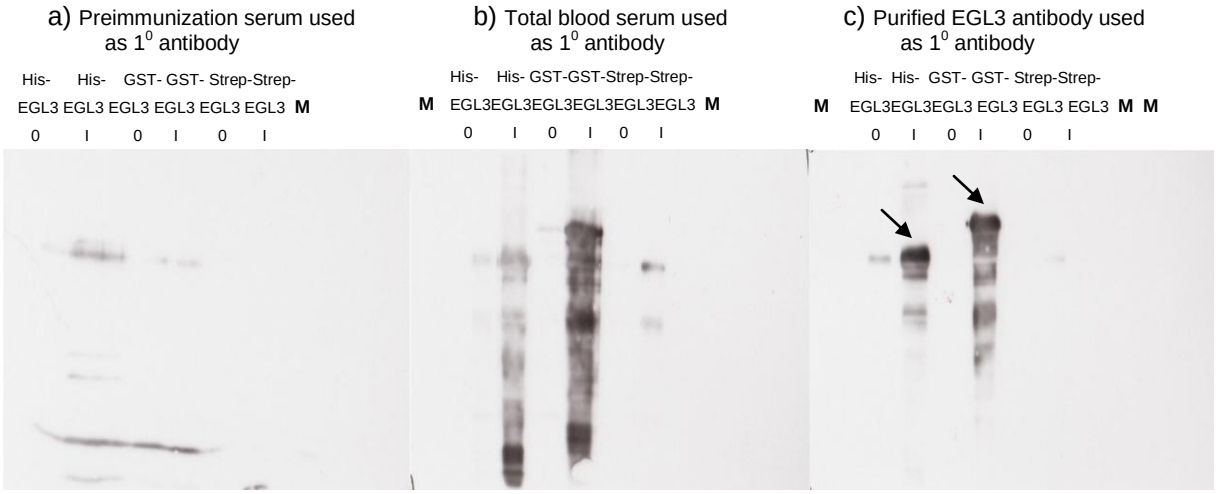

Figure 32 *Western blots of the antibodies when bacterially expressed proteins were used as samples.*

The samples loaded are the cell extracts before and after induction pelleted from 1 mL of the culture and boiled in SDS-Gel Loading Buffer after induction.

0 refers to the cells before induction, I refers to the cells after induction.

Detections were done by using preimmunization serum, total blood serum of the immunized animal or the purified antibody as primary antibodies. Different tagged EGL3 proteins were used as sample to specify the interference of the tags with antigen-antibody interaction. T7 promoter when using pDEST™17 vectors shows some leakage. As can be seen in the last blot antibody purification was successful to some extent. However, further polishing of the anti-EGL3 might be necessary for a better specificity.

Arrows indicate the His- and GST- tagged EGL3 binding specifically with the anti-EGL3 antibody.

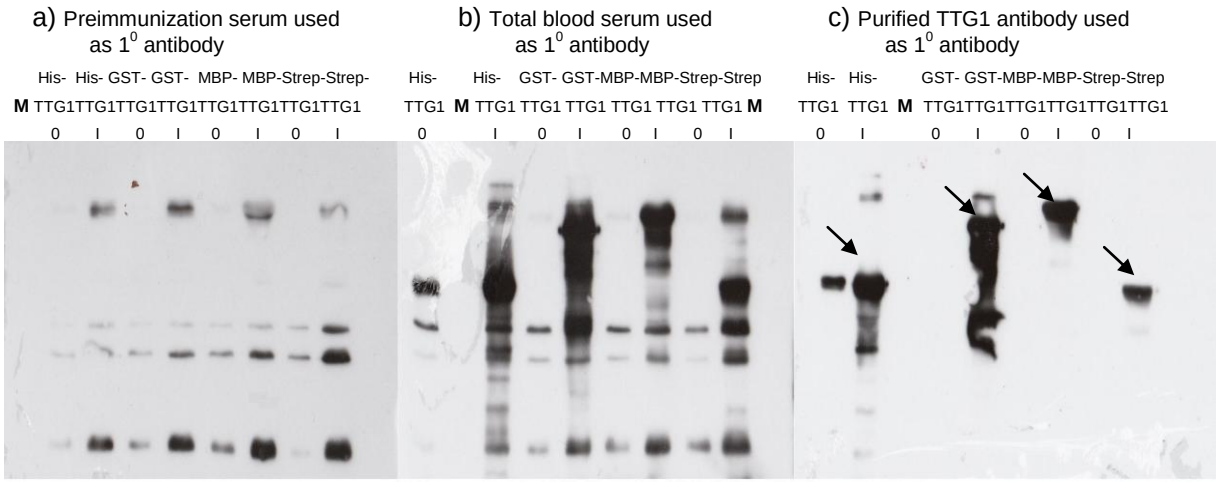

Figure 33 *Western blots of the antibodies when bacterially expressed proteins were used as samples*

The samples loaded are the cell extracts before and after induction pelleted from 1 mL of the culture and boiled in SDS-Gel Loading Buffer after induction.

0 refers to the cells before induction, I refers to the cells after induction.

Detections were done by using preimmunization serum, total blood serum of the immunized animal or the purified antibody as primary antibodies. Different tagged TTG1 proteins were used as sample to specify the interference of the tags with antigen-antibody interaction. T7 promoter when using pDEST™17 vectors shows some leakage. As can be seen in the last blot antibody purification was successful.

Arrows indicate the His-, GST-, MBP- and Strep- tagged TTG2 binding specifically with the anti-TTG2 antibody.

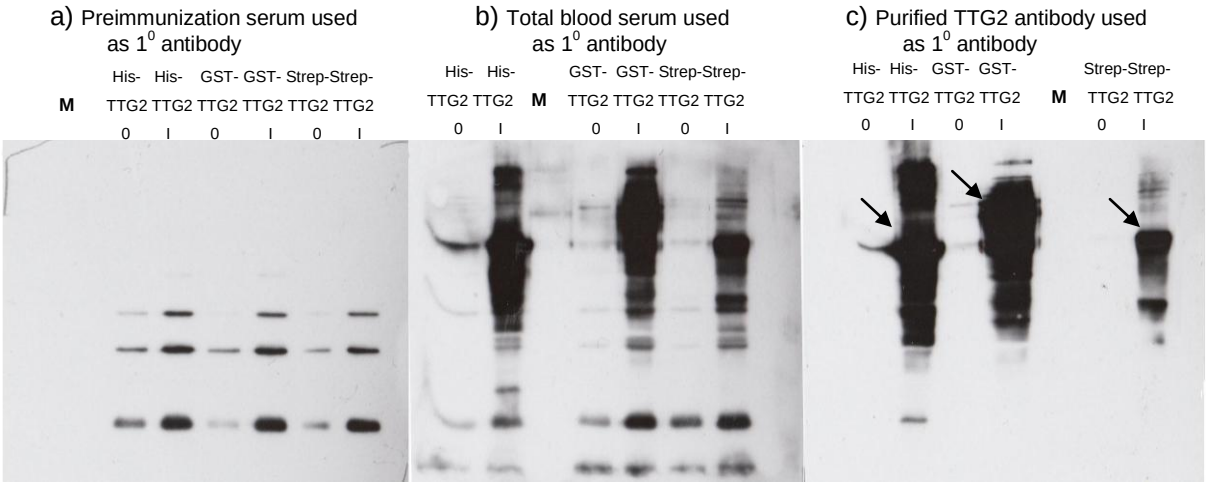

Figure 34 *Western blots of the antibodies when bacterially expressed proteins were used as samples.*

The samples loaded are the cell extracts before and after induction pelleted from 1 mL of the culture and boiled in SDS-Gel Loading Buffer after induction.

0 refers to the cells before induction, I refers to the cells after induction.

Detections were done by using preimmunization serum, total blood serum of the immunized animal or the purified antibody as primary antibodies. Different tagged TTG2 proteins were used as sample to specify the interference of the tags with antigen-antibody interaction. As can be seen in the last blot antibody purification was successful to some extent. However, further polishing of the anti-TTG2 is necessary for a better specificity.

Arrows indicate the His-, GST- and Strep- tagged TTG2 binding specifically with the anti-TTG2 antibody.

2.6.2. ANALYSES OF PURIFIED ANTIBODIES AGAINST PLANT PROTEINS

The specific antibodies of GL1, EGL3, TTG1, TTG2 and TRY were raised against bacterially expressed proteins. In order to check if proteins from *Arabidopsis* can be detected by these antibodies, one should test them against plants proteins. To do these tests, *Arabidopsis* plants containing HA- tagged fusions of these proteins in L*er* background expressed via the 35S promoter were used. These plants were selected by BASTA resistance and phenotypically for the presence of the constructs. The phenotypes observed were; trichomes at the leaf margins for plants bearing the *35S::GL1:HA*, glabrous for plants bearing *35S::TRY:HA*, increased trichome numbers for plants bearing *35S::GL3:HA* and *35S::EGL3:HA* , wildtypic phenotypes for *35S::TTG1:HA* and *35S::TTG2.* After 14 days of germination, the extracts of these whole *Arabidopsis* plants were prepared. These total plant extracts were then analysed by Western blot experiments using the purified antibodies as primary antibody. As positive controls, anti-HA antibodies were used to detect the tagged proteins in these plant extracts. Usage of the purified antibodies in titers of 1/1000 and 1/500 did not lead to any signal. On the other hand, the HA- tagged EGL3, TTG1 and TTG2 proteins could be detected successfully when a dilution of 1/100 of the purified antibody was used as the primary antibody. Western blot analyses corresponding to these experiments are shown in Figure 35.

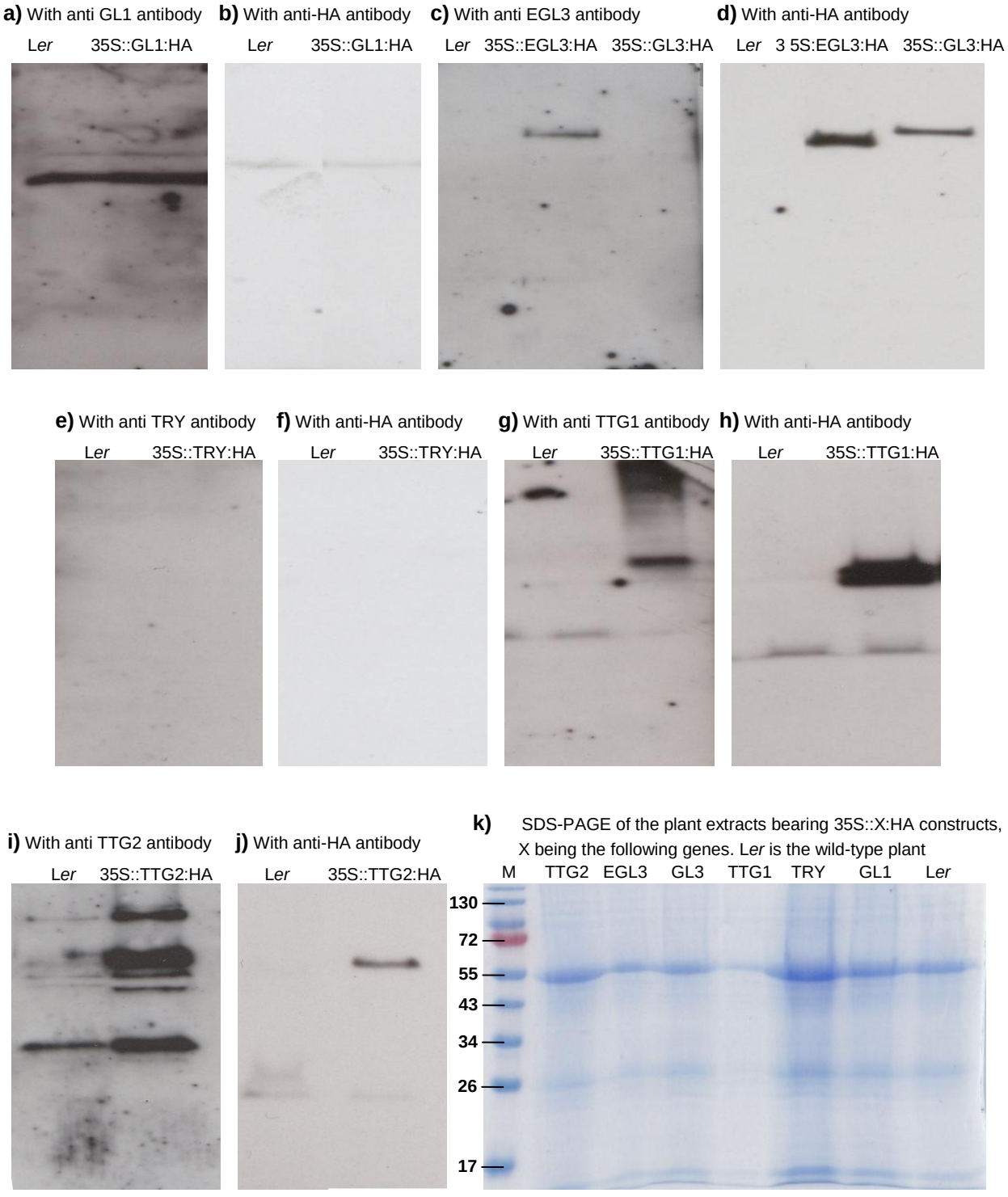

Figure 35 *Protein analyses from plant extracts containing the overexpression construct.*

Plant extracts were prepared from wild-type Ler *as well as* Arabidopsis *containing the 35S::GL1:HA, 35S::TRY:HA, 35S::GL3:HA, 35S::EGL3:HA, 35S::TTG1:HA, or 35S::TTG2:HA construct and analysed by Western blotting to test the functionality of the purified antibodies. In each blot wild-type plants were used as a negative control. Anti-HA antibody was used as positive control for each plant extract.*

k) The Coomassie staining of the plant extract to ensure equal loading by comparing the Rubisco band. The specific 1^0antibody used for each immunoblotting is indicated in the upper row of the subscription

M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown on the right side.

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2.7. THE KINETICS OF THE COMPETITION BETWEEN GL1 AND TRY FOR BINDING TO GL3

This work successfully shows that GL1 and TRY can interact *in vitro* with GL3. Current models assume that the formation of the activating complex occurs through the competition of MYB proteins GL1 and TRY for binding to the GL3 protein [17]. In order to calculate the binding coefficients of GL1 and TRY proteins to GL3, protein Surface Plasmon Resonance (SPR-Biacore) experiment was designed. However, with the conditions used for this experiment, binding of the GL3 protein to the surface neither with His- nor with a GST- tag was observed. The buffer used was HEPES, which was compatible with the chip surface. Nevertheless, resulting from the initial observations that indicate the proteins would not be stable in HEPES buffer, a high concentration of salt (300 mM NaCl) was used for Biacore. This could prevent the interactions of the proteins with the chip surface by hindering the His-sites. On the other hand, GL1 was non-specifically binding to the GSH surface when it was used as His- fusion and the GST-GL1 fusion protein was binding to the Ni-NTA chip surface. Increasing the salt concentration in this case could not prevent the non-specific interactions of the protein with the surface. This fact led to the conclusion that another coupling method should be used for Biacore experiments.

2.8. USE OF OTHER BIOCHEMICAL TOOLS TO STUDY PATTERNING PROTEIN FUNCTION

Current models involving the Lateral Inhibition Mechanism suggest that the inhibitors are produced in the trichome initiating cells and move from these cells to the neighbouring cells. The inhibitors then inhibit the trichome formation of these cells [7, 11, 62, and 80]. However, how this movement is achieved is still a question to be answered. One of the projects in our workgroup involves the microinjection of patterning proteins into tobacco leaves, in order to observe the movement of the patterning proteins. Microinjection experiments not only demonstrate the movement of the proteins but also can show whether the movement is actively mediated [70]. Purified N-terminal His- tagged GL3, TTG1, ETC3 and CPC proteins were labelled covalently (Figure 36). These proteins were then used by Rachappa Balkunde and Friedrich Kragler in microinjection experiments into tobacco mesophyll cells for analysis and comparison of their movements.

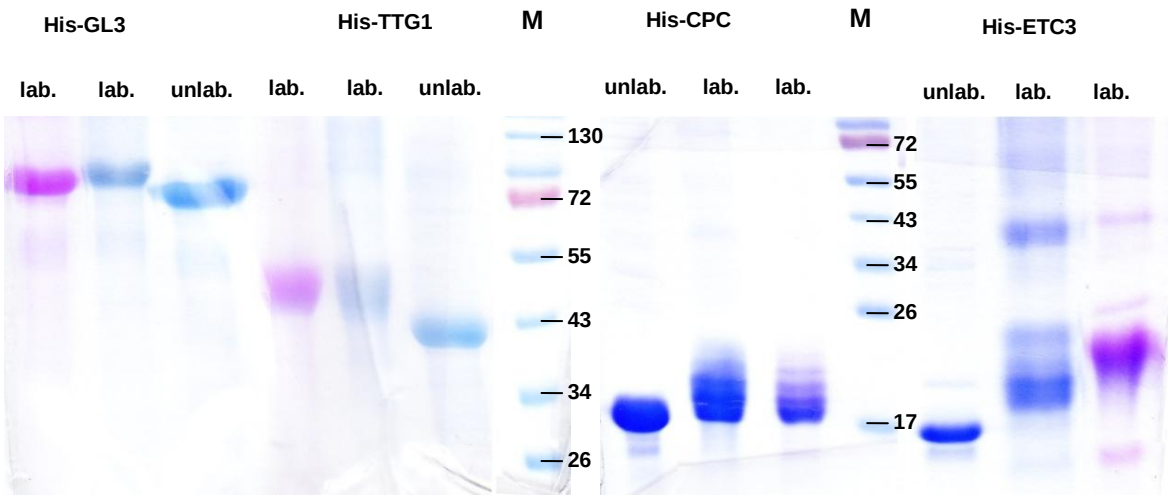

Figure 36 *SDS-PAGE showing the purified proteins after labelling.*
lab. corresponds to labelled protein
unlab. corresponds to unlabelled protein
M: PageRuler™ Prestained protein ladder, with corresponding MW values (kDa) shown for both ladders

3. DISCUSSION

Current knowledge indicates that a *de novo* patterning mechanism accounts for the proper establishment of *Arabidopsis* trichome pattern. This mechanism consists of the formation of an activator or inhibitor complex by the interaction of MYB-bHLH-WD40 proteins for determination of the trichome or non-trichome cell fate in *Arabidopsis* leaves. The reports on trichome initiation mechanism are all based on mutant plant analyses, genetics, and yeast two-hybrid interactions or its comparison with the root hair patterning system, which involves a similar patterning mechanism. However, the amount of biochemical research about trichome patterning is very limited. Until now, only the WER and CPC proteins were expressed in bacteria and purified [64, 65]; no other patterning protein was purified and analysed individually at protein level. Although some biochemical studies concerning the trichome patterning proteins were conducted, the data describing the specific protein properties and activities, direct interactions among these proteins and the functions of the native proteins is still not sufficient to explain trichome initiation process at a biochemical basis. Therefore, understanding the complete mechanism underlying the trichome patterning requires biochemical evidence in addition to the current molecular and genetic data.

3.1. EXPRESSION AND PURIFICATION OF THE PROTEINS AT A GLANCE

In order to conduct biochemical experiments, the principal requirement is the presence of proteins in hand. For this reason, initially several expression and purification methods were used to obtain pure proteins for the subsequent experiments in this study. Some expression systems attempted in this work did not yield production of the proteins at a desired level. Therefore, a systematic approach was utilised by means of changing the expression system used at each step. Only prokaryotic expression systems yielded the desired levels of the patterning protein expression tested in this work. Different tags were used for each protein. Usage of various tags helped handling, purification and detection of the proteins in the different experiments during this work. An overview of protein expression results in different expression systems and the ultimate purification success achieved in this study is given in Table 4.

Table 4 *Proteins and tags used for expression in this study*

+ indicates successful expression of the protein

- indicates no expression

NA indicates that expression in this system was not tested

IB indicates high protein concentration in inclusion bodies

S indicates high concentrations of soluble proteins

P *indicates successful purification*

Protein of Interest	Expression with no tag	Expression with C-term His- Tag	Expression with N-term His- Tag	Expression with N-term GST- Tag	Expression with N-term MBP- Tag	Expression with N-term Strep- Tag
GL1	+ IB, **P**	-	+ IB, **P**	+ S, **P**	+ S, **P**	-
GL3	-	-	+ IB, **P**	+ S, **P**	-	+
EGL3	-	-	+ IB, **P**	+ S, **P**	NA	+
TTG1	-	-	+ IB, **P**	+ S, **P**	+ S, **P**	+
TTG2	NA	-	+ IB, **P**	+ S, **P**	NA	+
TRY	+ IB, **P**	-	+ IB, **P**	+ S, **P**	+ S, **P**	-
CPC	+ IB	-	+ IB, **P**	+ S, **P**	NA	NA
WER	NA	NA	+ IB, **P**	NA	NA	NA
AtMYB23	NA	NA	+ IB, **P**	NA	NA	NA
ETC1	NA	NA	+ IB, **P**	NA	NA	NA
ETC2	NA	NA	+ IB, P	NA	NA	NA
ETC3	NA	NA	+ IB, P	NA	NA	NA

3.2. 3D STRUCTURE OF PATTERNING PROTEINS

To understand the native functions of proteins, solving their 3D structure plays a very important role. The cloning of the *MYB* genes acting as activators and inhibitors in trichome patterning pathway revealed that they contain conserved domains and the only difference between them is the lack of the transcription activation domain in inhibitors [23, 26, 27, 35, 45, 47, 49-51]. It has been found that the functional properties of MYB proteins are ensured by regulatory differences as well as the specificity at their protein levels [26, 37]. Nevertheless, how these similar proteins exert such different functions is still a question to be answered. Before working with the patterning proteins, homology modelling of the 3D structures of the activator and of the inhibitor -GL1 and TRY respectively, as representatives- was attempted. However, the lack of a reference protein structure for these proteins in literature made it impossible to model the 3D structures of these proteins. The closest MYB protein, the structure of which has been characterised, found from the PDB database [71b] is the RAD protein from *Antirrhinum* [58]. However, structural alignment of GL1 and TRY to RAD did not yield any similarities suitable for the modelling software.

When GL1 and TRY sequences are aligned to find another known structure in the PDB database, the first output comes out from a chicken MYB protein. Although MYB domain structure is crystallised and solved from several MYB proteins from the animal systems, homology modelling of GL1 and TRY in the Predict Protein Database also did not yield any results for the MYB protein found in chicken. Therefore, it would be of great interest to solve the 3D structures of these proteins. For this purpose GL1 and TRY proteins were expressed and purified using different expression systems. However, neither of the systems used in this work was successful enough to go further for crystallisation, due to the aggregation of GL1 and TRY. The aggregation of these proteins could be due to misfolding of them in the systems that were used, since the proteins were expressed in a prokaryotic system. If the aggregation problem is solved in future by using different expression systems (e.g., systems containing chaperones for correct folding of the protein [81]) it should be possible to solve the 3D structures of these proteins. Tominaga *et al*. have reported that the R3 MYB domain of the WER protein can replace the MYB domain of CPC, whereas the WER R3 domain cannot complement for the CPC MYB domain. This was explained by the evolution of R3 in WER and CPC protein by gene duplication from a single common ancestor. It is suggested that the differences in the functional properties of R3 type MYB genes arose from the loss of DNA binding regions of the

R2R3 type MYB proteins during the context of evolution [69]. It is also speculated that the evolution of these two different types of MYB genes from a common ancestor by gene duplications gave rise to new and different functions of these members of the same gene family [47]. The comparison of the structures of TRY -the CPC homologue- and GL1 -the WER homologue- can give more insights to the biochemical properties leading to this genetic drift. The comparisons of the tertiary structures of different regions such as amino acids preserved inside the loops or exposed to surfaces can better explain the susceptibility of these genes to mutations and the evolution of these proteins.

3.3. DO THE INTERACTIONS OF TRICHOME PATTERNING PROTEINS OCCUR IN A DIRECT MANNER?

The interactions for trichome initiation pathway have been identified at genetic levels which were then tested by yeast two-hybrid and BiFC assays until now [17, 62, and 69]. In this study, after coexpressing TRY and GL3 in a single colony, gel filtration was performed and these two proteins were found to be eluted together in the first elution fractions. The coelution of these two proteins indicates the *in vitro* formation of a strong TRY-GL3 complex.

Additionally, TTG1-GL3 and TRY proteins were also coexpressed in this work. This was done by cloning of the *GL3* and *TRY* into a single vector and cotransforming this vector with another construct containing the *TTG1* gene. Coexpression was achieved by the induction of these three proteins from a single bacterial colony. However, these three proteins were found to be insoluble when they were coexpressed. The GL1 and GL3 proteins were not successfully cloned into the same vector; therefore the proposed activator complex proteins could not be coexpressed in the same way as TTG1-GL3-TRY. Coexpression of the proteins building the proposed activator and inhibitor complexes for trichome patterning and subsequent gel filtration might provide a more detailed insight on whether these complexes can also form in bacteria.

The yeast two-hybrid, BiFC and coelution methods, which indicate interaction among patterning proteins, all represent an indirect interaction mechanism. However, these methods do not show whether these interactions can occur in a direct manner or whether intermediates are required for the formation of these complexes. The GST pull–down experiments conducted in this work show that

GL1-GL3/EGL3 and TRY-GL3/EGL3 interactions occur in a direct manner among the individual proteins.

The complex formation of GL1 protein together with GL3 and TTG1 proteins has been shown by Co-IP experiments from plants overexpressing the tagged GL1, GL3 and TTG1 proteins [82, 83]. However, these results also do not show the direct interaction of these three proteins, since other components might also be pulled-down together in these experiments. The GST pull-down experiments conducted in this work has shown that the direct formation of the GL1-GL3-TTG1 complex as well as GL1-EGL3-TTG1 complex can also occur *in vitro.* This indicates that the formation of the proposed activator complex for trichome patterning do not require any intermediate proteins. In order to analyse the actual components of the activator or inhibitor complex in *Arabidopsis,* one can pull-down the complex, using the specific antibodies produced. The pulled-down fractions can then be analysed in gel filtration experiments, where the size of the complex can be determined. On the other hand, the components of the actual activator or inhibitor complex for trichome patterning can also be analysed either by protein sequencing or after Western blot by using the specific antibodies. This will yield the actual components of the activator complex when GL1-GL3/EGL3-TTG1 specific antibodies and of the inhibitor complex when TRY-GL3/EGL3-TTG1 specific antibodies are used. A similar method was used to identify the components of the ESCRT complex [84].

3.4. NOVEL INTERACTION PARTNERS OF TRICHOME PATTERNING

One surprising interaction found in the GST pull-down experiments in this study was the interaction of GL1 with TRY. This interaction, although not reported by a yeast two-hybrid system, was also observed in the BiFC assay. These *in vitro* interactions of GL1-GL3/EGL3, TRY-GL3/EGL3 and GL1-TRY were published by Digiuni *et al.* in 2008 [62]. Digiuni *et al.* put forward a mathematical model where trichome patterning on *Arabidopsis* leaves is simulated by the formation of the active complex triggering the trichome cell fate. The GL1-TRY interaction gives an impulse to the concept of trichome patterning via three types of inhibition mechanisms. In the single competitive inhibition mechanism, TRY interacts with GL3 and inhibits the formation of the active complex for trichome cell fate. The second mechanism is the double competitive inhibition mechanism, which comprehends the TRY-GL1 interaction. In the last scenario, TRY binds to the active complex in an uncompetitive inhibition manner. The combination of the theoretical modelling and the experimental set-up comes to the conclusion that the single competitive inhibition

mechanism is most relevant for trichome initiation although all three types of inhibition mechanisms have the chance to play a role in the trichome system [62]. An experiment, where the binding and dissociation coefficients of GL1 and TRY proteins to GL3 protein are calculated and compared, can punctuate these inhibition scenarios. For this purpose, in this work it was attempted to conduct a SPR (Biacore) experiment to calculate the binding coefficients of GL1-GL3 and TRY-GL3 interactions. However, the sticky behaviour of the GST- and His- tagged GL1 protein precluded the differentiation of GL1 binding to chip surface or to the GL3 protein. An experimental setup where the non-specific binding of the GL1 protein is prevented might indicate a comparison of the GL1-GL3 and TRY-GL3 bindings. From time to time, the GL1 protein was showing non-specific interactions in other assays as well. This non-specific binding of the GL1 to the resin could be prevented by using detergents (Nonidet-P40) and other reagents such as BSA in the GST pull-down experiment that were conducted in this study. However, these reagents are not compatible with the Biacore experiments. For the Biacore experiments performed in this study, high salt concentration of 300 mM NaCl was used in order to prevent the non-specific binding to the chip surface. Besides preventing the non-specific interaction, this high concentration of salt might have also interfered with the interactions of GL1 and GL3 which were observed by other tools. Therefore, it will be helpful to use other methods for coupling of proteins to the Biacore chip surface, such as covalent binding for prevention of non-specific behaviour of proteins.

The GST pull-down experiments conducted in this study also resulted in the interaction of GL1 and TTG1 proteins. Although yeast two-hybrid has not yielded this result [Katja Wester, personal communication], it has been shown that *GL1* gene shows allele specific genetic interaction with *TTG1* gene [85]. A recent study also reports the interaction of GL1 protein with TTG1 in a Co-IP experiment [82]. The WD40 domain has been shown to form a beta-propeller structure which needs specific conditions for refolding properly. In this work, the TTG1 protein was expressed in a prokaryotic system. This might be a drawback for the proper refolding of TTG1. The GL1-TTG1 interaction observed in this work might not really occur naturally in *Arabidopsis* trichomes. On the other hand, the interactions shown in this work are *in vitro* interactions where only purified proteins were used. It may as well be the case that GL1 protein specifically binds to other components of the trichome activation pathway preferably, with a higher affinity than binding to TTG1. The activation of the trichome cell fate may be a result of the higher affinities of individual proteins to other proteins for the formation of the active complex. If this is the case for GL1 and TTG1 proteins binding to the GL3 protein, one may conclude

the following: TTG1 protein may also have the ability to bind to GL1 in single form, but in the presence of GL3, the GL1 and TTG1 may each prefer to bind to the GL3 protein forming the GL1-GL3-TTG1 active complex. Therefore the comparisons of binding coefficients rather than interactions of individual proteins might give a better explanation for the interaction preferences of the proteins in the trichome patterning mechanism. This can also be tested via yeast three-hybrid experiments by comparing the competition between TTG1 and other components of the trichome patterning [17, 62]

The cloning of the patterning proteins revealed that except TTG1 protein all other proteins are transcription factors. GL1, GL3, TRY, WER and CPC proteins have been also shown to localise to nucleus *in planta* observations [17, 64, and 68]. The interactions observed in this work such as GL1-TTG1 or GL1-TRY can also occur in the cell, whereas the determining factor for the preference of these interactions might be the actual subcellular localisation of these interactions. The interaction of GL1 with TTG1 was also observed in cytoplasm BiFC assay in *Arabidopsis*, whereas Fluorescence resonance energy transfer (FRET) experiments done in nucleus do not result in any interaction between these two proteins [Simona Digiuni, personal communication]. Other than the binding affinity preferences of the proteins determining the formation of activator or inhibitor complex for trichome initiation, the subcellular localisations of the interactions might also be important for initiation of trichome cell fate.

The protein interactions of TTG2 were also biochemically tested in this work. This protein was also always found to be non-specifically binding to the resins in the GST pull-down experiments. However, the signal detected after Western blotting of His-TTG2 pulled-down together with GST-TTG1 was higher than the non-specific binding of the His-TTG2 protein. This might show that these two proteins are able to interact with each other. However, another method should be applied to test this interaction. Therefore, His- and GST- tagged TTG2 and TTG1 proteins were coexpressed in a single colony and purified via the corresponding tag. The coelution of these two proteins after purification with respect to the corresponding tag also shows that TTG2 and TTG1 can interact with each other in bacteria as well as *in vitro*. In a similar manner, TTG2 was also coeluted together with the GL1 when they were coexpressed in a single colony. This coelution can actually be an artefact since GL1 protein was also found to be non-specifically binding to the chip surface in the Biacore. Alternatively, this can also be a new interaction which has not been observed before, as in the case of GL1-TRY interactions. This is another surprising interaction, since GL1 and TTG2 were not shown to interact in yeast two-hybrid

analyses [Martina Pesch, personal communication]. To find out whether TTG2-GL1 interaction is biologically relevant or not, one should test other proteins in this system, which are known not to interact with TTG2, as a negative control. One possible candidate for such a test is GL3, since yeast two-hybrid experiments do not show interaction between these two proteins [Martina Pesch, personal communication]. In order to assess the importance of the TTG2 interactions with GL1 and TTG1, one should first confirm precisely whether these interactions are really occurring *in planta*.

Howell *et al.* compare the techniques for *in vitro* protein-protein interactions and assess the parameters for such an interaction. It has been shown that usage of several tools for protein interactions can minimise the false positives [86]. Therefore, it is of great importance to test the novel interactions observed in this study by other techniques.

3.5. THE ANTIBODIES AGAINST TRICHOME PATTERNING PROTEINS

Until now, no antibodies against the GL1, GL3, EGL3, TTG1, TTG2 and TRY proteins have been reported in literature. The high levels of expressions in bacteria and the purification of these proteins achieved in this work allowed the production of specific antibodies against these trichome patterning proteins. This was achieved by the use of His- tagged GL1, EGL3, TTG1, TTG2 and TRY proteins. These pure proteins were used as antigens, so that one antibody specific for one conserved domain group of proteins could be obtained. After purification of the individual antibodies from the injected rabbit total blood serum, the antibodies were found to be specific for the individual bacterially expressed proteins. Although homologous proteins contain a high degree of identity in their protein sequences, no cross reactions of the anti-GL1 antibody with the GL1 homologous proteins WER and MYB23, and of the anti-TRY protein with the TRY homologous proteins CPC, ETC1, ETC2 and ETC3 were observed. Anti-GL1 and anti-TRY antibodies were also tested against mutated GL1 and TRY proteins that were shown not to interact with GL3 protein in yeast two-hybrid assays [Martina Pesch, personal communication]. These proteins contain single amino acid mutations that possibly prevent the proper functioning of the MYB domains. However, this single mutation does not mask the binding of the GL1 and TRY epitopes to these proteins. This could be an advantage when the aim is to assess antibody dependent assays comparing MYB and bHLH protein interactions. On the other hand, anti-EGL3 antibody showed cross reaction with the GL3 protein expressed in bacteria. This might be a drawback when it is

desired to conduct protein specific experiments for EGL3 protein. Nevertheless, the antibody purified for EGL3 protein can still be used to test the bHLH dependent experiments. It may be worthy to obtain pure GL3 antibody and to determine if anti-GL3 antibody can also cross-react with EGL3 proteins. When protein specific experiments are planned, one can still purify specific antibodies from the polyclonal EGL3 antibodies, specific for a region of the EGL3 protein that is not conserved in the GL3 protein. The signal detected in the Western blots when purified anti-TTG2 antibody is used against bacterially expressed TTG2 proteins revealed that the TTG2 antibody needs further purification.

It was of great importance to test the functionality of these purified antibodies against bacterially expressed proteins in reaction with plant proteins. Therefore, the purified antibodies were tested against plant specific proteins by Western blotting. The interaction of the antibodies produced against bacterially expressed antigens and the plant proteins revealed that these antibodies can successfully be used for plant protein specific experiments. Although anti-EGL3 antibody was shown to cross-react with bacterial GL3 protein, no signal was detected when plant GL3 protein was used with this antibody. The Western blots using the purified antibodies revealed that this work provided specific and pure antibodies against GL1, EGL3, TTG1, TTG2 and TRY proteins, which can successfully detect these proteins in *Arabidopsis*. These antibodies can be used in further experiments.

The absence of the specific antibodies for patterning proteins made it possible to work only with tagged proteins until now. Although the usage of tagged proteins should always be checked with rescue experiments, this situation may sometimes not reflect the real biochemical properties of the plant proteins. The tagged proteins for trichome patterning include the YFP- tags used for detection of the protein localisation and ChIP experiments [17, 64, 66, and 68] as well as the HA-tag [87].

The antibodies produced and purified in this work can be used for testing the protein-protein interactions as well as the protein-DNA interactions in the future, by techniques such as Far-Westerns, Co-IP and ChIP tools. They can also be utilised in studying the protein expression by immunofluorescence in order to visualise the subcellular distribution and the localisation of these patterning proteins without the need for a fusion tag.

3.6. MOVEMENT OF THE PATTERNING PROTEINS

In order to understand the trichome patterning mechanism, Meinhardt & Gierer model and the context of Lateral Inhibition have been widely used. One of the requirements for the Lateral Inhibition Mechanism to act in a system is the mobility differences between the activators and inhibitors [11, 38, and 80]. This suggests that one of the specific functional property differences of GL1 and TRY should be due to the differences in their mobility capabilities. As shown by particle bombardment experiments, TRY and CPC can move in *Arabidopsis* leaves, whereas GL1 cannot [62]. The comparison of the structures of activator and inhibitor proteins might provide information needed to study the mobility of these proteins. Moreover, this comparison might also lead the way for the discovery of some other intrinsic protein properties, which would reveal the unknown features of patterning mechanism.

Although the TRY and CPC proteins are thought to move via plasmodesmata, it is still not known how they are targeted to plasmodesmata or how the movement is achieved [62, 68]. The movement protein P30 from Tobacco mosaic virus, the movement of which is achieved via the plasmodesmata, was shown to require phosphorylation by a protein kinase [88].The tool PROSEARCH (Search Prosite Database for Patterns in a Protein Sequence) found at the San Diego Supercomputer Center (SDSC) Biology Workbench database [71f] also predicts the presence of protein kinase phosphorylation sites in both GL1 and TRY proteins. The purified GL1 and TRY proteins can be treated, as in the case of P30, to check whether they can also be phosphorylated in a similar manner. It may be of great value to test whether the movement of these proteins can be regulated by the similar phosphorylation/dephosphorylation mechanisms.

Microinjection has become a very important tool for observation of *in vivo* protein movement behaviour [70]. His- tagged GL3, CPC and ETC3 proteins purified in this study were covalently labelled with small dyes. These pure proteins could then be injected into plant cells and the behaviours could be observed under a microscope. It has been found that His- tag does not interfere with movement of patterning proteins [Friedrich Kragler, personal communication]. Kurata *et al.* have shown that the proper cell to cell transport of the CPC protein depends on its specific conformation [68]. The proteins that were used for microinjection experiments were obtained from inclusion bodies. The correct refolding of the proteins after inclusion body preparations is known to be a limiting step for biochemical analysis of proteins. The subsequent dialysis of the proteins in this work could successfully provide the

conformation which is required for movement in tobacco mesophyll cells, since this purification method did not interfere with the microinjection of these proteins [Friedrich Kragler and Rachappa Balkunde, personal communication] This work has provided the pure proteins of the trichome patterning which can be used for the comparison of the different movement of the different proteins.

3.7. THE INTERACTION OF PATTERNING PROTEINS WITH DNA *IN VITRO*

Until now, only WER and CPC proteins have been expressed in bacteria, purified and tested for their direct interaction with DNA [64, 65]. The cloning of the genes involved in trichome patterning has revealed that GL1, GL3/EGL3, TTG2 and TRY contain DNA binding domains [16, 18, 23, 42 and 47]. ChIP experiments using HA- and YFP- tagged proteins pointed out to the interaction of GL3 and TTG1 to target promoter sequences [66, 82, and 87]. However, the direct interaction of the target DNA sequences with individual proteins has not been shown yet, the only exception being WER.

Ongoing work includes the analysis of the promoter regions of trichome patterning proteins [Martina Pesch, personal communication]. This work is being done in order to check whether the putative transcription factors of trichome initiation pathway really have the ability to bind to the promoter sequences. The purified His- tagged and GST- tagged proteins of trichome patterning are prepared as described in this study. These pure proteins from this study have made it possible to perform EMSA [Martina Pesch, personal communication].

3.8. USAGE OF PROTEINS IN OTHER BIOCHEMICAL TESTS

It has been shown that phosphorylation plays an important role for movement of proteins through the plasmodesmata [88, 89, and 90]. Studies on animal MYB proteins also present the necessity of phosphorylation for proper functioning of these proteins [53]. The software PROSEARCH [71f] predicts phosphorylation sites at the similar position in both GL1 and TRY proteins. It would be of great interest to test whether GL1 and TRY proteins can be phosphorylated. This could be done by incubation of the pure proteins by plant extracts. As well as phosphorylation, other modifications such as ubiquitination or sumoylation can also play important roles for

regulation of the patterning proteins. The purified proteins in this work may be used for such *in vitro* modification assays in future.

Another important property of the patterning proteins for regulation of trichome cell fate could be the turnover rates of the activator and inhibitor proteins. The Western blot analyses using the protein specific antibodies purified in this work have shown that GL1 and TRY proteins could not be detected at the developmental stage when EGL3, TTG1 and TTG2 proteins were still detectable. This could be due to a degradation mechanism for controlling the tight regulations of activator and inhibitor MYB proteins. One candidate for such degradation mechanism is *KAKTUS (KAK)* gene -a putative E3 ligase- which might play a role in degradation of GL1 by the 26S proteosome [91]. The purified proteins in this work can also be used for *in vitro* degradation analysis and their degradation behaviours can be compared with each other. In such an experiment, purified proteins can be treated with wild-type and *kak* plant extracts to search for the KAK in degradation of specific proteins. On the other hand, different proteins can be treated with plant extract to compare their degradation rates with each other. This *in vitro* degradation assay was performed with His-GL1 and GST-GL1 obtained in this work and different degradation of GL1 was observed by different plant extracts [Jennifer Werner, personal communication]. Although, such an experiment may not exactly reflect the stability of the proteins in *Arabidopsis* leaves, it may give a respective value for activator and inhibitor stability comparisons. This may also be of great importance for the activation of trichome fate in trichome initials, provided that the activator complex or the components of the activator complex is more stable in these epidermal cells than in other epidermal cells.

3.9. SUMMARY AND OUTLOOK

This study presents a systematic approach to get a hold of the biochemical proof for trichome patterning mechanism in *Arabidopsis,* which, until now, has only been proposed by evidence from genetic investigations, yeast two-hybrid studies and comparison to similar patterning machineries. With this study, the patterning proteins were expressed in bacteria and purified successfully for the first time, by the usage of different tags. These expressed proteins were used for further experiments such as GST pull-downs to test the direct interactions among trichome patterning proteins. The production of the patterning proteins paves the way for these proteins to be used in other biochemical assays, which may be required in order to have more insight into trichome patterning pathway. Some examples to such assays are EMSA,

ChIP, microinjection and/or modification studies. Moreover, the antibodies produced in this work may be used in future for purposes where usage of specific fusion tags might interfere with the functionality of the proteins. Figure 37 illustrates a summary of the experimental procedures for future use of these pure proteins in order to enlighten the unknown processes determining the cell fate behaviour of the trichomes.

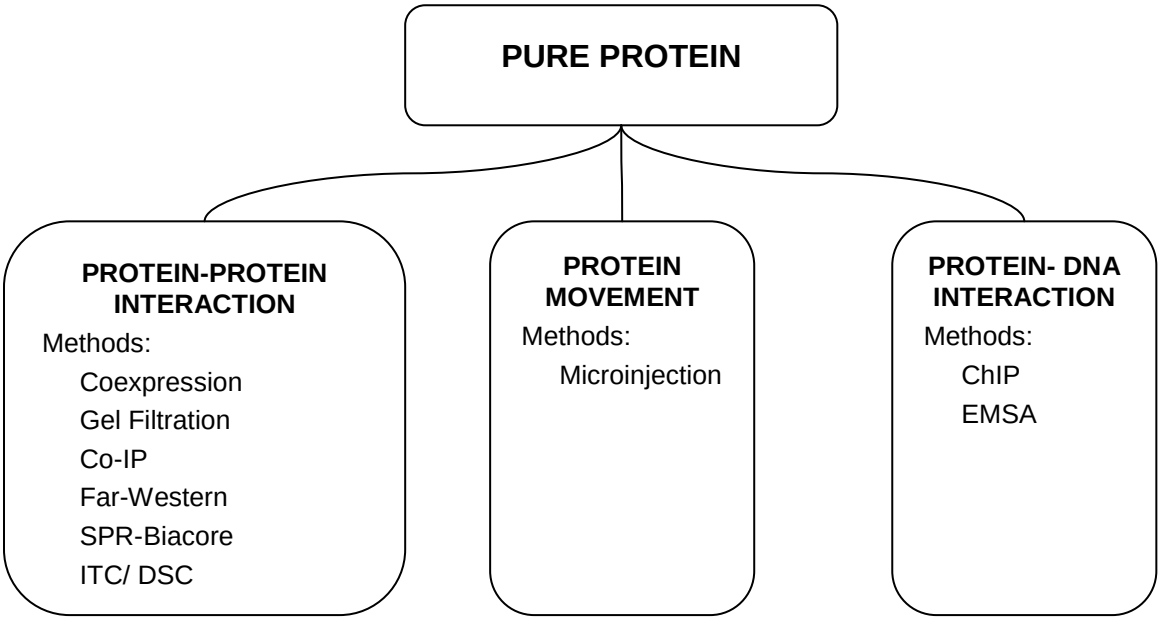

Figure 37 *Possible future uses of the proteins purified in this work*

4. MATERIALS AND METHODS

4.1. MATERIALS

4.1.1. ANTIBIOTICS AND CHEMICALS

In this study all the antibiotics, chemicals and reagents were attained from the companies Roth (Karlsruhe), Sigma (Deisenhofen), GE Healthcare, Qiagen (Dusseldorf), New England Biolabs (Frankfurt/Main), BioRad, Duchefa.

4.1.2. ENZYMES

All restriction enzymes were obtained from MBI-Fermentas (St. Leon-Rot). Gateway cloning enzymes were acquired from Invitrogen (Karlsruhe), Factor Xa was purchased from New England Biolabs (Frankfurt/Main)

4.1.3. PRIMERS

Primers were produced by Invitrogen (Karlsruhe). Oligonucleotides used in this work are as follows;

Forward for pET3a for *GL1* with NdeI restriction site:

GGAATTCCATATGAGAATAAGGAGAAGAGA

Reverse for pET3a for *GL1* with BamHI restriction site including the stop codon:

CGGGATCC CTAAAGGCAGTACTCAACAT

Forward for pET3a for *GL3* with NdeI restriction site:

GGAATTCCAT ATGGCTACCGGACAAAACAG

Reverse for pET3a for *GL3* with stop codon BamHI restriction site:

CGGGATCC TCAACAGATCCATGCAACCC

Forward for pET3a for *TTG1* with NdeI restriction site:

GGAATTCCAT ATGGATAATTCAGCTCCAGA

Reverse for pET3a for *TTG1* from 660 to silently mutate BamHI restriction site at 587:

GATCCTAACGGAACCATCAG

Forward from 581 for pET3a for *TTG1* to silently mutate BamHI restriction site at 587:

CTGATGGTTCCGTTAGGATC

Reverse for pET3a for *TTG1* with BamHI restriction site from the end with stop codon:

CGGGATCC TCAAACTCTAAGGAGCTGCA

Forward for pET3a for *TRY* with NdeI restriction site:

GGAATTCCAT ATGGATAACACTGACCGTCG

Reverse for pET3a for *TRY* with BamHI restriction site including the stop codon:

CGGGATCC CTAGGAAGGATAGATAGAAA

Forward for pET3a for *CPC* with NdeI restriction site:

GGAATTCCAT ATGTTTCGTTCAGACAAGGC

Reverse for pET3a for *CPC* from 200 to silently mutate BamHI restriction site at 189:

CGTCCCGGAATCCTTCCGGC

Forward for pET3a for *CPC* from 181 to silently mutate BamHI restriction site at 189:

GCCGGAAGGATTCCGGGACG

Reverse for pET3a for *CPC* from the end for BamHI restriction site including the stop codon:

CGGGATCC TCATTTCCTAAAAAAGTCTC

Forward for pMALC2 for *GL1* with XmnI restriction site:

GGAAGGATTTCAATGAGAATAAGGAGAAGAGAT

Reverse for pMALC2 for *GL1* with PstI restriction site including the stop codon:

GGGCTGCAGCTAAAGGCAGTACTCAACATCACC

Forward for pMALC2 for *GL3* with EcoRI restriction site:

TCAGAATTCATGGCTACCGGACAAAACAGA

Reverse for pMALC2 for *GL3* with PstI restriction site including the stop codon:

GGGCTGCAGTCAACAGATCCATGCAACCCT

Forward for pMALC2 for *TTG1* with XmnI restriction site:

GGAAGGATTTCAATGGATAATTCAGCTCCAGAT

Reverse for pMALC2 for *TTG1* with PstI restriction site including the stop codon:

GGGCTGCAGTCAAACTCTAAGGAGCTGCAT

Forward for pMALC2 for *TRY* with XmnI restriction site:

GGAAGGATTTCAATGGATAACACTGACCGTCGTCGC

Reverse for pMALC2 for *TRY* with PstI restriction site including the stop codon:

GGGCTGCAGCTAGGAAGGATAGATAGAAAAGCG

Forward for pMALC2 for *CPC* with XmnI restriction site:

GGAAGGATTTCAATGTTTCGTTCAGACAAGGCG

Reverse for pMALC2 for *CPC* with PstI restriction site including the stop codon:

GGGCTGCAGTCATTTCCTAAAAAAGTCTCTTCG

Forward for *GL3* for pETDuet™-1MCS(Multiple Cloning Site)1:

GGGGAATTCGATGGCTACCGGACAAAACAGA

Reverse for *GL3* for pETDuet™-1MCS1:

GAGCTCTCAACAGATCCATGCAACCCT

Forward for *GL3* for pETDuet™-1MCS2:

GGGCATATGGCTACCGGACAAAACAGAACA

Reverse for *GL3* for pETDuet™-1MCS2:

GGGGACGTCACAGATCCATGCAACCCTTTG

Forward for *TRY* for pETDuet™-1MCS1:

GGGGAATTCGATGGATAACACTGACCGTCGTCGC

Reverse for *TRY* for pETDuet™-1MCS1:

GGGGAGCTCCTAGGAAGGATAGATAGAAAAGCG

Forward for *TRY* for pETDuet™-1MCS2:

GGGCATATGGATAACACTGACCGTCGTCGC

Reverse for *TRY* for pETDuet™-1MCS2:

GGGGACGTCGGAAGGATAGATAGAAAAGCG

4.1.4. VECTORS

The following expression vectors were used in this study for bacterial expression.

pET3a	from New England Biolabs
pDEST™17	from Invitrogen
pGEX2TM-GW	This vector was kindly received from Imre Sommsich and Bekir Ülker
pASK3GW	This vector was kindly obtained from Marc Jakoby
pCDFDuet™-1, pETDuet™-1	from Novagen (Darmstadt)
pETFrame C	This vector was kindly obtained from Joachim Uhrig

4.1.5. BACTERIAL STRAINS

E.coli bacterial strains DH5α and TOP10(INVITROGEN) were used for clonings. Expressions were tried in *E.coli* strains Rosetta-gami™, Rosetta-gami™B(NOVAGENE), TOP10, BL21, and BL21DE3RIL(STRATAGENE). The best expression levels were obtained in BL21-CodonPlus(DE3)-RIL(STRATAGENE) strains therefore these strains were used for further expressions.

BL21-CodonPlus(DE3)-RIL strain[a]: *E. coli* B F– *ompT hsdS*(r_B– m_B–) *dcm*+ Tet[r] *gal* λ (DE3) *endA* Hte *[argU ileY leuW* Cam[r]]

4.1.6. BUFFERS AND REAGENTS

$CaCl_2$ Solution:	60 mM $CaCl_2$
	10 mM PIPES pH7
	15% glycerol
Tris-Lysis Buffer:	100 mM NaCl
	50 mM Tris-HCl pH 7.5
	2 mM EDTA
	1% Triton X-100
STE Buffer:	10 mM Tris-HCl pH8
	150 mM NaCl
	1 mM EDTA
PBS Buffer:	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	2 mM KH_2PO_4
PBST Buffer:	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	2 mM KH_2PO_4
	0.1% tween-20
TBS Buffer:	10 mM Tris-HCl pH7.5
	150 mM NaCl

TBST: 10 mM Tris-HCl pH7.5

150 mM NaCl

0.1% Tween-20

Column Buffer: 20 mM Tris-HCl pH 7.4

200 mM NaCl

1 mM EDTA

GST Binding Buffer: 50 mM Tris-HCl pH 7.9

1 mM EDTA

150 mM NaCl

1x SDS Gel Loading Buffer: 50 mM Tris-HCl pH 6.8

2%SDS

0.1% bromophenol blue

10% glycerol

100 mM β- mercaptoethanol

Tris-glycine Electrophoresis Buffer: 25 mM Tris

250 mM glycine pH8.3

0.1% SDS

Western blotting Cathode Buffer: 1X Arbeitslosung Roti-Blot 2K

20% methanol

Western blotting Anode Buffer: 1X Arbeitslosung Roti-Blot 2A

5% methanol

Coomassie Staining Solution: 0.25 g Coomassie brilliant blue R-250

50 mL methanol

40 mL H_2O

10 mL glacial acetic acid

Destaining Solution: 50 mL methanol

40 mL H_2O

10 mL glacial acetic acid

Ponceau Staining Solution: 0.5 g Ponceau

1 mL glacial acetic acid

H_2O up to 100 mL

IPTG Stock Solution: 1 M IPTG

4.2. METHODS

4.2.1. PREPARATION OF DIFFERENT BACTERIAL EXPRESSION VECTORS FOR DIFFERENT PATTERNING PROTEINS

GL1-pENTR™1A, WER-pENTR™1A, and all pDEST™17 constructs used in this study were kindly obtained from Martina Pesch. GL3-pDONR™201, TTG1-pDONR™201, MYB23-pDONR™201, TRY-pDONR™201, CPC-pENTR™1A constructs were kindly obtained from Ullrich Herrmann, EGL3-pDONR™201 construct was kindly obtained from Ilona Zimmermann, TTG2-pDONR™201 construct was kindly obtained from Bekir Ülker.

GL1, GL3, *EGL3, TTG1*, and *TRY* coding sequences containing the stop codons were recombined into pGEX2TMGW and pASK3GW vectors by LR recombination reaction. *TTG2* coding sequence with stop codon was recombined into pASK3GW vector and without stop codon was recombined into pGEX2TMGW vector. For cloning into pET3a, pCDFDuetTM-1, pETDuetTM-1 and pMALC2 expression vectors, the following primers were used for PCR reactions which were then ligated into the desired vectors by conventional cloning methods instructed by Sambrook and Russell [92]. Phusion™ Polymerase from Finnzymes or HiFi Taq Polymerase from Fermentas was used for PCR amplification reactions and reactions were performed according to supplier's protocols. TTG1pETFrameC and TTG2pETFrameC constructs were also kindly provided by Martina Pesch.

4.2.2. PREPARATION OF COMPETENT BACTERIA

A single colony of *E.coli* was inoculated into 50 mL LB medium and was grown o/n at 37°C with 250 rpm shaking. 2ml of the overnight culture was inoculated into 200 ml LB medium in a sterile 1L flask and was grown at 37°C shaking at 250 rpm to an OD of 0.375. The culture was aliquoted into four; 50 ml-prechilled, sterile polypropylene tubes and the tubes were left on ice 5 to 10 min. The cells were then centrifuged 7 minutes at 1600xg (3000 rpm) at 40°C. Supernatant was removed and each pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution. Cells were again centrifuged 5 minutes at 1100xg (2500 rpm) at 4°C. The supernatant was discarded and each pellet was resuspended in 10 ml ice cold $CaCl_2$ solutions which were then kept on ice for 30 minutes. The cells were again centrifuged for 5 minutes at 110xg at 4°C. The supernatant was discarded and each pellet was resuspended in 2 ml ice-cold $CaCl_2$ solution. The cells were then dispensed into prechilled, sterile polypropylene tubes and frozen immediately in liquid nitrogen. The cells were stored at -80°C

4.2.3. PREPARATION OF PLASMID DNA

DNA from *E.coli* was prepared by the Plasmid Miniprep Kit I from PEQLAB (Erlangen) according to the instruction manual of the manufacturer.

4.2.4. SEQUENCING OF THE DNA

Constructs prepared were sequenced by an ABI 310 Prism automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Big-Dye kit 3.1 (Perkin Elmer Applied Biosystems, Foster City, CA) was used for sequencing reactions.

4.2.5. EXPRESSION OF PROTEINS VIA IPTG INDUCIBLE T7 AND SP6 PROMOTER SYSTEMS

The bacterial cells containing the IPTG inducible constructs were grown until an OD_{600} of 0.5-0.8 and then the cultures were induced by a final concentration of 0.5mM IPTG for His- tagged, 0.1mM IPTG for GST- tagged and 0.3mM for MBP-tagged proteins. The induced cells were then grown further for 3 hours and cells were harvested by centrifugation at 7000xg for 7 minutes at 4°C.

4.2.6. LYSES OF INDUCED CELLS

The pellets from 50 mL of induced cells containing the His- tagged expression constructs were resuspended in 1 mL Tris-Lysis Buffer containing 200µg Lysozyme and incubated at room temperature for 70 minutes. The solution was then sonicated for 10 seconds 5 times and centrifuged for 5 minutes in bench top microfuge at 13200 rpm. The supernatant was kept and run on gel to check if the proteins are in this soluble fraction. The pellets were resuspended in 500 µL of Tris-Lysis Buffer and sonicated and centrifuged as described above for first washing. Washing was repeated once more and the pellet was resuspended in 250µL of 100mM $NaHCO_3$ 2% SDS solution by sonication and centrifuged for 5 minutes in bench top microfuge at 13200 rpm. The supernatant contains the solubilised inclusion bodies and proteins in the inclusion body prep were analysed by an SDS-polyacrylamide gel.

The cells containing the GST- fusion proteins were lysed as explained by Frangioni and Neel [79].

The lysis of the cells containing MBP- tagged fusion proteins was done as explained in supplier's protocols.

4.2.7. PURIFICATION VIA Ni-NTA RESIN

250 µL of inclusion body prep of His- tagged proteins were incubated with 500 µL of Ni-NTA resin in a column for 1 hour. The column was then washed for 6 times the column volume with PBS buffer. The elutions were done with PBS containing 150 µL 20 mM of EDTA of ten fractions.

4.2.8. PURIFICATION VIA GSH RESIN

The bacterial lysate from 100mL culture was incubated with 1mL of GSH resin for 1 hour at 4°C on a rotating platform. The resin was then washed 4 times with 10 times the resin volume of PBS buffer. The elutions were done with 50 mM Tris-HCl pH 8 containing 10 mM of reduced glutathione by incubating the resins with the elution buffer for 15 minutes at 4°C on a rotating platform. The elution step was repeated twice more to get a final of three elution fractions. Each elution was done with 1mL of elution buffer.

4.2.9. PURIFICATION VIA AMYLOSE RESIN

The bacterial lysate from 250 mL culture was incubated with 1mL of Amylose resin for overnight at 4°C in a column. The column was washed 10 times with twice the resin volume of column buffer. The elution was done with column buffer containing a final concentration of 10 mM Maltose in five elutions of each 150 µL elution buffer.

4.2.10. CLEAVAGE OF MBP- TAG FROM TRY PROTEIN

After purification through Amylose resin, MBP- tag was cleaved by Factor Xa by incubating at 4°C rotating for eight hours. For 50 µg of fusion protein 1 µg of protease was used. After cleavage, TRY protein was sequenced by peptide mass finger printing to check the integrity and correct cleavage of the protein.

4.2.11. MEASURING THE PROTEIN CONCENTRATIONS

Protein concentrations were measured by using the Bradford Reagent from Biorad as explained in the user manual for the reagent.

4.2.12. GST PULL-DOWN

The purified His- and GST- tagged proteins were dialysed against GST binding buffer. Equal amounts of GST- tagged proteins were mixed with His- tagged proteins and incubated half an hour at 4^0C on a rotating platform. For each 10 microgram of tagged proteins 50 mL of GSH resin was then added into the mixture and the proteins resin mixture was further incubated for 2 hours at 4^0C on a rotating platform. The resins were then washed with 2 mL of binding buffer 5 times and proteins were eluted by Glutathione Elution buffer or PBS containing 20 mM EDTA. The elution fractions were then analysed by Western blot using anti-His antibody as primary antibody.

4.2.13. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

The SDS-polyacrylamide Gels were performed according to instructions in Sambrook and Russell [92].

4.2.14. IMMUNOBLOTTING OF PROTEINS

After running them on SDS-PAGE, the proteins were transferred to PVDF membranes by a semi-dry blotting apparatus. The membranes were then blocked by PBST containing 5% milk powder. After the blots were blocked for at least 1 hour, they were incubated with PBST milk powder containing the primary antibody of specific dilutions (1/1000 anti-His antibody, 1/10000 anti-GST antibody, 1/1000 anti-Strep antibody). Then blots were washed 3 times with PBST for 15 minutes and they were incubated with PBST milk powder containing the secondary antibody. After incubation with secondary antibody for one hour, the blots were washed three times for 15 minutes with PBST and detection was done. Due to the reason that the secondary antibodies were coupled to HRP, detection was done by using the HRP substrate and subsequent chemiluminescence was detected.

4.2.15. PREPARATION OF ANTIBODIES

200 microgram of N-terminal His- tagged GL1, EGL3, TTG1, TTG2 and TRY proteins were injected into 2 different rabbits. After the second boost on the 30th day, the blood serum was tested against the purified protein samples which were at the same purification quality level as the injected antigens. Total blood serum from the rabbits which were infused with GL1, EGL3, TTG1 and TRY antigens were collected on the 61st immunisation day. TTG2 antigen was further immunised to the rabbits and after duration of 85 days of immunisation the total blood serum was collected from these animals. The immunisation and serum collections were done by the Pineda-Antikorper Service.

4.2.16. PURIFICATION OF ANTIBODIES

The polyclonal antibodies were raised against GL1, EGL3, TTG1, TTG2 and TRY proteins were purified for specific detection of the individual proteins. In order to do this, purified His- tagged proteins which were used for immunisation were run on gel and blotted against PVDF membranes. Proteins on the membrane were checked by Ponceau Staining, the protein band corresponding to the purified protein, was then cut from the rest of the membrane and staining solution was washed away with TBS. The membrane was blocked with1% BSA in TBS containing 0.05% Tween-20 for 2.5 hours at 4^{o}C. Total blood serum after immunisation was diluted 1 to 5 ratios with TBS buffer and membrane was incubated with this solution for 4 hours at 4^{o}C on a rotating platform. The membrane was then washed with TBS

and antibodies bound to the membrane were eluted with antibody elution buffer by incubation at 4°C for 1.5 minutes. Washing of the membrane and elution of the antibody was repeated once more and immediately after elution 1MTris-HCl pH8.0 was added to the eluted antibody. Then 1/100 dilution of 5% Naazide containing 10mg BSA were added to the antibody solution for long-term storage. Immunoblots were performed on bacterial cell extracts before and after induction with incubation of preimmune serum, whole blood serum after immunisation and purified antibodies of individual proteins as primary antibodies.

4.2.17. Test of Antibodies against Plant Proteins

Plants containing the HA-tagged plants expressed under 35S promoter were grown on soil for 14 days after sawing. BASTA was sprayed once a week following germination. Wildtype plants of *L.erecta* were used as negative control. Seeds for plants containing the HA-GL1, HA-GL3, HA-EGL3, HA-TTG1, HA-TTG2, HA-TRY were kindly provided by Martina Pesch. The plants were selected by phenotypic analysis and BASTA selection to ensure the presence of the overexpression constructs. Plants were collected and frozen in liquid nitrogen. The frozen plants were then grinded and 1:1 volume ratio of SDS-gel Loading Buffer: plant extract volume was then added and samples were boiled at 95^{0}C for 10 minutes. Samples were then analyses after Western blotting by using the purified antibodies as well as anti-HA antibodies as positive control. The antibody titer for anti-HA antibody was 1:2000. The antibody titers for the purified specific antibodies were determined to be 1:100.

4.2.18. Gel Filtration Chromatography

The Gel Filtration Chromatography Assays of GL1 and TRY expressed in pET3a vector were performed at Institute of Bioorganic Chemistry, Poznan, Poland.The Gel Filtration Chromatography Assays of TRY expressed in pMALc2 vector were performed at Institute for Biochemistry University of Cologne, Cologne, Germany. Superdex 200 16/60pg was used for this assay

4.2.19. Surface Plasmon Resonance Spectroscopy

Biacore experiments were performed at Institute for Biochemistry I, Center for Molecular Medicine, University of Cologne, Cologne Germany

4.2.20. Peptide Mass Fingerprinting

PMF was performed by Bioanalytical Laboratory, Zentrum für Molekulare Medizin Köln Zentrale Bioanalytik (ZBA), Cologne Germany

4.2.21. Coexpression of Proteins

The constructs containing the CDS of proteins that are desired to be coexpressed are transformed into a bacteria and a single colony is selected by growth on media containing the combination of antibiotics for the vectors used for transformation. The presence of the constructs in a single colony was tested by colony PCR using the gene specific primers for each construct. After growth of single colony bearing the desired constructs overnight in LB medium containing the proper antibiotics, this preculture was inoculated into LB plus antibiotic and cells were further grown until an OD_{600} of 0.6 to 1. The culture was then induced with a final IPTG concentration of 0.5mM for 3 hours at 370C shaking at 220 rpm. The cells were then harvested by centrifugation for 7 minutes at 7000x g at 4^0C. The inductions were then analysed by SDS-PAGE for which 1mL of cells were pelleted and dissolved in 100 µL of SDS gel loading buffer.

4.2.22. Affinity Purification of Coexpressed Proteins

Coexpressed His-GL3 and MBP-TRY were purified via Amylose resin. Same conditions were applied during this purification as in the case of MBP-TRY alone. Cells coexpressed GST-GL1 and His-TTG2-His, GST-TTG2-His and His-TTG1, were lysed after induction and incubated with GSH or Ni-NTA resins for single step affinity purification. In addition, double step affinity purification was performed where the cell lysates were first incubated with GSH resin and the elution fractions of these first purifications were incubated with Ni-NTA resin subsequently. The analyses of the purified proteins after affinity purification steps were done by Western blotting in which, anti-GST or anti-His antibodies were used for detection of the corresponding protein.

Photos were edited with GIMP, the GNU Image Manipulation Program, version 2.4.6.

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Burcu Dartan

LEBENSLAUF

PERSONLICHE DATEN

Name	BURCU DARTAN
Geburtsort	Kadikoy,Istanbul-Türkei
Geburtsdatum	09.02.1980

SCHULBILDUNG

09/1987 - 06/1991 Grundschule Faik Resit Unat, Istanbul-Türkei

09/1991 - 06/1995 Sekundare Schule Umraniye Anadolu Lisesi, Istanbul-Türkei

09/1995 - 06/1998 Gymnasium Umraniye Anadolu Lisesi, Istanbul-Türkei

STUDIUM

09/1998 - 06/2002 Mittel Ost Technische Universität, Ankara-Türkei

Bachelor Studiengang

Fachgebiet: Biologie

Neben fach: Chemie

09/2002 - 08/2004 Sabanci Universität, Istanbul-Türkei

Master Studiengang

Fachgebiet: Biologische Naturwissenschaft und Bioengineering

Thema: A Novel AP2 Domain Transcription Factor from *Lycopersicon esculentum* Functions in Tobacco Mosaic Virus Pathogenesis

10/2004 - 11/2008 Universität zu Köln, Köln

Promotionsarbeit, Botanisches Institut III, Prof. Martin Hülskamp

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