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CHARACTERIZATION OF PROTEIN-PROTEIN INTERACTIONS INVOLVED IN AUXIN SIGNALING PATHWAY IN TOMATO

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Résumé

La croissance et le développement des plantes sont fortement régulés par plusieurs hormones végétales, dont l'auxine qui joue un rôle prépondérant. La modification de l'expression de certains gènes en réponse à l'auxine est contrôlée par des interactions spécifiques entre les facteurs de transcription ARF (Auxin Response Factors) et les protéines Aux/IAA. Des études sur *Arabidopsis thaliana* ont aussi montré l'implication de corépresseurs de la famille TOPLESS pour réprimer les gènes cibles des ARF. Toutefois, cette régulation transcriptionnelle a surtout été caractérisée chez la plante modèle Arabidopsis et la validité de ce modèle n'a pas encore été confortée par l'étude d'autres modèles. La tomate (*Solanum lycopersicon*), espèce modèle tant pour les Solanacées que pour les plantes à fruits constitue une bonne alternative pour élucider les caractères généraux liés à la signalisation auxinique. Dans notre travail, nous avons d'abord mis en place des protocoles expérimentaux – double-hybride, *pull-down*, complémentation de fluorescence (BiFC, Bifluorescence Complementation) – permettant d'étudier les interactions protéines-protéines. Ces méthodes ont d'abord été validées sur des couples Aux/IAA – ARF étant connus chez la tomate pour leur implication dans le développement et la maturation des fruits (SlIAA9, SlARF8, SlIAA3, SlARF4, SlIAA27). L'utilisation du double hybride a également permis de construire une carte d'interactions entre les Aux/IAA et les ARF de tomate. Dans un deuxième temps, la disponibilité de la séquence du génome de la tomate a permis d'entreprendre une étude globale de la famille des corépresseurs TOPLESS. Cette étude a inclus : la caractérisation et le clonage des gènes, l'analyse de la séquence protéique, une analyse phylogénétique de la famille sur un ensemble de génome séquencés, la caractérisation du profil d'expression des différentes isoformes ainsi qu'une analyse comparative de leur capacité d'interaction avec les protéines Aux/IAA. Enfin, dans un dernier temps, nous avons souhaité construire des premiers outils permettant d'entreprendre une recherche non-ciblée de nouveaux partenaires interagissant avec les protéines ARF ou Aux/IAA en partant de protoplastes de cellules BY-2 de tabac exprimant de façon transitoire des gènes codant des protéines chimères (*tagged*

proteins). Même si ce travail reste préliminaire, il a pu notamment illustrer l'importance de l'intégrité des noyaux pour la stabilité des Aux/IAA, même en l'absence d'auxine.

Abstract

The plant hormone auxin plays a central role in plant growth and development. The specific Aux/IAAs and Auxin Response Factors (ARFs) interactions are involved in auxin signaling pathway to regulate the auxin-responsive gene expression. Studies in *Arabidopsis* showed that TOPLESS family (TPLs) also was recruited by some Aux/IAAs to repress the function of ARFs. The whole machinery of the auxin signaling pathway is not clear yet, and most of this knowledge comes from the research on Arabidopsis. As a reference for Solanaceae and fleshy fruit plant, tomato (*Solanum lycopersicon*) is a good alternative model to better understand general traits of the auxin regulation process. In our work, we first established in our labs three experimental protocols – Yeast two-Hybrid, Pull-down and Bifluorescence complementation to unravel protein-protein interactions. These methods were first challenged on specific Aux/IAA and ARF proteins that were already characterized as major actors in fruit tomato development or ripening (SlIAA9, SlARF8, SlIAA3, SlARF4, SlIAA27). This also enabled us to build an ARF-Aux/IAA interaction map. In a second part, taking advantage of the tomato genome sequence, we carried a whole-genome study on tomato TOPLESS family. This investigation included gene cloning and characterization, protein sequence analysis, phylogenetic analyses, expression pattern and construction of protein-protein interaction maps. In a last part, we developed tools to start a non-targeted approach aiming at identifying new potential partners or protein complex involved in auxin signaling pathway using BY-2 tobacco cell protoplasts transiently expressing tagged-proteins. Although this study is still preliminary, it demonstrated the importance of nucleus integrity for Aux/IAA stability even in absence of auxin.

摘 要

植物生长素激素在植物的生长和发育过程中扮演着非常重要的角色。一些特定的生长素响应蛋白 IAA 与生长素响应因子 ARF 的相互作用在生长素信号途径中起了关键的作用，这些相互作用调控着下游生长素响应基因的表达情况。越来越多关于拟南芥的研究表明，一些 IAA 蛋白可以通过招募 TOPLESS 家族蛋白的互作方式共同抑制 ARF 蛋白的活性。很多关于生长素信号传导途径的认识主要来自于对拟南芥植物的研究工作，但具体的生长素信号传导机制到目前还没有完全清楚。为了更好的弄清楚生长素调控的机制，特别是对于其它植物例如果实的成熟调控研究，番茄这种茄科属类又能提供新鲜果实的植物是一个很好的研究的对象。在我们的工作里，我们首先为实验室建立起了三种有效的研究蛋白质互作的方法：Yeast 酵母双杂交、Pull-down 实验、双分子荧光互补技术。这些方法首先被用来检测一些已经报道过的与番茄果实发育相关的蛋白之间的互作情况，例如 IAA9、ARF8、IAA3、ARF4 和 IAA27。随后，关于整个番茄 IAA 蛋白家族与 ARF 蛋白家族之间的互作也得到了相应的检测。论文第二部分，得益于基因组测序的研究进展，我们展开了对番茄 TOPLESS 家族系统全面的研究。研究内容包括它的基因信息、蛋白信息、进化分析、表达模式分析以及蛋白质互作分析等。论文最后部分工作主要是依据 BY-2 烟草原生质体瞬时表达系统，通过表达已知的标签蛋白，探寻和获取生长素信号传导过程中未知和潜在的蛋白复合体。尽管最后这项工作只是一个开端，但它已经暗示了 IAA 家族蛋白只能在完整的细胞核中保持稳定全长。

Publications

Articles:

Yanwei Hao*, **Xinyu Wang***, Xian Li, Carole Bassa, Isabelle Mila, Corinne Audran, Elie Maza, Zhengguo Li, Mondher Bouzayen, Benoit van der Rest and Mohamed Zouine. **Genome-wide Identification, phylogenetic analysis, expression profiling and protein-protein interaction properties of the *TOPLESS* gene family members in tomato.** J. Exp. Bot., paper accepted, in press. (* First co-authors)

Zhiqiang Xian, Yingwu Yang, Wei Huang, Ning Tang, **Xinyu Wang** and Zhengguo Li. **Molecular cloning and characterization of SlAGO family in tomato.** BMC Plant Biology 2013 Sep. 13:126

Deng W, Yang Y, Ren Z, Audran-Delalande C, Mila I, **Wang X**, Song H, Hu Y, Bouzayen M, Li Z. **The tomato SlIAA15 is involved in trichome formation and axillary shoot development.** New Phytol. 2012 Apr. 194(2): 379-90.

Poster presentation:

Bassa Carole, Delalande Corinne, van der Rest Benoît, **Wang XinYu**, Buscaille Pierre, Bouzayen Mondher. **Sl-IAA27 protein is involved in tomato root and fruit development and in leak chlorophyll content.** Jacques Monod Conference on „Evolution and plant developmental regulatory mechanisms". April-30-May 4th 2011, Roscoff, France

Xinyu Wang, Yanwei Hao, Isabelle Mila, Carole Bassa, Corinne Audran-Delalande, Mohamed Zouine, Benoit Van DER REST and Mondher Bouzayen. **Identification and Characterization of the TOPLESS Gene Family in Tomato.** 06/11/2012. Grand Auditorium-UPS-Toulouse, France.

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

3-AT: 3-Amino-1,2,4-triazole

A. thaliana: Arabidopsis thaliana

AA: Amino acid

ABA: Abscisic acid

ABP1: Auxin binding protein 1

AD: Activation domain

AFB: AUXIN RECEPTOR F-BOX

ARF: Auxin response factors

ASP: Asparaginases

At-: Arabidopsis thaliana

AuxREs: Auxin response elements

Aux/IAA : Auxin/Indole-3-Acetic Acid

BD : Binding domain

BiFC: Bimolecular fluorescence complementation

bp : Base pair

B. rapa: Brassica rapa

BSA: Bovine Serum Albumin

CaMV: Cauliflower Mosaic Virus

cDNA: Complementary deoxyribonucleic acid

CDS: Coding sequence

CTD: C-terminal dimerization domain

CTLH: C-terminal to Lissencephaly homology domain

DBD: DNA binding domains

dNTP: Deoxyribonucletides

EAR: Ethylene-responsive element binding factor-associated Amphiphilic Repression

EDTA: Ethylenediamine tetraacetic acid

ER: Endoplasmic Reticulum

ERF: Ethylene response factor

GA: Gibberellic acid or gibberellin

GFP: Green Fluorescent Protein

G. max: Glycine max

HDAC: Histone deacetylase

IAA: Indole-3-acetic acid

IFs: Transcriptional factors

JA: Jasmonic acid

LisH: Lyssencephaly homology domain

LR: Lateral root

LUG/LUH: LEUNIG/LEUNIG HOMOLOG

M. guttatus: Mimulus guttatus

MR: Middle region

NAA: α-Naphthalene acetic acid

N. benthamiana: Nicotiana benthamiana

NLS: Nuclear localization signal

ORF: Open reading Frame

O. sativa: Oryza sativa

PCR: Polymerase chain reaction

P. patens: Physcomitrella patens

PPIs: Protein-protein interactions

P. trichocarpa: Populus trichocarpa

qRT-PCR: Quantitative reverse transcription Polymerase chain reaction

RD : Repression domain

S. bicolor: Sorghum bicolor

SCF: SKP1-Cullin-F-box

SFLC: Split firefly luciferase complementation

S. lycopersicum: Solanum lycopersicum

S. moellendorffii: Selaginella moellendorffii

S. tuberosum: Solanum tuberosum

Taq: Thermus aquaticus DNA polymerase

TIR1: Transport Inhibitor Resistant 1

TL: Yeast selection medium without Trp and Leu

TLH: Yeast selection medium without Trp, Leu and His

TLHA: Yeast selection medium without Trp, Leu, His and Ade

TPL: Topless

TPR: Topless-related

Tris: Tris (hydroxymethyl) aminomethane

V. vinifera: Vitis vinifera

WT: Wild-type

Y2H: Yeast two-hybrid

YFP: Yellow Fluorescent Protein

Z. mays: Zea mays

Content

General introduction of the thesis

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The phytohormone auxin affects many plant development processes, from cell level to tissue level, which includes cell division, elongation, rhizogenesis, apical dominance and organ patterning. Auxin rapidly alters the expression of hundreds of genes within minutes (Chapman and Estelle 2009; Goda et al. 2008). *Aux/IAAs* gene family comprises some of the most important early auxin-inducible genes. After the perception of auxin in the nucleus, the proteins of Aux/IAA family, Auxin Response Factor family (ARFs) and Topless family (TPLs) play key roles in the auxin signaling pathway: ARF being transcription factors that activate or repress the auxin-responsive gene expression, Aux/IAA repressing the activity of ARF and having its turnover controlled by the presence or absence of auxin (Guilfoyle and Hagen 2007; Szemenyei et al. 2008). More over, studies in Arabidopsis showed that TOPLESS family (TPLs) is also recruited by some specific Aux/IAAs to repress the function of ARFs (Szemenyei et al. 2008; Li et al. 2011a; Causier et al. 2012). This emerging model of auxin signaling pathway is not completely deciphered, and most of its knowledge comes from the research on Arabidopsis, which is not a suitable model to analyze fruit development. To understand better the auxin regulation process on other plants, the tomato which is a reference species for *Solanaceae* and fleshy fruit plants constitutes a good alternative model of study.

In our lab, Sl-IAA and Sl-ARF families in tomato were characterized and the result was published recently (Audran-Delalande et al. 2012, Fu et al. *in preparation*). As protein-protein interactions between members of Aux/IAA, ARF, or TOPLESS multigenic families are believed to be crucial for auxin mediation, the work described in my PhD thesis aimed at elucidating the specificities of interactions between different isoforms. In addition, we also developed tools to explore the existence of some new potential partners or protein complex involved in auxin signaling pathway in tomato.

The Chapter I gives a general review about the studies of auxin regulation machinery. Most of the protein components involved in auxin regulation during auxin synthesis, transportation, perception and signaling regulation will be introduced in this Chapter. Chapter

II focuses on the development of protein-protein interactions studies that were applied in the frame of Sl-IAAs and Sl-ARFs families. Several approaches were combined – yeast two-hybrid (Y2H), pull-down, bimolecular fluorescence complementation (BiFC) in order to revisit the Aux/IAA-ARF interaction map or to focus on more specific pairs of partners that were studied in the lab and that are known to be involved in fruit development or ripening. Chapter III is dedicated to molecular and functional characterization of six *Sl-TPL* genes. Our analysis focused on the identification, evolutionary relationships and expression patterns of each member of the tomato TPL family. Moreover, we used yeast two-hybrid approach to establish the framework of TPL/IAA and TPL/ARF protein-protein interactions. These results will provide a framework for further studies to better understand the potential functions of TPL proteins in tomato plants, especially during the flower and fruit development.

At last, since Aux/IAA and ARFs act both upstream (as an element of auxin perception) and downstream (as members of the activation and/or repression complexes) in the auxin perception cascade, we considered the development of non-targeted approaches to fish new proteins involved in auxin signaling which are detailed in chapter IV. Indeed, in Eukaryotes, transcription regulating complexes involve a large set of proteins that typically comprises TRANSPORTER INHIBITOR RESPONSE 1 (TIR1)/AUXIN-SIGNALING F-BOX (AFB), AUXIN/INDOLE ACETIC ACID (Aux/IAA), AUXIN RESPONSE FACTOR (ARF), and TOPLESS (TPL)/TPL-RELATED (TPR) (Lee et al. 2013). It is likely that in auxin signaling, either Aux/IAA, ARF or TOPLESS interact with several partners among the transcription machinery complexes. This led us to the *in vivo* production of specific Sl-IAAs and Sl-ARFs proteins fused with GFP-tag (IAA3-GFP, IAA9-GFP and ARF8a-GFP). This approach, although remaining incomplete, opens the perspective to give us more useful information and to discover the existence of other protein-protein interactions involved in plant signaling.

Chapter I: Bibliographic review

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1. Auxin in plant

1.1 Roles of auxin

In 1880, a plant substance that enables the modulation of plant shoot elongation and allows tropic growth toward light was observed by Charles Darwin. It was the first observation of auxin regulation of plant development (Darwin 1880). This substance had been isolated and called auxin (Thimann 1977). Several molecules displaying auxin activity, such as indole-3-butyric (IBA), 4-Chloroindole-3-acetic (4-Cl-IAA) and indole-3-propionic acid (IPA) were discovered latter. Nevertheless, the major form in plants is indole-3-acetic (IAA) (Ozga et al. 2002; Van Huizen et al. 1997; Woodward and Bartel 2005, Zolman et al. 2000).

Indole-3-acetic acid is a small tryptophan-derived phytohormone that regulates many plant growth and developmental processes including embryogenesis (Hamann et al. 2002; Weijers et al. 2006), tropic growth (Holland et al. 2009), leaf formation (Scarpella et al. 2010), stem elongation (Vernoux et al. 2010), root elongation (Overvoorde et al. 2010), and fruit development (Sundberg and Ostergaard 2009). The plasticity of plant development and its responsiveness to a multitude of environmental situations suggest that regulatory mechanisms are very complex. Indeed auxin starvation of cells leads to the arrest of cell division which can be restored by application of auxin (Stals and Inze 2001; Inze and De Veylder 2006). Inactivation of auxin perception pathway leads to cell-cycle arrest (David et al. 2007) or displays aberrant cell division in the suspensor during embryo development (Chen et al. 2001a). Auxin was also shown to induce rapid cell elongation in stem and hypocotyl segments (Rayle and Cleland 1992), to increase expansion of some leaf tissues and to display altered cell expansion (Chen et al. 2001b). Study of root growth in response to different auxin concentration treatment showed that low auxin concentration stimulates root growth but high

level of auxin plays an inhibitor for root elongation (Mulkey et al. 1982; Golaz and Pilet 1987). Gradient of auxin transporter distribution in the root strongly regulates the root elongation. The root development, notably the lateral root (LR) initiation, has also been a large object of study (Friml et al. 2002; Krecek et al. 2009; Dubrovsky et al. 2001; Dubrovsky et al. 2009). The presence of auxin is essential for organ initiation (Heisler and Jonsson 2007) and the formation of lateral leaves (Reinhardt et al. 2000; Reinhardt et al. 2003). Auxin also regulates floral meristems which controls floral organs formation and spatial distribution (Cheng and Zhao 2007).

1.2 Multiple proteins involved in auxin metabolism and mediation

Auxin synthesis

The whole auxin regulation mechanism in plant involves several programs: auxin metabolism (biosynthesis, production of auxin-conjugates and catabolism), auxin transport, auxin perception and auxin signaling pathway.

In Arabidopsis, analysis revealed that different pathways of auxin biosynthesis involve a key amino acid: the tryptophan (Trp) (Bartel 1997). Recent studies showed Trp dependent and independent pathways both contribute to IAA biosynthesis in shoots but only Trp dependent way is predominant in roots (Kiyohara et al. 2011; Ehlert et al. 2008). Auxin can be produced both in shoot and root (Cheng et al. 2006; Stepanova et al. 2008; Petersson et al. 2009). The localization of auxin synthesis plays a role in the regulation of auxin function, such as to modulate gradient directed polarity in root hair development in Arabidopsis (Ikeda et al. 2009). The environment and development signal also could change the auxin synthesis behavior (Tao et al. 2008).

Auxin transport

After auxin synthesis, it will be transported to be present in other tissues in plant. Auxin is transported actively in a polar way from one part to another (Blakeslee et al. 2005), that is essential for the initiation and maintenance of polarized plant growth (Carrier et al. 2008;

Wang et al. 2011). Auxin transport needs carriers (Blakeslee et al. 2005; Carrier et al. 2008). Decades of studies revealed that the auxin transport carriers comprise AUXIN RESISTANT 1/LIKE AUX1 (AUX/LAX), PINFORMED (PIN), PIN-LIKES (PILS) and ATP-binding cassette transporters/multi-drug resistance/P-glycoprotein (ABCB/PGP) (Bennett et al. 1996 ; Palme and Galweiler 1999 ; Noh et al. 2001).

The AUXIN BINDING PROTEIN 1 signaling pathway

For auxin perception and signaling pathway, specific binding proteins and interactions between these proteins are needed. The first auxin binding protein described was called AUXIN BINDING PROTEIN 1 (ABP1) (Hertel et al. 1972; Lobler and Klambt 1985). It is a small glycoprotein on the outer leaflet of the Endoplasmic Reticulum (ER) membranes and in the plasma membrane (Napier et al. 2002). Loss of ABP1 function confers embryonic arrest, associated with misorientation of cell division planes and defects in cell elongation leads to the abortion of the plant embryo (Chen et al. 2001a). On the other hand, overexpression of ABP1 causes an auxin-dependent expansion in the size of differentiated cells that are normally nonresponsive (Jones et al. 1998). Repression of ABP1 through cellular immunization via an antibody-deriving polypeptide blocking ABP1 demonstrated its critical role in cellular division and expansion (David et al. 2007; Braun et al. 2008). More recently, different authors elucidated some mechanisms involved in the ABP1-auxin mediation pathway. First, it was found that ABP1 could bind and activate calcium permeable ion channels (Shishova and Lindberg 2010). Moreover, analysis of heterozygous mutants showed an inhibition of auxin transporters PIN1 triggered by auxin-binding on ABP1 (Robert et al. 2010; Effendi et al. 2011). At last, auxin-binding on ABP1 regulates the activity of two GTPases ROP2 and ROP6 (Sauer & Kleine-Vehn, 2011).

The TIR1 signaling pathway

In 2005, Transport Inhibitor Resistant 1 (TIR1) protein was confirmed to function as an auxin receptor (Dharmasiri et al. 2005; Kepinski and Leyser 2005). TIR1 is part of F-box protein family and forms SCF protein complex (SKP1-Cullin-F-box), implying that regulation

of protein stability is a crucial part of auxin signaling (Gardozo and Pageno 2004). TIR1 binds auxin at physiologically relevant concentrations (Kd ~20–80 nM), and this binding of auxin with TIR1 is required to stimulate the interaction between TIR1 and Aux/IAAs (Dharmasiri et al. 2005a; Kepinski and Leyser 2005). This interaction with auxin does not change the shape of its receptor to modulate its activity (Dharmasiri et al. 2005a). SCF complex is related to the auxin signaling pathway which involves more complex proteins such as Aux/IAA family (IAAs), Auxin Response Factor family (ARFs) and Topless family (TPL). We will introduce these proteins family in following parts, and present the recent studies about how these proteins work and interact with each others.

2. Aux/IAA, ARF and TOPLESS families as key players in TIR1-mediated signaling

During plant growth and development, auxin perception occurs in nucleus to regulate downstream gene expression. The interactions between Aux/IAA family (IAAs), Auxin Response Factor family (ARFs) and Topless family (TPLs) play key roles in this auxin signaling pathway to control the target gene expression. The TPLs function as corepressors by interacting with Aux/IAAs, and the specific interactions of Aux/IAAs and ARFs proteins are involved in auxin signaling pathway to regulate the auxin-responsive gene expression (Guilfoyle and Hagen 2007; Szemenyei et al. 2008).

2.1 Aux/IAAs family

In plant, auxin perception and signaling involve several components, among which auxin/indole-3-acetic acid (Aux/IAA) proteins play a pivotal role in the auxin signaling pathway. Biochemical and genetic studies indicated that Aux/IAA generally function as transcriptional repressors of auxin-regulated genes expression (Tiwari et al. 2001; Tiwari et al. 2004). This repression is notably revealed by the use of cells or protoplasts transiently expressing a *GFP* reporter gene under the control of the DR5 Auxin inducible promoter

(Tiwari et al. 2004). The *Aux/IAA* genes are a class of primary auxin-responsive genes which could be rapidly induced by auxin (Theologis et al. 1985; Oeller et al. 1993; Yamamoto and Yamamoto 1998). The function of Aux/IAA proteins has first been investigated through the characterization of gain-of-functions mutants with auxin-linked phenotypes. A mutation in At-*IAA14* alters hypocotyl gravitropic response and the mutant does not display lateral root formation any longer (Fukaki et al. 2002). The At-iaa19 mutant displays similar phenotypes with altered hypocotyl gravitropism and lateral root formation (Tatematsu et al. 2004). The At-iaa28 mutant displays a reduced apical dominance with short inflorescence stems, but the root phenotype is similar to At-iaa18 mutant with a reduced lateral root formation (Rogg et al. 2001). More recently, a study showed that *At-IAA17* can inhibit the timing of floral transition under short days light conditions (Mai et al. 2011). Strikingly, while gain-of-functions mutants were essential for the elucidation of Aux/IAA function, few phenotypes were obtained through loss-of-function Arabidopsis mutants, thus suggesting an important functional redundancy among Aux/IAA family members (Overvoorde *et al*. 2005).

Aux/IAA proteins are direct targets of TIR1 through the interaction of Aux/IAA domain II and TIR which is possible mediating in the presence of auxin (Dharmasiri et al. 2005a; Dharmasiri et al. 2005b; Kepinski and Leyser 2005; Tan et al. 2007). Mutation of Aux/IAA domain II stabilizes the IAA protein in absence or presence of auxin (Dreher et al. 2006). The binding of auxin and TIR1 will lead to the degradation of Aux/IAA proteins, then the release of auxin response factors (ARFs) which could regulate downstream gene expression. Studies showed Aux/IAA are short-lived and nuclear-localized proteins (Hagen and Guilfoyle 2002; Liscum and Reed 2002).

Aux/IAA genes belong to a large gene family observed in all land plant (Kalluri et al. 2007; Wang et al. 2010a). There are 29 members of this genes family in Arabidopsis, 31 members in rice and maize (Liscum and Reed 2002; Jain et al. 2006; Wang et al. 2010b). In recent studies in our lab, structures and functional characterization of the tomato *Aux/IAA* genes were carried out. There are 25 members in tomato *Aux/IAA* gene family (Audran-Delalande et al. 2012). Canonical Aux/IAA proteins have four conserved amino acid sequence motifs which are called domain I, II, III and IV. Some of the proteins lacking one or

more of these domains are also included in this family (Fig. 1). Each domain plays different role in the auxin signaling pathway, and they are related to the interaction with other transcriptional protein factors. This interaction relationship will be introduced in another part (Chapter I, 3).

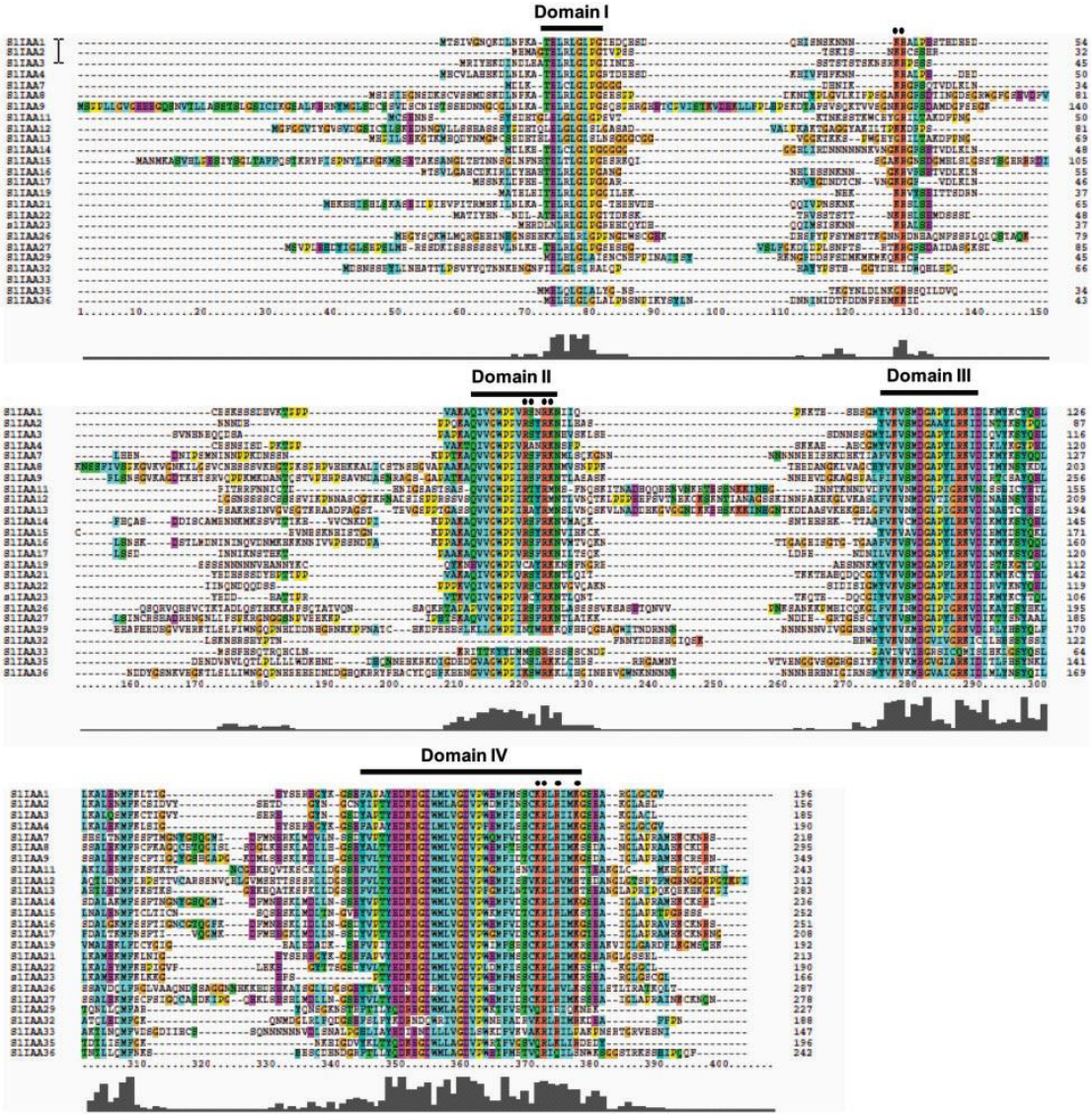


Fig. 1 Multiple sequence alignment of the full-length Sl-IAA proteins. Alignment was obtained with ClustalX and manual correction. Conserved domains of Aux/IAA proteins are underlined. From Audran-Delalande *et al*. 2012.

2.2 ARFs family

In the auxin signaling pathway, Auxin response factors (ARF) family functions by

activating or repressing auxin response genes. ARFs are transcription factors which could bind to TGTCTC auxin response elements (AuxREs) found in promoters of early auxin response genes, such as *GH3s*, *SAURs* and *Aux/IAA* family genes (Ulmasov et al, 1999). Numerous studies revealed that ARF family genes play a pivotal role in plant developmental processes, such as apical hook formation (Li et al, 2004), embryo patterning (Rademacher et al, 2012), lateral root growth (Marin et al, 2010), leaf expansion and senescence (Lim et al, 2010), floral organ abscission and petal growth (Ellis et al, 2005; Varaud et al, 2011), fruit set and development (Goetz et al, 2007; Goetz et al, 2006; Guillon et al, 2008) as well as various responses to environmental stimuli. ARF family genes are also involved in the hormone cross-talk between auxin and ethylene (Li et al, 2006), auxin and gibberellins (de Jong et al, 2011) and also auxin and ABA (Liu et al, 2011).

Since the cloning of the first *At-ARF1* gene from Arabidopsis, 23 other members of this family from Arabidopsis, 25 from rice, and 31 from maize have been identified (Ulmasov et al. 1997; Hagen and Guilfoyle 2002; Okushima et al. 2005; Xing et al, 2011; Wang et al. 2007). More recently, 22 ARF genes have also been identified from tomato (*Solanum lycopersicum*) genome comparing with *At-ARF* genes (Kumar et al, 2011; Wu et al, 2011).

A typical ARF protein contains four protein sequence parts: an N-terminal DNA binding domain (DBD), a middle region (MR) that functions as activation domain (AD) or repression domain (RD), and two carboxy-terminal Aux/IAA domain (CTD) (Fig. 2) (Guilfoyle and Hagen, 2001). The ARF DBD domain is identified to bind to TGTCTC Auxin response elements (AuxREs) on the promoter of auxin regulated gene (Ulmasov et al, 1999b). The ARF MRs is located between the DBD domain and CTD domains. The ARF ADs are rich in glutamine (Q), serine (S), and leucine (L) residues while ARF RDs are rich in proline (P), serine (S), threonine (T), and glycine (G) residues (Guilfoyle et al. 1998; Ulmasov et al. 1999a, Ouellet et al. 2001; Tiwari et al. 2003). The ARF C-terminal Aux/IAA domains (CTD) are also found in Aux/IAA proteins which are called domain III and IV. These domains are known to contribute to formation of either ARF/ARF homo- and hetero-dimers or ARF/Aux-IAA hetero-dimers (Guilfoyle and Hagen 2001, 2007).

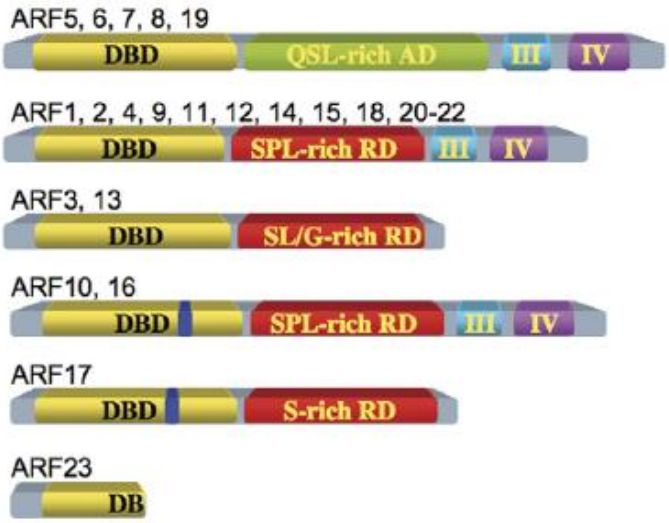


Fig. 2 The classification and structures of the ARF protein family in Arabidopsis (From Guifoyle and Hagen, 2007).

2.3 TPLs family

In plants, two major families of transcriptional factors (TFs) co-repressors of the GROUCHO family have been identified: LEUNIG/LEUNIG_HOMOLOG (LUG/LUH) and the TOPLESS/TOPLESS-RELATED (TPL/TPR) groups (Liu and Karmarkar 2008). In my PhD study, we focused on the TPL/TPR family. TPL/TPRs co-repressors are recruited by transcription factors that can repress or activate target genes expression or switch between repression and activation. Recent studies revealed that TPL/TPRs have a range of functions in plant, including roles in embryo development (Long et al. 2002), plant immunity (Zhu et al. 2010), meristem fate (Kieffer et al. 2006; Gallavotti et al. 2010), auxin and jasmonic acid signaling (Szemenyei et al. 2008; Pauwels et al. 2010).

There are five members in Arabidopsis TPL/TPR family: TPL and TPR1 to 4, they were first described as direct interactors of the Arabidopsis homeodomain transcription factor WUSCHEL (WUS) (Kieffer et al., 2006). WUS is expressed in the organizing center at the base of the shoot apical meristem, where it signals to the overlying stem cells to maintain their meristematic fate as part of a well-studied feedback loop that controls meristem homeostasis (Laux et al. 1996; Brand et al., 2000; Schoof et al., 2000; Sablowski, 2007). Canonical TPL/TPR proteins have several conserved amino acid sequence motifs: N-terminal

region of TPL/TPR which contains a Lissencephaly (LisH) domain as well as a "C-terminal to LiSH" (CTLH) domain, and the C-terminal containing two WD40-repeat domains (Kieffer et al., 2006). The LisH domains have been shown to promote protein-protein interaction (Cerna and Wilson, 2005). Studies revealed that TPL/TPR proteins interact almost exclusively with transcription factors (TFs), such as IAAs and ARFs, many of which have previously been implicated in transcriptional repression (Causier et al. 2012).

3. Protein-protein interactions (PPIs) in auxin signaling

The elucidation of TIR1 function and the establishment of a link between auxin perception and transcription regulation enabled the construction of a new model of auxin signaling which represented a considerable change of paradigm (Lee et al. 2013). In this model, the protein-protein interactions between Aux/IAA, ARF and TOPLESS appeared as major actors.

3.1 Model of auxin signaling pathway in the nucleus

The similar conserved domains III and IV present in IAAs and ARFs proteins allow the formation of Aux/IAA-ARF heterodimers. Auxin signaling is mainly regulated by these interactions between Aux/IAA and ARF proteins (Fig. 3). When the auxin concentration is low, Aux/IAAs can interact with ARFs to inactivate them by recruiting the TPL corepressor. Therefore in the absence of auxin, ARFs does not activate the transcription of their target genes (Guilfoyle and Hagen 2007; Szemenyei et al. 2008). When auxin concentration is increased, auxin promotes the interaction between Aux/IAA proteins and SKP1-Cullin-F-box (SCF) complex through the binding to the auxin transport inhibitor response 1 (TIR1) or to its paralogs AUXIN RECEPTOR F-BOX (AFB) proteins. The association of Aux/IAAs to the SCF complex leads Aux/IAAs to become ubiquitinated and targeted for proteolysis (Dharmasiri et al. 2005a; Dharmasiri et al. 2005b; Kepinski and Leyser 2005; Leyser 2006; Tan et al. 2007; Chapman and Estelle 2009). The degradation of Aux/IAAs results in the

„release" of ARFs which can then activate the transcription of target genes via binding to the Auxin Responsive Elements (AuxRE) present in the promoter regions of auxin-regulated genes (Hagen et al. 1991; Ulmasov et al. 1997; Hagen and Guilfoyle 2002).

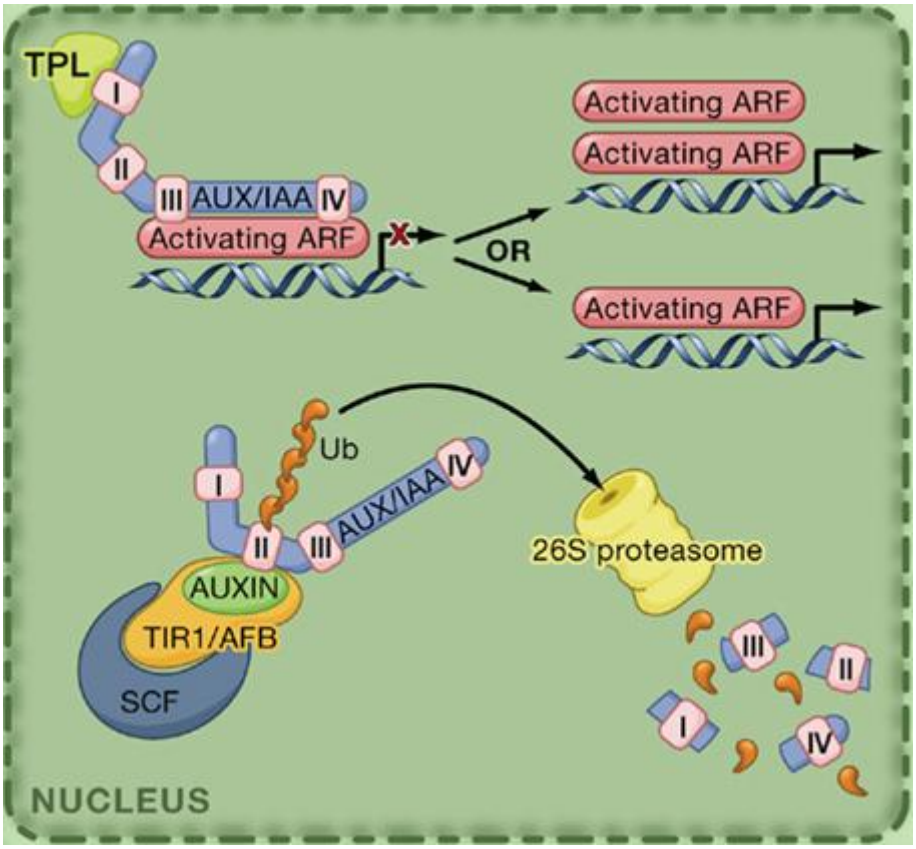


Fig. 3 Auxin signaling is mainly regulated by the interactions between Aux/IAA and ARF proteins (Vanneste and Friml 2009).

The protein ubiquitination machinery implies that regulation of protein stability is a crucial part of auxin signaling. In this scenario, Aux/IAAs are recruited by SCF/TIR1 and interact directly through their domain II with TIR1 in an auxin-dependent manner. They are then ubiquitinated and thus marked for degradation (Gray et al., 2001). It has been shown that auxin is the only regulatory element in this equation. TIR1 binds auxin at physiologically relevant concentrations (Kd ~20–80 nM), and auxin binding to TIR1 is required to stimulate the interaction between TIR1 and Aux/IAAs (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Interestingly, auxin does not change the conformation of TIR1 but acts as a "molecular glue" by filling a hydrophobic cavity at the interaction interface (Fig. 4), thereby enhancing

TIR1-Aux/IAA interactions (Tan et al., 2007). Moreover, there are at least five TIR1- related F box proteins (Auxin-Binding F box [AFB]1-5) also interacting in an auxin-dependent manner with Aux/IAAs (Dharmasiri et al., 2005b). The auxin signaling pathway looks very short and simple, but the number of genes involved at each level is large. Thus cells can have many different possible outputs of auxin signaling depending on the developmental context.

Fig. 4 Auxin does not change the conformation of TIR1 but acts as a "molecular glue" by filling a hydrophobic cavity at the interaction interface (Tan et al., 2007).

3.2 PPIs between IAAs, ARFs, and TPLs

The interactions of Aux/IAA, ARF and TPL play a central role in the transcriptional regulation of auxin signaling pathway. In Arabidopsis, Aux/IAA proteins (18 to 36 kD) contain 4 conserved domains: domain I, II, III and IV (Liscum and Reed 2002). Domain I of Aux/IAA contains a conserved Leu-rich (LxLxL) motif that is similar to the EAR (Ethylene-responsive element binding factor-associated Amphiphilic Repression) found in ERFs and several other transcriptional regulators (Tiwari et al. 2003; Kagale et al. 2010). Aux/IAA Domain II is responsible for the stability of Aux/IAA proteins (Dreher et al. 2006; Worley et al. 2000) and directly binds to the TIR1/AFB auxin receptors in an auxin-dependent manner. Domains III and IV of the Aux/IAA proteins shares a homology with the C-terminal domain (CTD) of ARF proteins. Through an interaction between domains III and IV and the CTD, Aux/IAA can form a heterodimer with ARF (Kim et al. 1997; Muto et al. 2006; Ulmasov et al. 1997b).

In Arabidopsis, protein-protein interactions between the 29 Aux/IAAs and 23 ARFs have been deeply investigated. Vernoux et al. (2011) performed a large scale analysis of the interaction between Aux/IAA and ARF using a yeast-two hybrid system. 433 interactions among the 1,225 tested combinations between Aux/IAAs and ARFs were revealed. These studies show that the majority of Aux/IAAs are able to interact with ARF activators while ARF repressors display very few interactions (Fig. 5). To date, the only ARF repressor which

is able to interact with different Aux/IAA genes is AtARF9. Another large-scale analysis of Aux/IAAs and ARFs were performed by split firefly luciferase complementation (SFLC) assay. That study also demonstrated there are specific pairs of interactions between Aux/IAAs and ARFs (Li et al. 2011b). These studies indicated that ARF repressors may more act as a competition for AuxRE element binding with ARF activators rather than be directly involved in auxin signaling through Aux/IAA regulation (Weijers et al. 2005; Vernoux et al. 2011). Moreover, the analysis of Aux/IAA and ARF mutants in Arabidopsis show putative interactions between these two protein families. Indeed, AtIAA12 could interact with ARF5, regulating embryonic root formation (Weijers et al. 2005) and the specific interaction between AtIAA14 and AtARF7 or AtARF19 is essential for the inactivation of lateral root formation (Fukaki et al. 2006). These studies suggest that specific interactions among the Aux/IAAs and ARFs define the gene expression profile during development and consequently determine the developmental specificity.

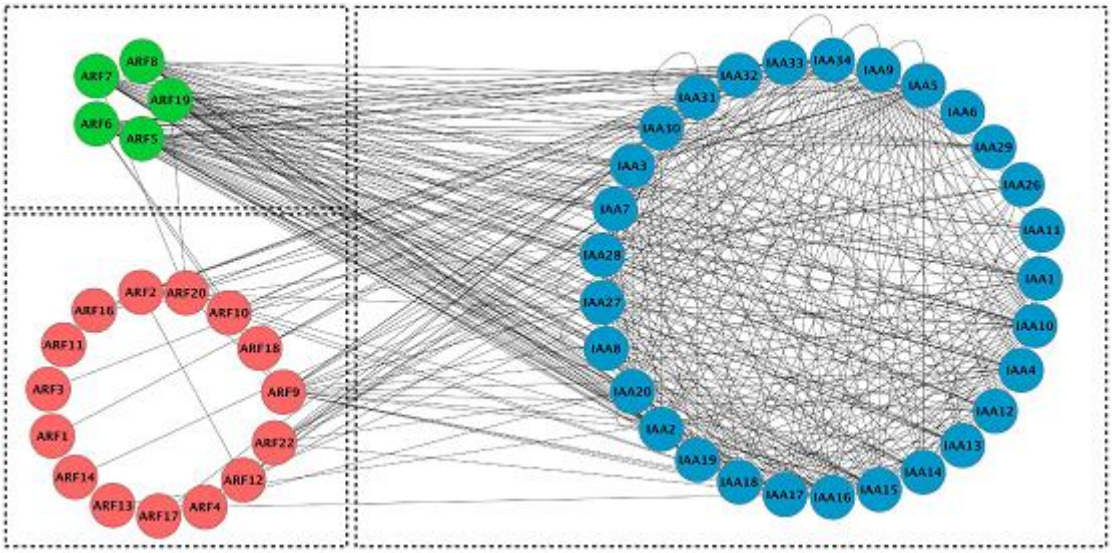


Fig. 5 The *Arabidopsis* ARF-Aux/IAA interaction map. In Arabidopsis, most of Aux/IAAs are able to interact with ARF activators while ARF repressors display very few interactions in Arabidopsis. (Vernoux *et al*. 2011)

Studies showed that domain I of Aux/IAA could interact with transcriptional co-repressors, which are called TOPLESS (TPL), to form a complex to repress the transcriptional function of ARF (Szemenyei et al. 2008). A mutation in the LxLxL motifs of

domain I of the stabilized Aux/IAA mutant repressors lead to the constitutive activation of ARF, resulting in a strong-auxin phenotype (Li et al. 2011a). This finding suggests that the LxLxL motif of domain I is essential for its repressor activity via the TPL interaction. More studies also proved the TPL proteins play the function of co-repressors, and suggested that Aux/IAA recruits TPL to strongly repress ARF activity (Szemenyei et al. 2008; Causier et al. 2012). Earlier studies (Szemenyei et al., 2008; Arabidopsis Interactome Mapping Consortium, 2011; Barry et al. 2012), identify 20 of the 29 Arabidopsis Aux/IAA proteins as interaction partners of the TPL/TPRs (Aux/IAA1, -2, -3, -4, -6, -7, -8, -9, -10, -11, -12, -13, -14, -16, -17, -18, -19, -26, -27, and -28). In addition, we have seen that ARFs repressing transcription do not interact with any Aux/IAA proteins. The repression mechanism has not been determined until now. But there is still another notable thing is that repressor-ARF proteins, such as ARF2 and ARF9, interact directly with TPL/TPR proteins (Causier et al. 2012a), suggesting a mechanism for repression and implicating TPL/TPR co-repressors in both forms of ARF-mediated repression (Fig. 6).

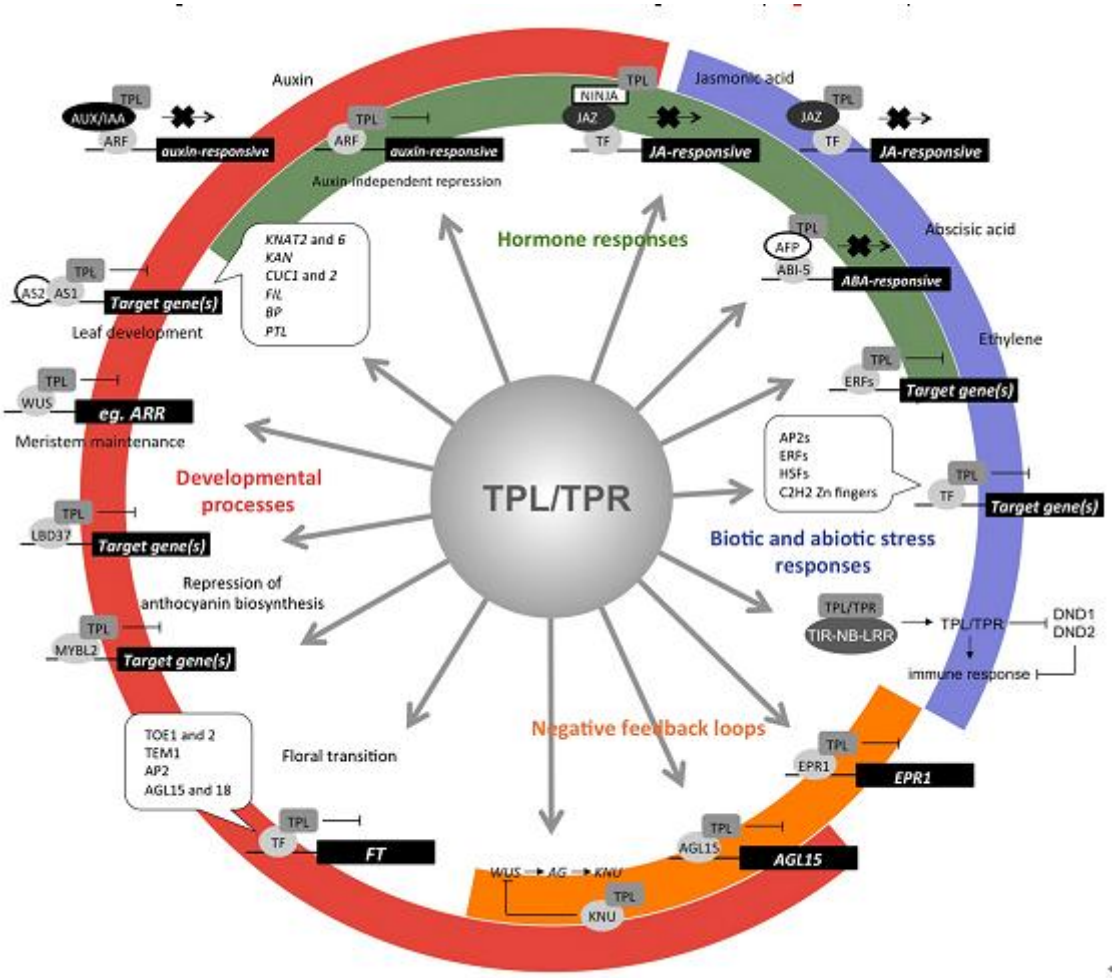


Fig. 6 Multiple involvement of TOPLESS proteins. The TPL family acts as general co-repressors in diverse biological pathways. The interactome data place the TPL/TPR family of co-repressors at the center of many biological processes (Causier et al. 2012a).

Up to now, there are still a lot of questions that need to be investigated about these major actors. Are there specific couples between tomato Aux/IAA, ARF and TPL protein families? Are some of them redundant? Do other potential or new proteins exist and be involved in the interaction map of tomato Aux/IAA, ARF and TPL? What is (are) the correct model(s) which could indicate the step-by-step interaction relationship of these major actors to switch on or off the target gene expression?

Chapter II: Protein-protein interactions (PPIs) between Sl-IAAs and Sl-ARFs family

Chapter II: Protein-protein interactions (PPIs) between Sl-IAAs and Sl-ARFs family

1. Introduction

1.1 General introduction

In the general introduction, we have seen that the Aux/IAA-ARF heterodimer formation allowed by the similar conserved domains III and IV present in IAAs and ARFs proteins establish a primary link between auxin perception and the regulation of the transcription by the different ARF transcription factors.

In Arabidopsis, protein-protein interactions between the 29 Aux/IAAs and 23 ARFs have been studied by high-throughput assays including yeast two-hybrid system (Y2H), bimolecular fluorescence complementation (BiFC) and split firefly luciferase complementation (SFLC) assay (Vernoux et al. 2011; Li et al. 2011b; Arase et al. 2012). In rice (Oryza sativa L.), interactions between 14 integrated Os-ARF and 15 Os-IAA proteins were tested using yeast two-hybrid system (ChenJia et al. 2010). Besides, pull-down assay and co-immunoprecipitation (Co-IP) were also mentioned in these articles to be complementary methods for those high-throughput screenings. The overview of these PPIs maps indicated Aux/IAAs preferentially interact with themselves and with the activator ARFs, but weakly with repressor ARFs. In contrast to the Y2H assays, the SFLC assay using the protoplast system gave rise to somewhat contradictory results. For example, ARF5 (an activator ARF) did not interact with IAA6 and IAA9 in the Y2H assay whereas ARF5 strongly interacted with them in the SFLC assay (Li et al. 2011b). ARF9 was the only repressor ARF that interacted with different Aux/IAAs in the Y2H assay, but it interacted only with IAA28 in the SFLC assay (Li et al. 2011b). Another study showed IAA8 was able to interact with ARF C-terminal domains in both Y2H and BiFC assays, but the two

methodologies display differences, for example concerning the capacity of IAA8 to interact with some repressor ARFs (Arase et al. 2012).

Contradictory information in ARF-Aux/IAA interactions revealed by different PPI methodologies could be due to the variation of ARF or Aux/IAA concentrations between a native molecular and cellular environment (*in planta*) and an *in vitro* environment (yeast cell, protoplast). Additional factors - cofactor, other protein - absent *in vitro* might also be involved in the ARFs-Aux/IAAs interactions in plant cells. It also reminds us that those potential high throughput methods have diverse merits and defects. Y2H, as heterologous systems, may lack plant co-factors or subcellular compartments necessary for specific PPIs, leading to false positive and negative results. Although BiFC allows the visualization of subcellular localizations of PPIs, but the external light source used in the BiFC assay will also excite the auto fluorescence of plant cells which could make the false positive result. And also, BiFC cannot reflect the dynamics of a given PPI in a real-time manner because of the irreversible reconstitution and slow maturation of the fluorescent protein. The pull-down assay can strongly confirm direct interaction between two proteins. Unless performed on plant tissue, it may also miss indirect interactions and new potential partners. Besides, pull-down assay is not suitable for a large scale PPIs analysis because of lot of time is needed to optimize the expression and extraction conditions for different proteins.

At the time the PhD work started, there was still no report about the interactions between the whole Sl-IAAs family and Sl-ARFs family and no comprehensive picture was yet provided in other species (Shen et al. 2010 and Vernoux et al. 2011 being published later). Early release of tomato genome sequences enabled the identification in tomato of 25 Sl-IAAs members and 23 Sl-ARFs members genome. Besides, several pairs of Sl-ARF and Sl-IAA were already under investigation in the GBF laboratory, notably the Sl-IAA9 and the Sl-ARF8 pair. Indeed, *in situ* hybridization experiments revealed that a tissue-specific gradient of IAA9 expression is established during flower development; upon pollination, this gradient is released and it triggers the initiation of fruit development (Wang *et al*. 2005, 2009). By contrast, the *Sl-ARF8* expression symmetrically increased just after pollination and the overexpression of Sl-ARF8 in transgenic tomatoes yield a parthenocarpic phenotype similar

to Sl-IAA9-antisense plants (Yang and Zouine, unpublished results, Fu PhD thesis, *in preparation*).

In order to identify specific interactions between Sl-IAAs and Sl-ARFs, and to better understand the potential mechanism of how these two families work together during the auxin signaling pathway, I started my PhD research on the identification of interactions for some specific Sl-IAAs and Sl-ARFs pairs by using different methods including yeast two-hybrid (Y2H), pull-down assay and Bimolecular fluorescence complementation (BiFC). Then we checked the whole family interaction map between each Sl-IAA and Sl-ARF member. Besides, some specific Sl-IAAs and Sl-ARFs have been chosen to make different deletions, and to see how the removal or modifications of special domains affect the interaction of Sl-IAAs and Sl-ARFs.

1.2 The aim of the work in Chapter II

These early steps of the PhD work also implied that we needed to establish some useful protocols for the protein-protein interactions research which were never used in our lab, such as: Yeast two-hybrid (Y2H), Pull-down assay and Bimolecular fluorescence complementation (BiFC). After being developed, these methods were used to (1): Find and confirm some specific interactions between Sl-IAA and Sl-ARF by using different protocols. (2) Describe the large scale protein-protein interactions map between Sl-IAA family and Sl-ARF family by Y2H assay and assess the specificity of the different isoforms. (3) Reveal the role of some canonical Sl-IAA domains which might be involved in the PPIs of Sl-IAA and Sl-ARF.

2. Materials and Methods

For protein-protein interactions (PPIs) assay, three methods were selected and optimized for the first time in our lab, including Yeast two-hybrid (Y2H), Pull-down assay and Bimolecular fluorescence complementation (BiFC). Some protocols for protein expression and extraction were also optimized in our experiment.

2.1 Protocol of yeast two-hybrid (Y2H)

In our study, Y2H was used to test many different protein-protein interactions. Here we would like to explain the principle and method of Y2H. This protocol mainly refers to the Y2H User Manual (Nontechnical).

Principle of the yeast two-hybrid assay

In Y2H system, a bait gene is expressed as a fusion to the GAL4 DNA-binding domain (BD-bait), while another gene or cDNA is expressed as a fusion to the GAL4 activation domain (AD-prey; Fields and Song, 1989; Chien et al. 1991). When bait and prey proteins interact, the BD and AD proteins are brought into proximity, thus activating transcription of reporter genes (Fig. 7). This technology can be used to identify novel protein interactions, confirm suspected interactions, and define interacting domains.

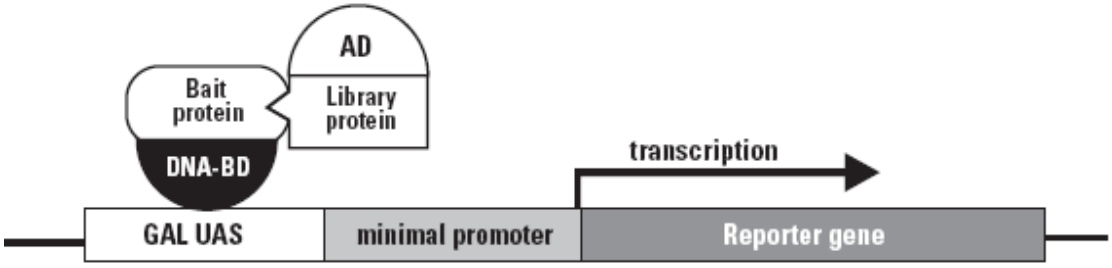


Fig. 7 Principle of the two-hybrid assay. When bait and prey proteins interact, the BD and AD proteins are brought into proximity, thus activating transcription of reporter genes.

Yeast strain and report gene

Yeast strain AH109 is auxotrophic for three amino acids (His, Leu, Trp) and adenine (Ade) and contains three reporters --- *ADE2*, *HIS3*, and *lacZ* --- under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes (Fig. 8). If the BD-bait and AD-prey proteins interact, the AD domain will activate the expression of the reporter genes and the strain will grow on a medium lacking adenine and histidine. The auxotrophy on leucine and tryptophan is used to select the yeast co-transformed with pGADT7 and pGBKT7 derived vectors (see below).

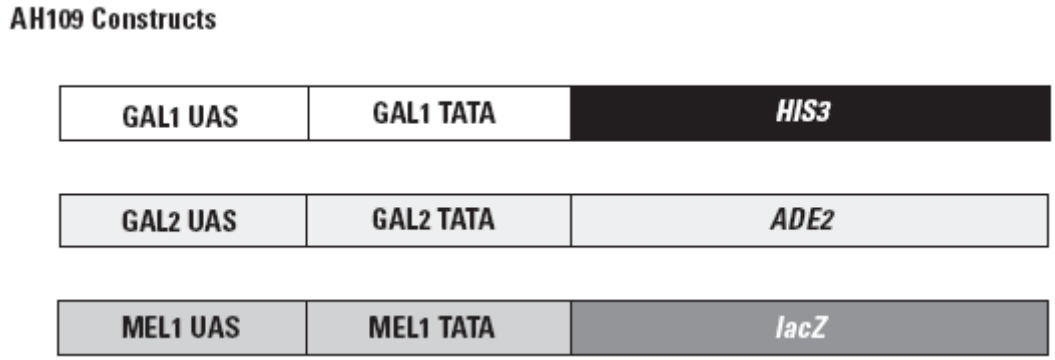


Fig. 8 AH109 reporter genes. Yeast strain AH109 contains three reporters --- *HIS3*, *ADE2*, and *lacZ* --- under the control of distinct upstream activating sequences (UASs) and TATA boxes.

Method of Y2H experiment

For Y2H experiments, coding sequences of bait and prey were introduced into pAD and pBD vectors, two plasmids deriving from the pGADT7 and pGBKT7 vectors (Clontech, Matchmaker™ GAL4 Two-hybrid system) in which an A*ttR* recombination site was introduced, thus allowing the use of Gateway® recombination technology (Invitrogen). Y2H vectors pAD and pBD modifications were performed by Dr Laurent Deslandes (LIPM CNRS-INRA Toulouse) and kindly provided by Dr Laurent Deslandes and Dr Hua Wang (LRSV Université Paul Sabatier).

Consequently, for bait and prey protein cDNA cloning, each template was firstly cloned into plasmid pDONR-207 (Invitrogen) using BP Clonase II (Invitrogen), and after cloned into the Y2H vector (BD and AD) by using LR Clonase II (Invitrogen). pBD Y2H vector (BD-bait) was used for the bait-protein construction. It contains a functional copy of the *Trp1* gene, thus restoring in AH109 tryptophan autotrophy. pAD Y2H vector (AD-prey) was used for the prey-protein construction. It contains a functional copy of the *Leu2* gene, thus restoring in AH109 leucine autotrophy. Then, the BD-bait and AD-prey were co-transformed into yeast AH109, co-transformants being selected on culture lacking both tryptophane and leucine. If there is no interaction between the bait and prey protein, yeast will grow only on the selection medium lacking Trp and Leu. If there was an interaction, yeast can grow on the selection medium lacking Trp, Leu, His and Ade. Technical details of yeast transformation protocol are

further developed in Appendix (A1) in this thesis.

For positive control of Y2H experiments, yeasts were co-transformed with the pBD-p53 (BD-p53) and the pAD-SV40T (AD-T7) vectors (Clontech).

2.2 Protocol of protein expression and extraction in *E. coli* strain

Before pull-down assay, some specific GST and His tagged proteins were expressed and extracted from *E. coli*-expression system.

Plasmid construction

For plasmid construction, the destination vector pDEST15 (Invitrogen) generating GST-fusion proteins was used for bait, and the pDEST17 (Invitrogen) vector generating poly-His tagged proteins was used for prey. For bait and prey protein cDNA cloning, first, the template was cloned into plasmid pDONR-207 (Invitrogen) using BP Clonase II (Invitrogen), and after cloned into the destination vector (pDEST15 and pDEST17) by using LR Clonase II (Invitrogen).

***E. coli* strain for protein expression**

Bait and prey proteins were then produced in *E. coli*. In order to optimize the expression condition for different proteins, three strains of *E.coli* were tested: the BL21-DE3-LysS strain (Invitrogen), the BL.21AI strain (Invitrogen) and the Rosetta Strain (Novagen), a derived BL21 strain designed to enhance the expression of eukaryotic proteins by containing codons rarely used in *E. coli*. These *E. coli* strains carry a chromosomal insertion of a cassette containing the T7 RNA polymerase (T7 RNAP) gene, allowing tagged-protein expression under the control of T7 promoter. For BL.21AI strain, the L-arabinose (final 0.2%) is needed to induce the protein expression. If a T7 expression vector containing the *lac*I gene is used (BL21-DE3, Rosetta), the IPTG also should be added at variable concentrations for induction.

Proteins expression and extraction

Recombinant protein production was optimized by playing on different conditions, such as: *E. coli* strain, culture temperature, induction time and rotating speed. Bacteria were lysed with a French pressure cell press in a 10% glycerol Tris/NaCl or PBS buffer and soluble and insoluble protein fractions were collected after centrifugation. More details of the protocol for protein expression and extraction could be checked in Appendix (A2) in this thesis.

2.3 Protocol for western blot

Protein samples are loaded on SDS-PAGE gel. After running the gel, proteins were transferred to Nitrocellulose membrane. Antibody dilution ratio was optimized for each antibody and protein (Table 1). The membrane is exposed with ECL-advance kit (GE Healthcare) by following the instruction book. More details of the protocol for western blot could be checked in Appendix (A3) in this thesis.

Table 1 The antibody dilution ratio for western blot assay.

Tagged-protein	Primary antibody*	Secondary antibody**
GST alone	1:20000	1:20000
GST-IAA3	1:20000	1:20000
GST-IAA9	1:20000	1:20000
His-ARF8a	1:5000	1:20000
HisARF8a-del	1:5000	1:20000

*Mouse monoclonal anti-GST/His (Sigma)

**Anti-mouse IgG (Sigma)

2.4 Protocol of pull-down assay

Pull-down system was used to test the *in vitro* protein-protein interactions in our study. The experiment protocol mainly refers to the MagneGST Pull-Down System User Manual (Promega).

Principle of pull-down assay

GST pull-down is an important tool for validation of suspected protein-protein interactions or for discovering novel protein interactions. In our study, GST pull-down uses a GST-fusion protein (bait) bound to glutathione (GSH)-coupled particles to affinity purify any proteins (prey) that interact with the bait from a pool of proteins in solution. Bait and prey proteins were produced in bacteria as described above.

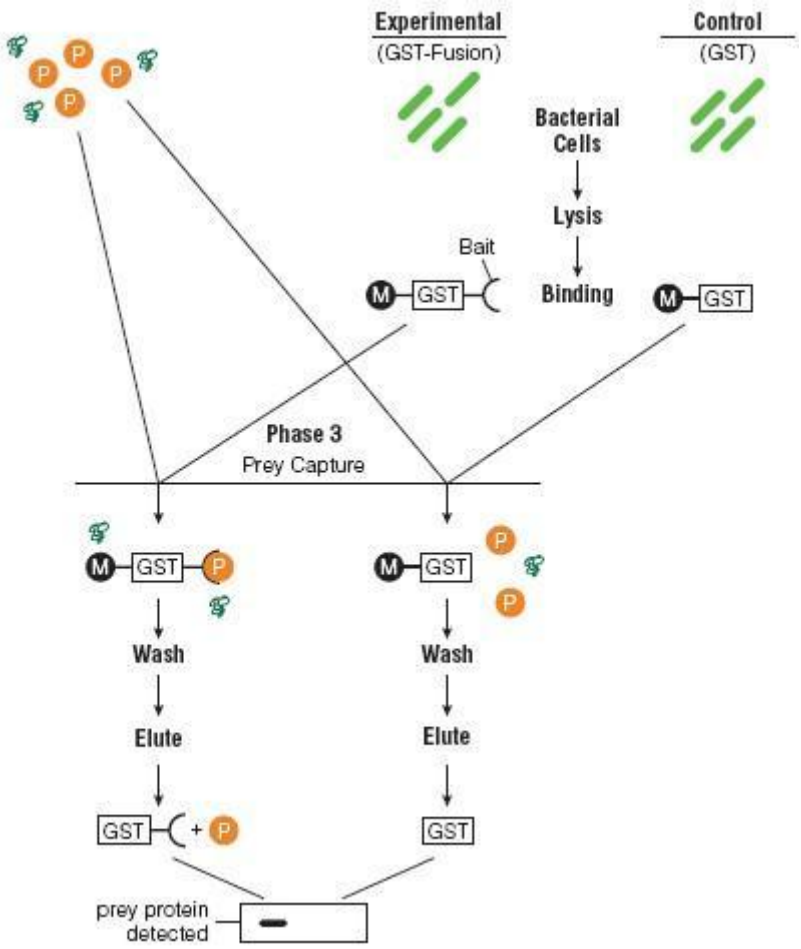


Fig. 9 Principle of the pull-down assay. The MagneGST Pull-Down System provides GSH-linked magnetic particles that allow simple immobilization of GST-fusion bait proteins from bacterial lysates. Prey proteins are then co-immobilized on the bait protein attached to the MagneGST (GSH) Particles (from Promega Magne-GST handbook).

Method of pull-down experiment

Pull-down protocol is divided into three phases: 1) the expression of GST-fusion-bait and another tagged-prey proteins; 2) the GST-fusion-bait protein is immobilized onto the MagneGST Particles; and 3) the prey protein is added to the MagneGST™ Particles carrying bait and captured through bait-prey interaction. The potential captured prey protein will be detected by western blot. If there is an interaction between the bait and prey protein, western blot result would reveal a band corresponding with the prey protein. For a negative control, we can either produce the GST alone, using a pGEX-3X vector or express a non-specific protein containing the same tag than the prey. More details of the protocol for pull-down could be checked in Appendix (A4) in this thesis.

2.5 Protocol of bimolecular fluorescence complementation (BiFC)

Bimolecular fluorescence complementation (BiFC) has been proven as a valuable tool to study protein-protein interactions in living cells. This method is now frequently used in plant sciences and is likely to develop into standard techniques for the identification, verification and in-depth analysis of polypeptide interactions (Bhat et al. 2006; Montse et al. 2007; Emiko et al. 2012; Fumi et al. 2012).

Principle of BiFC

The BiFC (also known as "split YFP") assay is based on the observation that N- and C-terminal sub-fragments of GFP (or derivatives thereof, e.g. YFP) do not spontaneously reconstitute a functional fluorophore. However, if fused to interacting proteins, the two non-functional halves of the fluorophore are brought into tight contact, refold together and generate the novo fluorescence. Thus, by BiFC, the interaction status of two protein interaction partners can be easily monitored via fluorescence emission upon excitation with a suitable wavelength (Fig. 10).

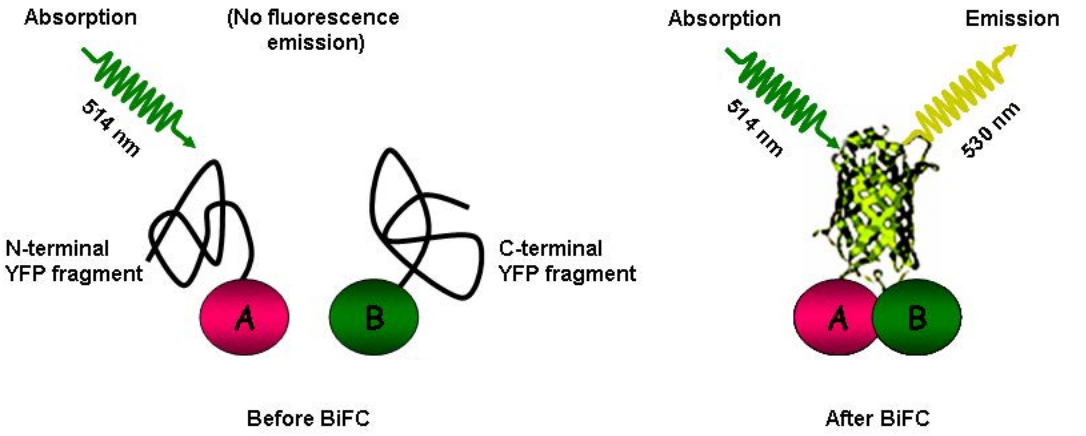


Fig. 10 Principle of the BiFC assay. The scheme depicts the principle of the BiFC assay, exemplified by a split YFP fluorophore. Proteins A and B are fused to N- and C-terminal fragments of YFP, respectively. In the absence of an interaction between A and B, the fluorophore halves remain non-functional. Following interaction between A and B, a functional fluorophore is reconstituted which exhibits emission of fluorescence upon excitation with an appropriate wavelength. (Bhat et al. 2006)

Plasmid construction for BiFC

The coding sequences of two potentially interacting proteins (protein-A and -B) which were tested by BiFC have been cloned into the expression vector pAM-35SS-YFPn-GWY and pAM-35SS-YFPc-GWY by the gateway cloning technology (Invitrogen). The N terminal of protein-A was fused to the N terminal half of YFP (nYFP) to yield nYFP-A. Another N terminal of protein-B was fused to the C-terminal half of YFP (cYFP) to yield cYFP-B.

For the construction of nYFP or cYFP fusion genes, which were driven by the CaMV 35S promoter for BiFC experiments, full-length cDNAs of protein-A and -B were amplified by PCR and cloned into pDONOR 207 using the Gateway BP recombination reaction (Invitrogen). After verifying the nucleotide sequence of A and B by sequencing, Gateway LR recombination reaction (Invitrogen) was used to transfer the A and B full-length cDNAs into the BiFC pAM-35SS-YFPn-GWY and pAM-35SS-YFPc-GWY respectively. These two vectors were kindly provided by Laurent Deslandes (LIPM, INRA Toulouse).

BiFC experiment protocol

To examine a possible interaction between protein-A and protein-B i*n planta*, we further conducted bimolecular fluorescence complementation (BiFC) assays using a transient expression system in tobacco protoplasts. The tobacco protoplasts were co-transfected with 10 μg of nYFP- and cYFP-fusion plasmids by the PEG transient method (Appendix A5). YFP fluorescence was observed by confocal microscopy 16 hours after transformation and excitation at 488 nm. The emission was captured between 530 to 570 nm wavelength. The YFP fluorescence was also quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA). By comparing with the autofluorescence background, the average fluorescence intensity of the cell population will indicate if there is an interaction between the tested protein-A and protein-B. More details of the protocol for BiFC could be checked in Appendix (A6) in this thesis.

2.6 Plasmid construction

Plasmid construction for Y2H

Tomato *IAA3* and *IAA9* coding sequences were cloned into Y2H pBD-vector respectively as bait protein, while *ARF4* and *ARF8a* were cloned into Y2H pAD-vector respectively as prey protein. An AD-ARF8a-del construct containing only the N-terminal part of ARF8a and lacking the AD region, domains III and IV was used as a negative control (Fig. 11).

Fig. 11 The construction of AD-ARF8a-del without the middle region, domain III and IV.

Plasmid construction for pull-down

Tomato *IAA3* and *IAA9* coding sequences were cloned into pDEST15 vector respectively as GST-tagged bait protein, and *ARF4*, *ARF8a* and *ARF8a-del* were cloned into pDEST17 vector respectively as His-tagged prey protein. pGEX-3X plasmid which could yield the GST protein alone was used as negative control.

Plasmid construction for BiFC

Tomato *IAA*-1, 2, 3, 4, 7, 9 and 11 coding sequences were cloned into pYFPn-vector (YFPn-IAAs), and *ARF4*, *ARF8a* and *ARF4-del* were cloned into pYFPc-vector (YFPc-ARFs).

Plasmid construction for PPIs map between whole Sl-IAA and Sl-ARF family

Tomato *IAA*-1, 3, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 19, 22, 26, 27 and 29 were cloned into Y2H pGBG-vector respectively as BD-bait protein.

Tomato *ARF*-1, 2a, 2b, 3, 4, 5, 6, 7, 8a, 9a, 9b, 10a, 10b, 16a, 16b and 17 were cloned into Y2H pGAD-vector respectively as AD-prey protein.

Plasmid construction for Sl-IAA deletion

Specific IAA9 domains (I, II, III and IV) were deleted from the full length protein (Fig. 12). These IAA9 deletions were cloned into BD-vector as a bait protein: BD-IAA9-2R, BD-IAA9-3R and BD-IAA9-3F. AD-ARF8a was chosen as the prey protein.

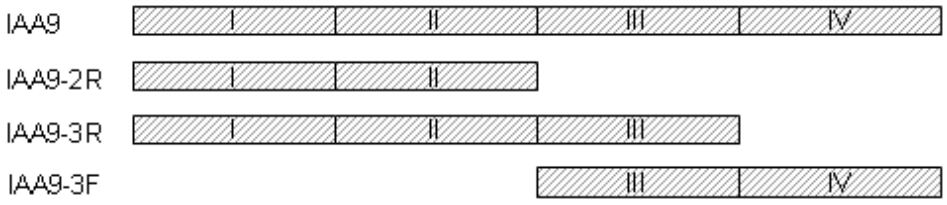


Fig. 12 Specific truncated IAA9 of the domain I, II, III or IV. All constructs were cloned as a bait protein: BD-IAA9-2R, BD-IAA9-3R and BD-IAA9-3F

2.7 Expression of GST- and His- tagged proteins

GST-IAA3, GST-IAA9, His-ARF8a-del and GST-alone (pGEX) were expressed in Rosetta or in BL21DE3-pLysS strains. His-ARF8a was expressed in BL.21AI. The optimized expression conditions for each protein are listed in Table 2. Protocol and materials refer to Appendix (A2). As a large quantity of overexpressed proteins accumulated in inclusion bodies, the IPTG inductor was removed in the experiments performed with *Rosetta* strains. All proteins were detected by western blot.

Table 2 The optimized expression conditions for each GST- or His- tagged protein.

	E. coli strain	Culture Temperature (℃)	Induction	Rotation (rpm)	Expressing time (h)
pGEX	Rosetta	28	No IPTG	250	3
GST-IAA3	Rosetta	28	No IPTG	250	3
GST-IAA9	Rosetta	28	No IPTG	250	3
GST-IAA3	BL21DE3-pLysS	28	IPTG 0.1M	250	4
GST-IAA9	BL21DE3-pLysS	28	IPTG 0.1M	250	4
His-ARF8a	BL.21AI	18	Arabinose 0.2% [W/V]	200	4
His-ARF8a-del	Rosetta	15	No IPTG	200	Over night

3. Results

We have seen that in tomato, the Aux/IAA and the ARF families contain 25 and 22 members respectively (see Chapter I.2). Among these members, Sl-IAA9 (Wang *et al*. 2005, 2009), Sl-ARF4 (Jones *et al*. 2002, Sagar *et al*. 2013), Sl-ARF8a (Yang *et al*. in preparation) have been extensively studied since they are highly expressed during fruit development or ripening and since the alteration of its expression triggers modifications in fruit biology. Sl-IAA3 is involved in both Auxin and Ethylene regulations and also showed high accumulation during fruit ripening (Chaabouni *et al*. 2009). This strong interest for these members prompted us to use them as initial targets to develop different PPI approaches. In a second step, we decided to build a larger screen of PPI among the whole ARF and Aux/IAA families to get access to a complete picture and address the question of Aux/IAA or ARF specificities.

3.1 Development of PPIs methods on specific AuxIAA-ARF pairs

3.1.1 Y2H shows some specific Sl-IAAs/Sl-ARFs interaction

For Y2H assay, all tested partners were listed in Table 3. BD-p53 and AD-T7 interaction was used as a positive control (Clontech). BD-IAAs interaction with AD-T7 or

ARF8a-deletion was used as negative controls. Protocol and materials refer to Part 2.1 of this Chapter.

The result of Y2H (Fig. 13) showed that both IAA3 and IAA9 interacted with ARF8a. Neither IAA3 nor IAA9 interacted with ARF4. The P53-T7 pair interaction was used as a positive control. The absence of interactions between bait-IAAs + prey-T7 or bait-IAAs + ARF8a-del (truncated form of ARF8a, see above) were used as negative control.

Table 3 List of specific BD-IAAs and AD-ARFs for Y2H assay.

	BD	AD
1	P53	T7
2	IAA3	T7
3	IAA3	ARF4
4	IAA3	ARF8a
5	IAA3	ARF8a-del
6	IAA9	T7
7	IAA9	ARF4
8	IAA9	ARF8a
9	IAA9	ARF8a-del

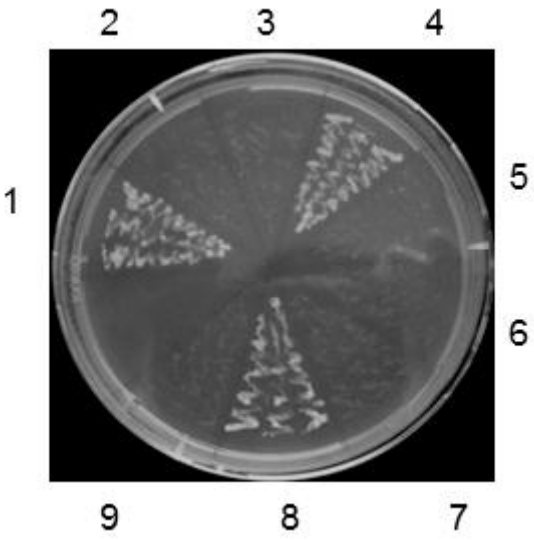


Fig. 13 The Y2H result of specific IAAs and ARFs. Yeast grows on the plate means that there are interaction between P53/T7 (1), IAA3/ARF8a (4) and IAA9/ARF8a (8). Selected medium is TLHA plate (Appendix A1).

3.1.2 Pull-down showed some specific Sl-IAAs/ Sl-ARFs interaction

Before pull-down assay, GST-IAA3, GST-IAA9, His-ARF8 and ARF8a-del proteins were expressed in *E. coli* expression system. During our experiment, we found that each protein needed different conditions for expression. It really took us a lot of time to optimize each condition for these proteins. From the Coomassie blue staining gel (Fig. 14) we could see GST-tagged proteins look easier to be expressed comparing with His-tagged proteins. All expression conditions of these proteins are listed on Table 2, and the western result is showed on Figure 14. Unfortunately, up to now, we have not found the appropriate conditions to obtain any soluble GST-ARF4 or His-ARF4 protein.

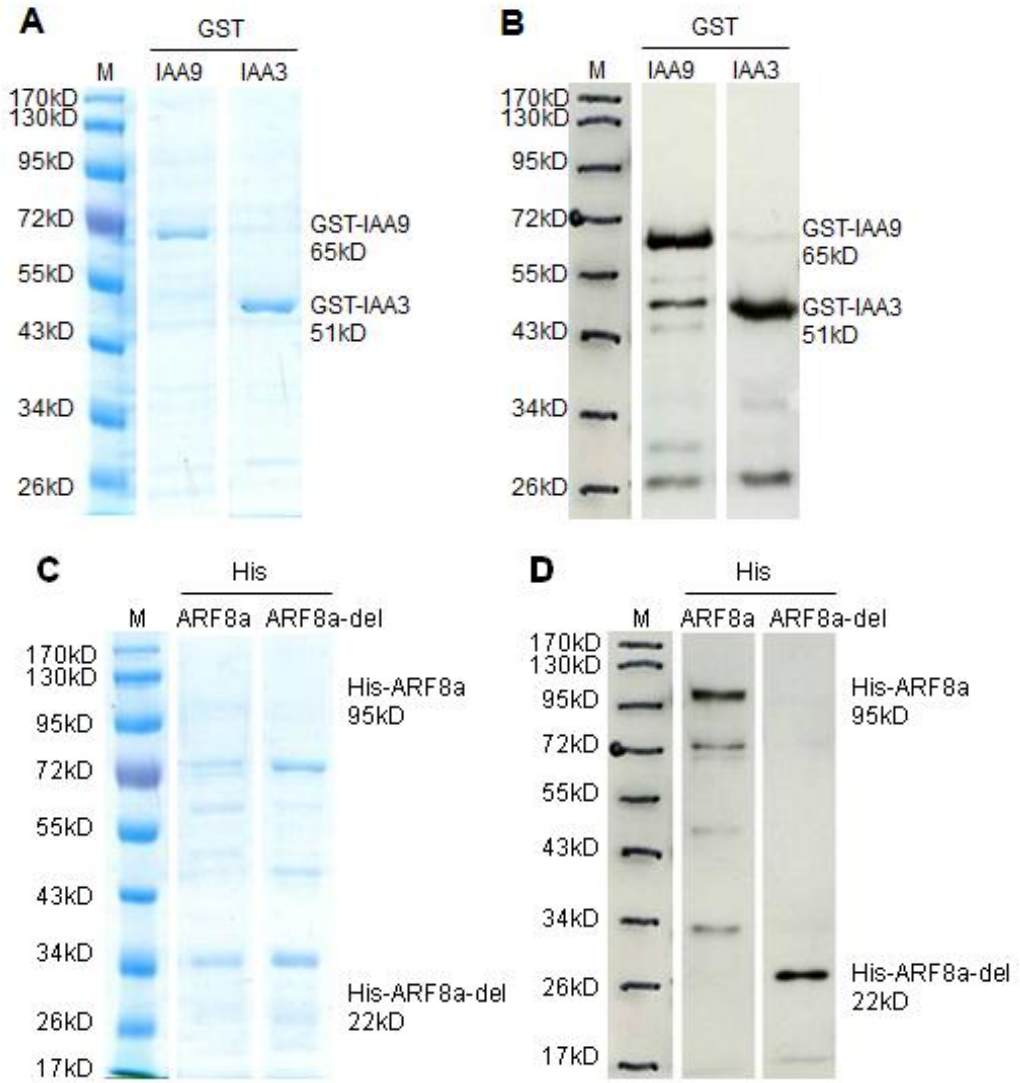


Fig. 14 Coomassie blue staining gel and western blot result for GST-IAAs and His-ARFs.

Different GST-IAAs and His-ARFs partner assessed for pull-down assay are listed on Table 4. The pull-down result (Fig. 15) confirmed that IAA3 and IAA9 did interact with ARF8a. The GST-alone did not interact with ARF8a or ARF8a-del. There were also no interaction between any IAAs and ARF8a-del.

Table 4 The list of specific GST-IAAs and His-ARFs for pull-down assay.

	GST	His
1	GST	ARF8a
2	GST	ARF8a-del
3	IAA3	ARF8a
4	IAA3	ARF8a-del
5	IAA9	ARF8a
6	IAA9	ARF8a-del

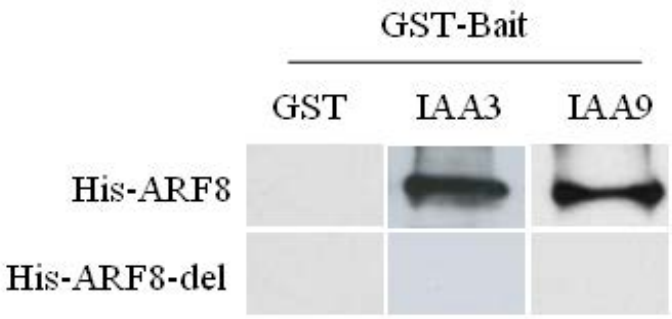


Fig. 15 Evidence of IAA3 and IAA9 interaction with ARF8 by pull-down. The bands of western blot indicated that IAA3 and IAA9 did interact with ARF8a respectively.

3.1.3 BiFC showed some specific Sl-IAAs/Sl-ARFs interaction

Protoplasts co-transfected with YFPn-IAAs and YFPc-ARFs plasmids were assayed for fluorescence by using confocal microscope. Empty vector of YFPn and YFPc plasmids were used as negative control. If there was an interaction between testing protein partner, clear fluorescence should be observed from protoplast compared with the autofluorescence background and negative control, such as the observed nuclear fluorescence indicated the interaction between YFPn-IAA9 and YFPc-ARF8a (Fig. 16).

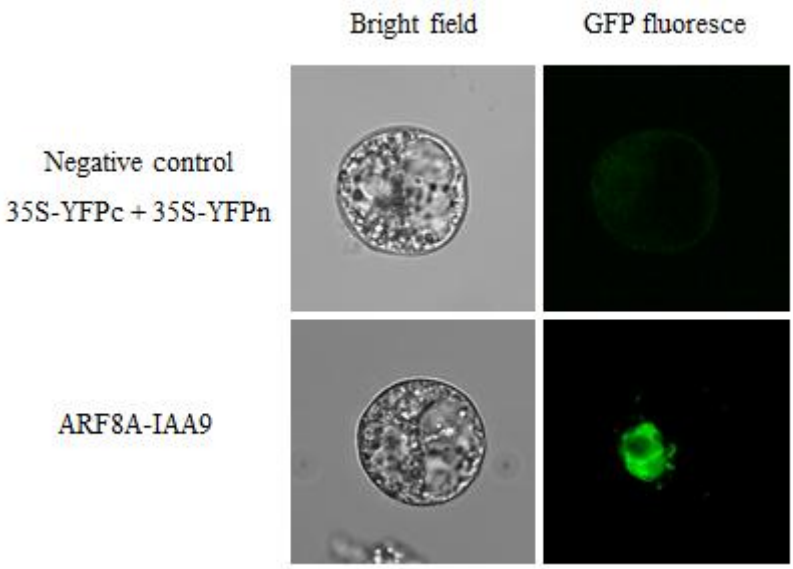


Fig. 16 Example of BiFC result. Clear nuclear fluorescence could be observed from tobacco protoplast compared with the background and negative control. Fluorescence indicated the interaction between ARF8a and IAA9.

Table 5 shows the specific YFPn-IAAs and YFPc-ARFs partner for BiFC assay. BiFC result (Fig. 17) showed that most of Aux/IAAs (IAA1, 2, 3, 4, 7, 9) interact with ARF8a and ARF4 while they do not interact with a truncated form of ARF4 (ARF4-del) that lacks domains III and IV (Fig. 17C). In contrast to Y2H results, IAA9 showed interaction both with ARF8a and ARF4 in BiFC assay. This is different from the result of IAA9/ARF4 interaction testing in Y2H. As BiFC is an *in vivo* system, other proteins may take part in the interaction between IAA9 and ARF4 in of tobacco cells. As a first interpretation, we can conclude that IAA9 could interact directly with ARF8a whereas IAA9 could not interact directly with ARF4. I*n vivo*, IAA9, ARF4 and other proteins can be assembled to form a functional protein complex.

Table 5 The list of YFPn-IAAs and YFPc-ARFs for BiFC assay.

	YFPn-							
YFPc-ARF8a	Empty	IAA1	IAA2	IAA3	IAA4	IAA7	IAA9	IAA11
YFPc-ARF4	Empty	IAA1	IAA2	IAA3	IAA4	IAA7	IAA9	IAA11
YFPc-ARF4-del	Empty	IAA1	IAA2	IAA3	IAA4	IAA7	IAA9	IAA11

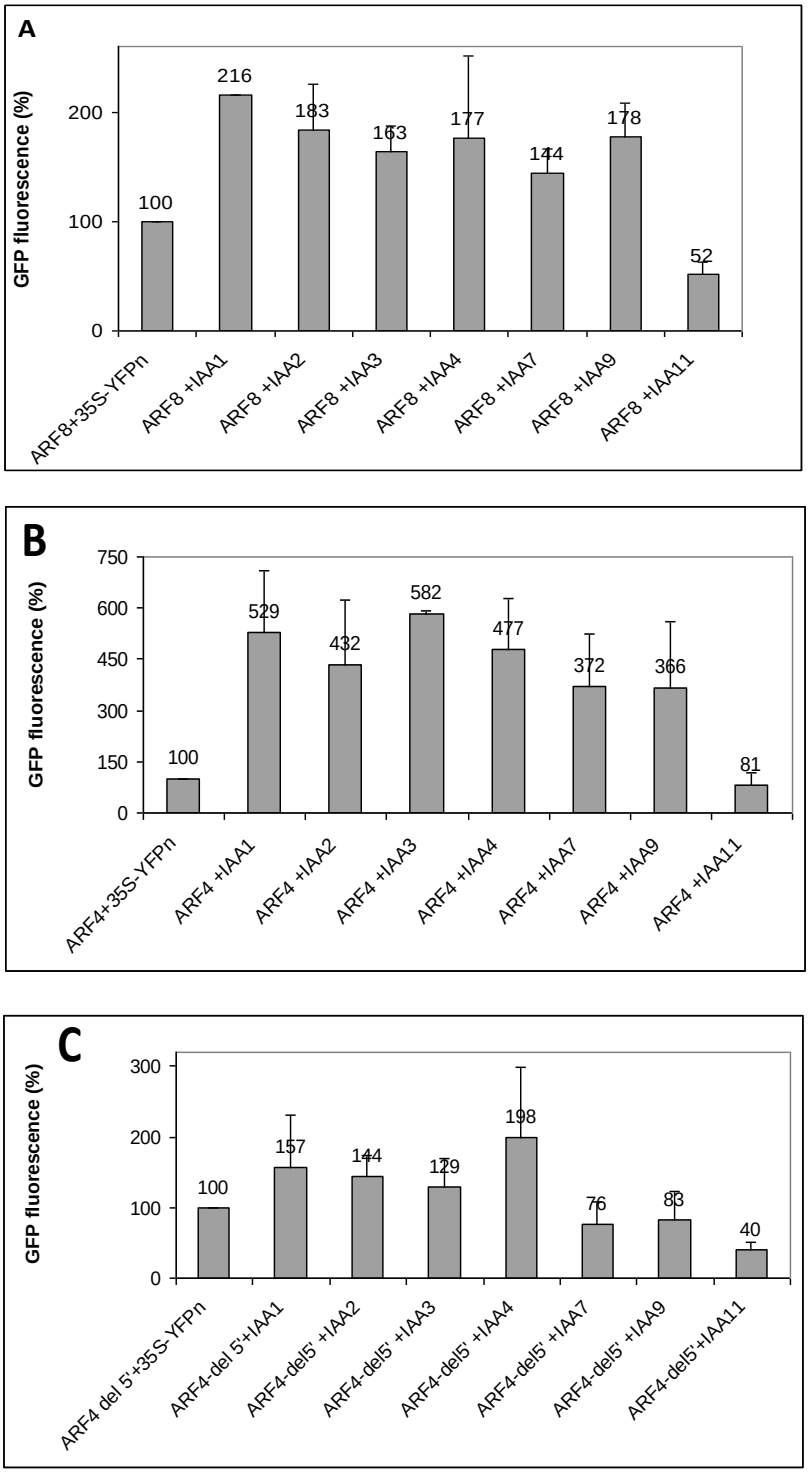


Fig. 17 BiFC result for specific IAAs and ARFs. Most of Aux/IAAs (IAA1, 2, 3, 4, 7, 9) interact with ARF8a (A), ARF4 (B) and ARF4-del (C). To quantify the YFP fluorescence, samples were analyzed by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA) on the cytometry and cell sorting platform.

3.2 PPIs between whole Sl-IAAs and Sl-ARFs family

Among the three methods tested above, Y2H seemed the most well-suited to be developed in wider PPI screens. Y2H was first used to test the interaction relationship between the whole family members of tomato IAAs and ARFs. The interactions between BD-IAAs and AD-ARFs were tested with all different partners respectively:

BD-IAA1, 3, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 19, 22, 26, 27 and 29

AD-ARF1, 2a, 2b, 3, 4, 5, 6, 7, 8a, 9a, 9b, 10a, 10b, 16a, 16b and 17

Figure 18 shows the interaction result of all BD-IAAs and AD-ARFs proteins. All ARFs activators (ARF5, 6, 7 and 8a) showed an interaction with all Aux/IAAs except IAA11. Parts of ARFs repressors, ARF1, 2a, 2b, 4 and 16a, showed an interaction with a few members of Aux/IAAs family. There were no interaction observed with ARF3, 9a, 9b, 10a, 10b, 16b and 17 by using this Y2H assay system.

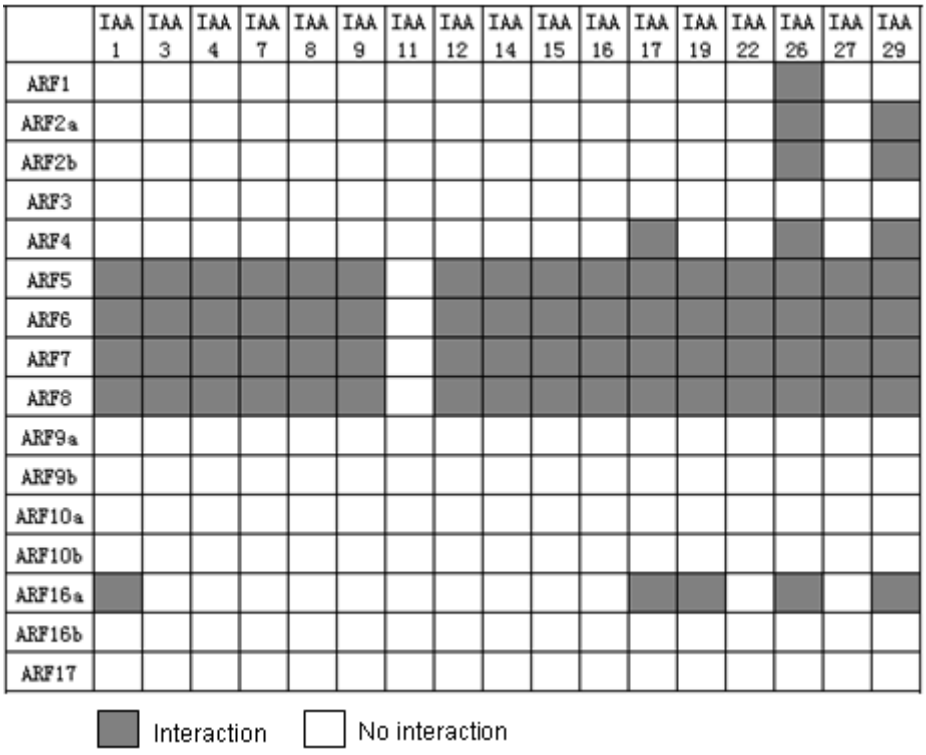


Fig. 18 PPIs result of the whole IAAs family and ARFs family. Black grid means there is an interaction between the two partners. Blank grid means there is no interaction.

3.3 Parts of canonical Sl-IAA domains affect the interaction of Sl-IAA and Sl-ARF

Up to now, dimerization domains of Aux/IAAs referred to as domain III and IV are considered to be responsible for the interaction between Aux/IAAs with ARFs. In our experiment, BD-IAA9-2R, BD-IAA9-3R and BD-IAA9-3F (Fig 12) were used as bait proteins. AD-ARF8a was chosen as the prey protein. BD-p53 and AD-T7 interaction was used as positive control. Interactions between different IAA9-deletions and ARF8a were assayed by Y2H (Table 6). IAA9-del-2R (domain I and II) and IAA9-del-3R (domain I, II and III) showed no interaction with ARF8a, but IAA9-del-3F (domain III and IV) and IAA9 full length did interact with ARF8a (Fig. 19). This result confirms that IAA9 domains III and IV are essential for the interaction between IAA9/ARF8a. Similar conserved domains III and IV also exist in some ARF proteins (The C-terminal domain; CTD). IAA9 and ARF8a interact with each other via domain III and IV to form a dimer.

Table 6 The list of specific BD-IAAs and ARFs for Y2H test.

BD	AD
1. P53	T7
2. IAA9	T7
3. IAA9	ARF8a
4. IAA9-2R	ARF8a
5. IAA9-3R	ARF8a
6. IAA9-3F	ARF8a

Fig. 19 PPIs result of the IAAs deletions and ARF8a. Black grid means there is an interaction between the two partners. Blank grid means there is no interaction.

4. Conclusion and discussion

Three methods were used in our study to identify some specific interaction of Sl-IAA and Sl-ARF: Yeast two-hybrid (Y2H), Pull-down and Bimolecular fluorescence complementation (BiFC). All methods confirmed that IAA3 and IAA9 interact with ARF8a. The Y2H result showed Sl-IAA3 and Sl-IAA9 do not interact with Sl-ARF4. On the contrary, BiFC result suggests that Sl-IAA3, Sl-IAA9 and a broad set of Aux/IAAs also interact with ARF4. Why it happened? There is one thing noticeable that BiFC is more like an *in vivo* system, the protoplast providing a *in planta* environment which could provide other plant proteins necessary for the interaction. In a BiFC experiment it is still likely that other proteins take part in the protein complex. This hypothesis should be checked further. Alternatively, these contradictory results may suggest the existence of modifications triggered by the plant cellular context (phosphorylation or other post-translational modifications) necessary to enable an ARF4-Aux/IAA interaction.

Of all the tested methods, Y2H protocol was well-suited as a high throughput method. During our experiment, the Y2H could show us very clear and reproducible results. For pull-down assay, we felt that the biggest difficulty was to express and purify each protein. Table 2 shows some optimized conditions of expression for each protein. Up to now, we still cannot obtain enough quality and quantity of ARF4 for pull-down assay. BiFC is also a rapid method. The key step which could affect the BiFC experiment is the quality of protoplast transformation. Thus, the reproducibility of the results was often the main problem.

As the more efficient and stable, Y2H method was used to test at large scale interactions within the whole Sl-Aux/IAA and Sl-ARF families. In our study, the combination between 17 Sl-IAA members and 16 Sl-ARF members were tested. The most obvious result is that all activator-Sl-ARFs (ARF5, 6, 7 and 8a) showed interaction with all Sl-IAAs except Sl-IAA11 and Sl-IAA29. This dominant trait was also observed in other large-scale of the IAA and ARF interaction patterns in Arabidopsis (Vernoux et al. 2011) or in rice (Shen et al. 2010). Indeed, the study in Arabidopsis also showed that nearly all the At-IAAs were able to interact with At-ARF activators while At-ARF repressors display very few interactions (Fig. 6). For the

repressor Sl-ARF in our study, very few of them showed interactions with Sl-IAAs. Even some of them showed no interaction with Sl-IAAs, such as Sl-ARF3, 9a, 9b, 10a, 10b, 16b, 17. To date, in Arabidopsis the only At-ARF repressor which is able to interact with different At-IAA genes is At-ARF9. But this trend was not observed in tomato Sl-ARF9. Contrary to our results on Sl-IAA9 in tomato, At-IAA9 display very few interactions, even with activator ARF (Vernoux et al. 2011), however, At-IAA8, its closest paralog that emerged from a recent duplication (Audran-Delalande et al. 2012) displayed a very similar PPI pattern than Sl-IAA9, thus suggesting that this difference may be the consequence of recent evolutive events. All these studies demonstrated the broad interaction capacity between Aux/IAAs and ARFs. It also indicated that ARF repressors may act more as a competition for AuxRE element binding with ARF activators rather than being directly involved in auxin signaling through Aux/IAA regulation.

In Arabidopsis, the specific interactions among ARFs and Aux/IAAs were demonstrated by the high-throughput Y2H assay where Aux/IAAs preferentially interact with themselves and the activator ARFs, but weakly with repressor ARFs (Vernoux et al. 2011). Slight amino acid differences in domain III and IV between At-ARF activators and repressors was proposed to provide the molecular clue for the differential interaction between At-IAAs and At-ARF activators or repressors (Guilfoyle and Hagen, 2012). In contrast to the Y2H assays, the split firefly luciferase complementation (SFLC) assay using the protoplast system gave some contradictory results. For example, At-ARF9 was the only repressor ARF that interacted with diverse At-IAAs in the Y2H assay, but it interacted only with At-IAA28 in the SFLC assay (Li et al. 2011b). At-ARF5 (activator) did not interact with At-IAA6 and At-IAA9 in the Y2H assay whereas At-ARF5 strongly interacted with them in the SFLC assay (Li et al. 2011b). As discussed for our BiFC experiments, these differences in the interaction patterns between At-ARFs and At-IAAs could be due to the different molecular and cellular environments between yeast and plant cells, implying that additional factors might be implicated in the native ARFs-Aux/IAAs interactions in plant cells. Another factor to be considered is the concentration of ARFs and Aux/IAAs in the cell for their native interactions.

As we learnt before, typical Aux/IAA has 4 classical domains: domain I, II, III and IV.

Domains III and IV of the Aux/IAA proteins shares a homology with the C-terminal domain (CTD) of ARF proteins. Through an interaction between domains III and IV and the CTD, Aux/IAA can form a heterodimer with ARF (Kim et al. 1997; Muto et al. 2006; Ulmasov et al. 1997b). In our study, three deletions of Sl-IAA9 were cloned. IAA9-2R lacking domain III and IV and IAA9-3R lacking domain IV both showed no interaction with ARF8a. As expected, only IAA9-3F which contains only domain III and IV showed clearly interaction with ARF8a. However, there is also one thing noticeable when Y2H was performed to identify the interaction between IAA9/IAA9-3F and ARF8a, we always found the yeast containing IAA9-3F + ARF8a grew a little bit slowly and that the number of cotransformants was smaller than observed on the yeast transformed with pBD-full-length-IAA9 + pAD-ARF8a. This suggests that, although IAA domain III and IV are essential for the interaction with some ARF, the Aux/IAA domain I and II could also affect the interaction efficiency perhaps by stabilizing the whole structure of the Aux/IAA protein.

To compare with Arabidopsis, the C-terminal domain III and IV found in both ARFs and Aux/IAAs enables the formation of homo- or heterodimers: ARF-ARF, Aux/IAA-Aux/IAA, or ARF-Aux/IAA (Kim et al. 1997; Ulmasov et al. 1999b). A recent comparative analysis on the secondary structure of domain III and IV suggests there is a molecular preference in these interactions (Guilfoyle et al. 2012). In this study, domain III and IV of ARFs and Aux/IAAs were found to have a similar secondary structure with the PB1 (Phox and Bem 1) domain, which includes an ubiquitin like β-grasp fold. Three different types of PB1 domains can interact with each other in different combination where variations in certain changed amino acid residues in the N-terminus and C-terminus contribute to the type-specific interactions between different types of PB1 (Sumimoto et al. 2007). Those changes in residues are also found in domain III and IV of ARFs and Aux/IAAs and were proposed to similarly contribute to the diverse interactions for ARFs and Aux/IAAs (Guilfoyle et al. 2012). This model also supports the idea that interactions between ARFs and Aux/IAAs occur specifically but not randomly.

Chapter III: Genome-wide analysis and interaction properties of the *TOPLESS* gene family members

Chapter III: Genome-wide analysis and interaction properties of the *TOPLESS* gene family members

1. Introduction

In this chapter, a second partner crucial in auxin signaling was investigated, the TOPLESS protein and its paralogs. An exhaustive yeast two-hybrid screen, completed with the analysis of the multigenic family in tomato constituted the core of the manuscript „Genome-wide analysis and Identification, phylogenetic analysis, expression profiling and protein-protein interaction properties of the TOPLESS gene family members in tomato" submitted to Journal of Experimental Botany. In complement to the manuscript presented below, the ARF-TOPLESS protein-protein interactions were also studied and the specificity of one TOPLESS isoform was also challenged using different mutagenesis strategies.

2. Manuscript „Genome-wide Identification, phylogenetic analysis, expression profiling and protein-protein interaction properties of the TOPLESS gene family members in tomato"

2.1 Introduction

It is now well admitted that transcriptional co-repressors play crucial roles in a broad range of plant development processes (Liu and Karmarkar, 2008; Krogan and Long, 2009). In land plants, the Groucho (Gro)/Tup1 family of co-repressors includes TOPLESS/TOPLESS-RELATED (TPL/TPR) and LEUNIG/LEUNIG HOMOLOG (LUG/LUH) (Conner and Liu, 2000; Kieffer et al., 2006; Long et al., 2006). TPL proteins have been shown to be involved in multiple signaling pathways in higher plants, including

hormone-signaling pathways (auxin, jasmonic acid, abscisic acid and ethylene), meristem maintenance, floral induction, biotic stress and circadian oscillator mechanism (Causier et al., 2012a, b; Liu et al., 2008; Pauwels et al., 2010; Szemenyei et al., 2008, Zhu et al., 2010, Wang et al., 2013).

The first *TPL* gene was identified in Arabidopsis as responsible for the semi-dominant *tpl-1* embryo development mutation resulting in altered polarity, ranging from fused cotyledons to complete replacement of the shoot with a second root (Long et al., 2002, 2006). Subsequently, the *TPL* family in arabidopsis was found to comprise five members that seem to act redundantly (*TPL*, *TPR1*, TRP2, TRP3, and TRP4). Indeed, a quintuple loss of function, in which all five TPL/TPR genes were inactivated by mutation or RNA interference, is required to phenocopy the *tpl-1* phenotype (Long et al., 2006).

It was established that though TPL proteins are lacking a DNA-binding activity, they are incorporated into transcription complexes by interacting with transcription factors to repress gene expression in various processes. This inhibition of the expression of target genes is mediated by the recruitment of histone deacetylases (HDACs) into transcription complexes, and by changing the chromatin state from active to inactive (Long et al., 2006; Liu and Karmarkar, 2008; Krogan and Long, 2009; Krogan et al., 2012). Interaction between the TPL/TPR co-repressors and transcription factors depends on the Lissencephaly (LisH) and the C-terminal to LisH Homology (CTLH) domain of TPL (Szemenyei et al., 2008; Gallavotti et al., 2010) and on a small conserved protein motif found in transcription factors. This motif is known as the ethylene response factor (ERF)-associated amphiphilic repression (EAR) domain (Ohta et al., 2001), with the consensus sequence (L/F)DLN(L/F)xP (Ohta et al., 2001; Hiratsu et al., 2004). Recently, the Arabidopsis TPL/TPR interactome framework revealed that the TPL co-repressors are able to interact with various transcription factors harboring different repression domains (Causier et al., 2012a). Among these TPL-interactants, the transcriptional repressors involved in auxin signaling (i.e. Aux/IAA and Auxin Response Factors (ARFs) families) have been well documented. In Arabidopsis, the discovery that TPL is recruited by Aux/IAA proteins to suppress the expression of auxin-responsive genes in the absence of auxin, revealed a crucial role for TPL in mediating the inhibitory effect of

Aux/IAA on ARF-regulated transcription (Szemenyei et al., 2008). Large interactome studies in Arabidopsis identified 20 of the 29 At-IAA proteins as interacting partners of the TPL/TPRs (Arabidopsis Interactome Mapping Consortium, 2011; Causier et al., 2012a). Besides, a large-scale analysis of the interaction between Aux/IAA and ARF in the Arabidopsis shoot apex revealed that the vast majority of the Aux/IAAs interact with all the ARF activators and show very limited interactions with ARF repressors (Vernoux et al., 2011). However, recent study showed that repressive ARF proteins, such as ARF2 and ARF9, can interact directly with TPL/TPR proteins, suggesting a mechanism for repression implicating TPL/TPR co-repressors in both forms of ARF-mediated repression (Causier et al., 2012a).

The release over the past years of several plant genome sequences has offered a possibility to investigate a large set of multigenic families at the genome scale. In this context, the tomato genome is of special interest since (1) tomato has emerged as a model plant, for fleshy fruit development and (2), a reference species for Solanaceae family and also for the taxum of Asterids, particularly since the majority of sequenced Dicot genomes belongs to Rosids. Noteworthy, the structure of several multigenic families involved in auxin perception and responses have been examined in tomato (Wu et al., 2011, Kumar et al., 2011, Audran-Delalande et al., 2012, Pattison & Catalá, 2012, Wu et al., 2012a&b, Kumar et al., 2012, Ren et al., 2011), thus shaping an exhaustive picture of auxin signalization complementary to Arabidopsis model plant. However, beside the plant model Arabidopsis, the TPL gene family has been so far poorly described.

To fully characterize the molecular biology and evolution of the tomato TPL family and to understand its possible functions, we identified and characterized 6 Sl-TPL genes. Our analyses focused on the identification, evolutionary relationships and expression patterns of each member of the tomato TPL family. Moreover, we used yeast two-hybrid approaches to establish the framework of TPL/Aux/IAA protein-protein interactions. These results will provide a framework for further studies to better understand the potential functions of TPL proteins in tomato plants, especially during the flower and fruit development.

2.2 Materials and Methods

2.2.1 Isolation and cloning of Sl-TPL genes

The full length coding sequences of 6 Sl-TPLs were amplified from mature green fruit cDNA. The primers used were as follows:

TPL1_attb1: 5‟-ATGTCATCTCTCAGTAGAGAGCTT-3‟;

TPL1_attb2: 5‟-TCATCTTGGTGCTTGATCGGAGC-3‟;

TPL2_attb1: 5‟-ATGTCTTCCTTGAGTAGGGAACTG-3‟;

TPL2_attb2: 5‟-TCACCTTGAAGGTGTTTCTGATG-3‟;

TPL3_attb1: 5‟-ATGTCTTCTCTTAGCAGAGAATTG-3‟;

TPL3_attb2: 5‟-TCATCTTTGAACTTGGTCAGCAG-3‟;

TPL4_attb1: 5‟-ATGACTTCTTTA AGCAGAGAGCTG-3‟;

TPL4_attb2: 5‟-CTACCTTGATGCTTGATCAAGACC-3‟;

TPL5_attb1: 5‟-ATGAGGCATTTTGATGAAATGGT-3‟;

TPL5_attb2: 5‟-CTACCTTTGAGGTTGATCTGAAT-3‟;

TPL6_attb1: 5‟-ATGTCTCTTAGTAAGGACCTTAT-3‟;

TPL6_attb2: 5‟-CTATATTGGTTGCTCATTGGTAA-3‟.

After amplification, Sl-TPLs were cloned into pDONOR207 vector using gateway method (Invitrogen) and were fully sequenced.

2.2.2 Subcellular localization of Sl-TPLs

For localization of the Sl-TPL proteins, the Sl-TPL CDS sequences were cloned by Gateway technology as a C-terminal fusion in frame with yellow fluorescent protein (YFP) into the pEarlyGate104 vector, and expressed under the control of the 35S CaMV promoter. The empty vector pEarleyGate104 was used as control. Protoplasts were obtained from tobacco suspension-cultured (Nicotiana tabacum) BY-2-cells and transfected according to the method described previously (Leclercq et al., 2005). YFP localization by confocal microscopy was performed as described previously (Audran-Delalande et al., 2012).

2.2.3 Expression analysis of Sl-TPL genes

Total RNA extraction, DNA contamination removal, cDNA generation of 8 tomato tissues (root, stem, leaves, bud, flower, mature green fruit, breaker fruit, and red fruit) and qRT-PCR were performed according to methods previously described (Audran-Delalande et al 2012, Pirrello et al., 2006). The primer sequences are as follows: TPL1F: 5‟-TGTTCGTTCTAGGAGACTAACCAG-3‟ and 5‟-TPL1R: AAGACAAACCTTCCCTTCCGA-3‟; TPL2F: 5‟-CCTGTAAATACGCCTCTTGCT and TPL2R: 5‟-ACTGGTTGGAATGGACTGTG-3‟; TPL3F: 5‟-CACTTTCTGCTCCAATAACCT-3‟ and TPL3R: 5‟-TCCATCTGTCAACCCAACTG-3‟; TPL4 F: 5‟-CCTTCTAACCCAAGCTCCAG-3‟ and TPL4R: 5‟-ATAAACTCCGCCATCAGTAAGTC-3‟; TPL5F: CGTCTATTGTAACCCATCCACTC-3‟ and TPL5R: 5‟-AGAAGTTACACCATGAGGACCC-3‟; TPL6F: 5‟-ACTGGACTAGCATTCTCTAACAC-3‟ and TPL6R: 5‟-TTGAATT CCACACCACTATCTGAG-3‟. Actin was used as internal reference. The relative fold differences (Sl-TPL6 as a reference gene) for each sample were calculated using the formula 2-ΔΔCt. Three independent RNA isolations were used for cDNA synthesis and each cDNA sample was subjected to real-time PCR analysis in triplicate.

2.2.4 Bioinformatics analyses

Sl-TPLs were searched using BLAST queries on Genomic (Chromosome v2.40) and transcript database (cDNA itag 2.4) available on SGN website (http://solgenomics.net/tools/blast/index.pl). Exons and introns were deduced from the ITAG 2.3 annotation. For Sl-TPL5 (Solyc07g008040), the „predicted annotation‟ missing the N-terminal extremity was completed with an additional exon (from position 2754093 to 2754173 on SL2.40ch07 chromosome 7 annotation). Protein domains were first predicted on the prosite database protein (http://prosite.expasy.org/). The prediction of the WD40 segments was refined using the PF00400.27 pfam Hidden Markov Model with an i-value threshold at

0.1. For i-values > 0.1, the prediction of WD40 position was deduced from the sequence alignment of the different TPL isoforms.

Nuclear Localisation Signal (NLS) analysis prediction was performed with "cNLS Mapper" (http://nls-apper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Kosugi et al, 2009). NLS prediction scores above 5.0 were considered as positive.

2.2.5 Evolutionary analyses

Phylogenetic analyses and distance matrices were built using the Mega5 package (Tamura et al., 2011). Full length amino acid sequences were aligned using the ClustalW algorithm. For the overall phylogeny, an initial tree encompassing sequences from *Physcomitrella patens*, *Selaginella moellendorffii*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Arabidopsis thaliana*, *Solanum lycopersicon*, *Nicotiana benthamiana*, *Populus trichocarpa*, *Glycine max* and *Mimulus guttatus* was performed using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together was calculated in the bootstrap test (500 replicates). The topology was further confirmed using the Maximum Likelihood method. Ultimately, a simplified tree was performed by limiting the number of genomic sets as the topology remained unchanged. Trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The following genome annotations were used for phylogenetic analyses: *P. patens* (Phypa1_1.FilteredModels, Rensing et al., 2008); *S. moellendorffii* (Lycophyte Selmo1_GeneModels_FilteredModels3, Banks et al., 2011); *A. thaliana* (TAIR10, Swarbreck et al., 2008); *P. trichocarpa* (Eudicot Populus.trichocarpa.v2.0, Tuskan et al., 2006); *V. vinifera* (12X March 2010 release, Glycine max Glyma1_pacId, Schmutz et al., 2010); *O. sativa* (MSU Rice Genome Annotation (Osa1) Release 6.1, Ouyang et al., 2007); *Z. mays* (ZmB73_4a.53_working_translations, Schnable et al., 2009); *S. bicolor* (Sorbi1_GeneModels_Sbi1_4_aa, Paterson et al., 2009); Tomato (ITAG2.3_release, 2012); *B. rapa* (Chiifu-401-42, Wang et al., 2011); *Eucalyptus grandis* (Egrandis_201, http://www.jgi.doe.gov/); *M. guttatus* (Mguttatus_140, http://www.jgi.doe.gov/); *N.*

benthamiana (Niben.genome.v0.4.4, Pallas et al., 2012); *S. tuberosum* (PGSC_DM_v3.4, Xu et al., 2011).

2.2.6 PPIs assay of Sl-TPLs and Sl-IAAs families by Y2H

Tomato TPL genes were amplified and cloned into pDBD (BD-TPLs) vector (Clontech). Similarly, Sl-IAA target genes (IAA1 (JN379431), IAA3 (JN379433), IAA4 (JN379434), IAA7 (JN379435), IAA8 (JN379436), IAA9 (JN379437), IAA11 (JN379438), IAA12 (JN379439), IAA14 (JN379441), IAA15 (JN379442), IAA16 (JN379443), IAA17 (JN379444), IAA19 (JN379445), IAA22 (JN379447), IAA26 (JN379449), IAA27 (JN379450) and IAA29 (JN379451)) were inserted in pGAD (AD-IAAs) vectors (Clontech). Diploids were selected on medium lacking Trp and Leu (TL), and interactions were validated by the use of HIS3 and ADE2 reporter genes on medium lacking Trp, Leu, His, and Ade (TLHA). Manipulation and analysis of Y2H protocol refers to Appendix A.1, and all experiments were repeated 3 times independently. For Sl-TPL1 lacking LisH, the coding sequence was truncated at position +112 nucleotide.

2.3. Results

2.3.1 Identification and cloning of TPL-related genes in tomato genome

In silico search was performed on the tomato genome and transcript databases (http://www.solgenomics.net/) using Arabidopsis TPL and TPL-related sequences (TPR) as queries for BLAST searches. While the initial screen identified nine ORFs as predicted to encode putative TPL-like proteins (Sl-TPLs) only six corresponded to full-length proteins containing all canonical motifs that define the TPL proteins (Table 7). The full-length cDNA of the six Sl-TPLs was further confirmed by RT-PCR amplification indicating that the corresponding coding sequences (CDS) range from 3396 bp to 3669 bp with deduced protein sizes ranging from 1131 to 1222 amino acids (Table 7).

Table 7. Main structural features of the tomato Sl-TPL family members.

Nomenclature	Gene			Predicted Protein		Domains		
Sl-TPLs	iTAG Gene ID	Exons	Introns	Length	MW (kDa)	LisH	CTLH	WD-40 repeats
Sl-TPL1	Solyc03g117360.2.1	25	24	1131 aa	124.676	4-36	34-92	411-632/832-957
Sl-TPL2	Solyc08g076030.2.1	25	24	1136 aa	124.60	4-36	34-92	341-668/834-959
Sl-TPL3	Solyc01g100050.2.1	25	24	1132 aa	124.676	4-36	34-92	343-669/871-955
Sl-TPL4	Solyc03g116750.2.1	26	25	1133 aa	124.318	4-36	34-92	413-634/839-964
Sl-TPL5	Solyc07g008040.2.1	24	23	1134 aa	124.82	4-36	34-92	398-639/881-965
Sl-TPL6	Solyc08g029050.2.1	33	32	1222 aa	134.181	3-35	33-91	531-664/934-1060

Structural analysis of the six Sl-TPL genes showed that they display similar numbers of introns (23 to 25) and exons (24 to 26) except for Sl-TPL6 which is longer than the other TPL members (Table 7). Pairwise comparison of the six Sl-TPL protein sequences showed that the percent identity among family members ranges from 44% to 75%. Protein domain searches in Pfam database (http://pfam.sanger.ac.uk/) indicated that all Sl-TPLs display the conserved LisH and CTLH domains and two domains containing several WD40 repeats: WD40-repeat-1 and WD40-repeat-2 with 7 and 5 WD40 segments, respectively (Fig. 20 and 21). The CTLH domain and the WD40-repeat-1 are separated by a proline-rich region.

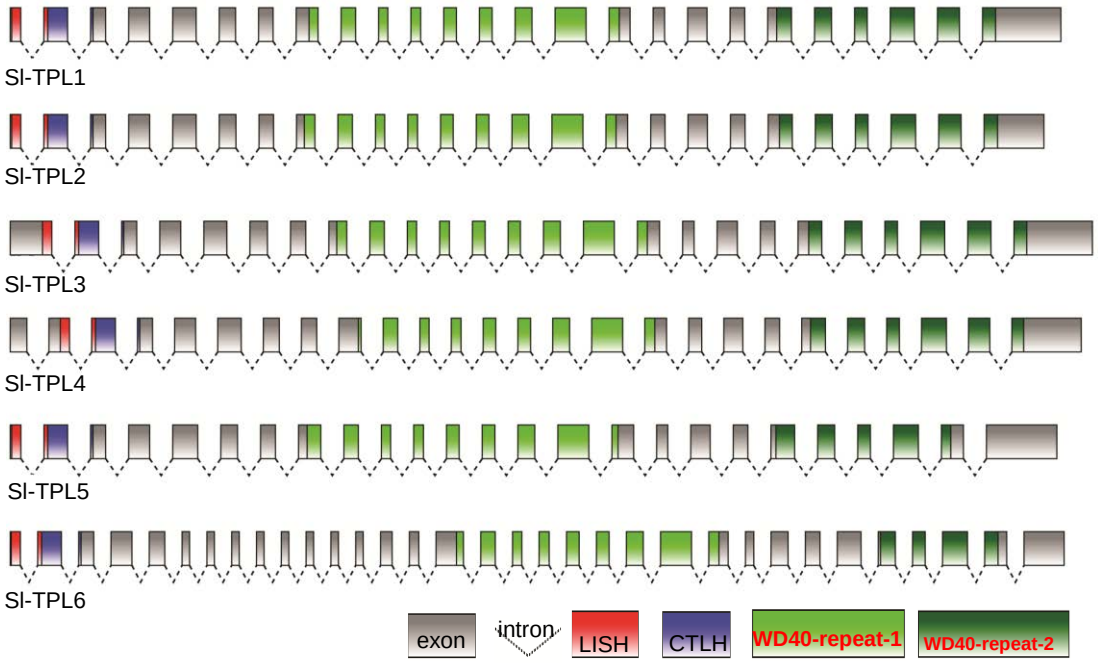


Figure 20. Gene structure of the 6 tomato *TOPLESS* genes. The grey boxes represent exons, the dotted lines represent introns, the red box the LisH domain, the blue box the CTLH domain, the light green boxes the WD40-repeat 1 and the dark green boxes the WD40-repeat 2. The figure was achieved using the FancyGene software (http://bio.ieo.eu/fancygene/).

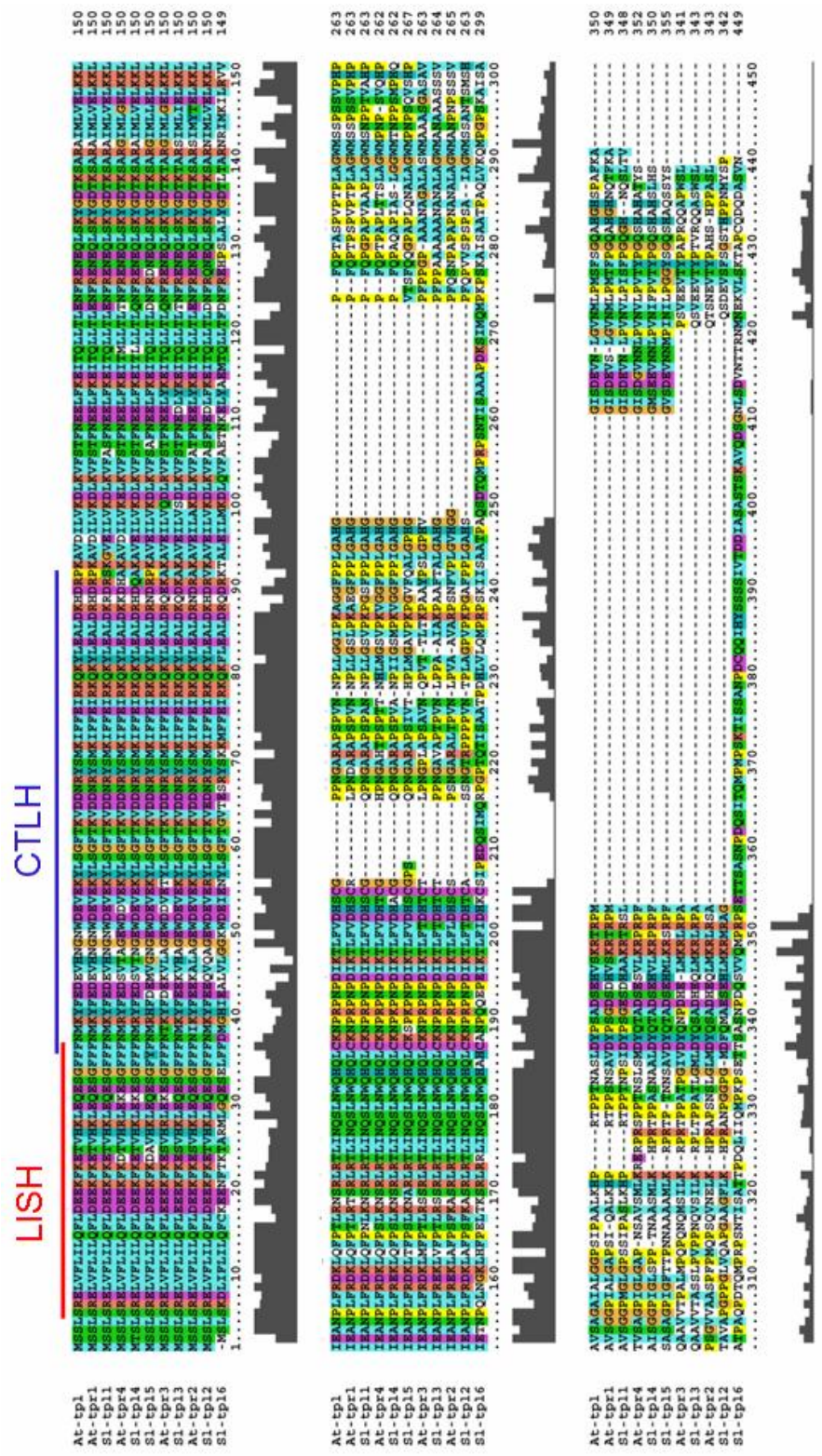


Figure 21. Multiple sequence alignment of full-length Sl-TPL proteins. Conserved domains are underlined. The red line represents LisH domain. The blue line represents CTLH domain. The light green line represents the WD40-repeat 1 domain, the dark green line represents WD40-repeat 2 domain.

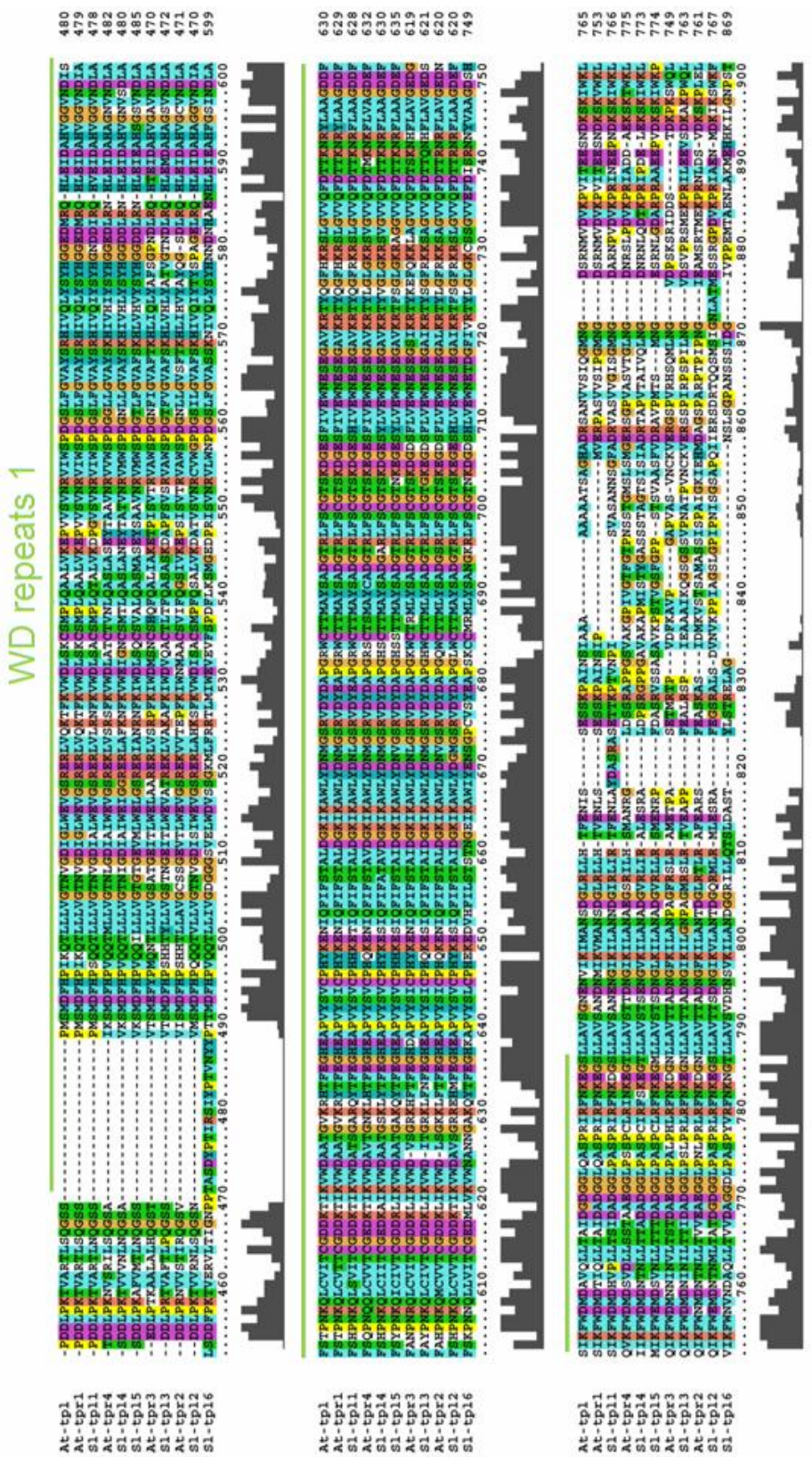


Figure 21. (Continuance)

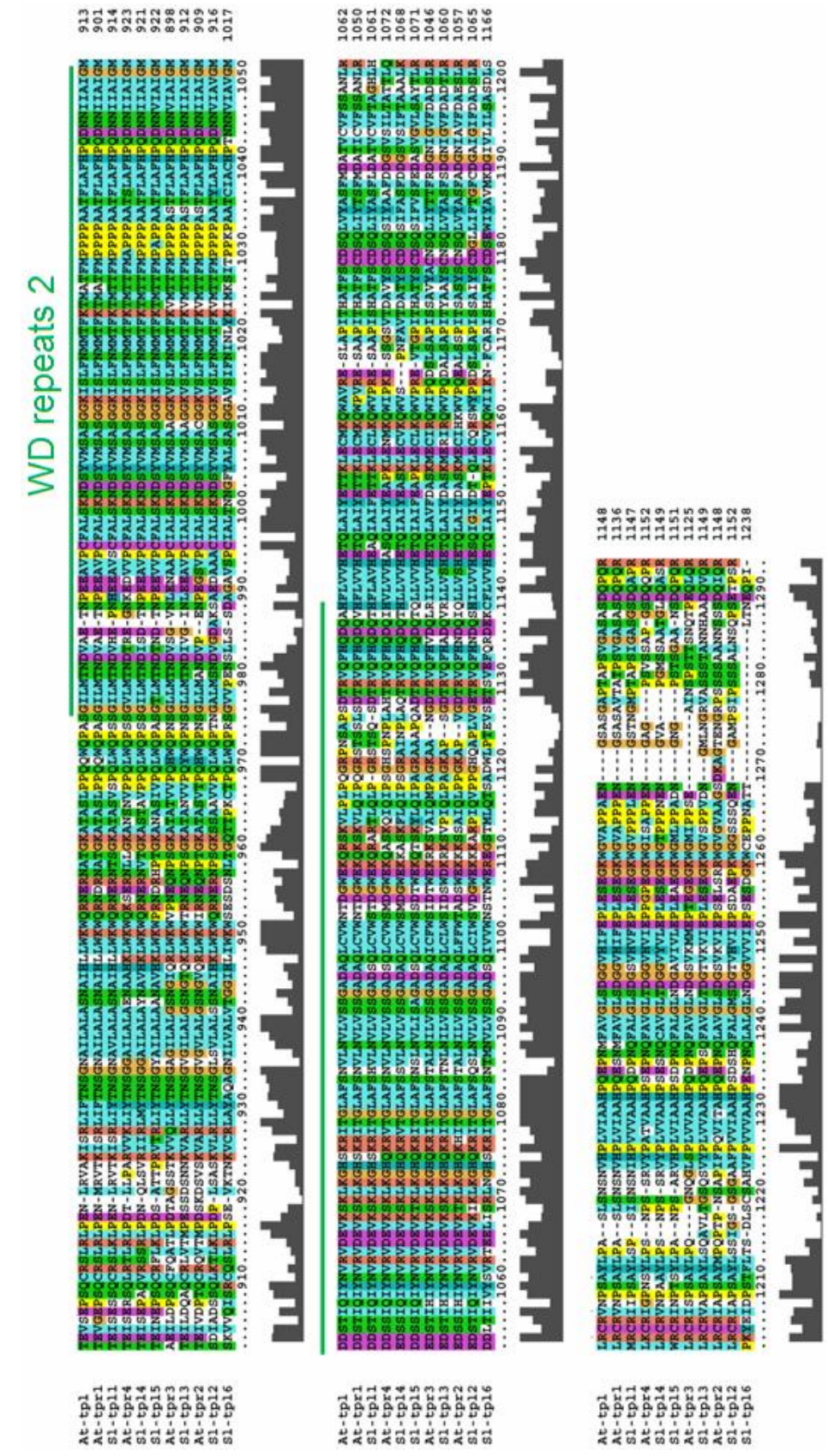


Figure 21. (End)

Tomato TPLs are distributed on four chromosomes. Two Sl-TPLs (Solyc03g116750, Solyc3g117360) on chromosome 3, two (Solyc08g076030.2.1, Solyc08g029050.2.1) on chromosome 8, one (Solyc01g100050.2.1) on chromosome 1 and one (Solyc07g008040.2.1) on chromosome 7. Three additional truncated-TPL sequences lacking the LisH and CTLH domains among which two are located on chromosome 3 (Solyc03g117370, Solyc03g117410) and one on chromosome 1 (Solyc05g016070).

The number of „full-length" TPL genes in tomato falls in the range found in other plant genomes which varies in Angiosperms from 4 members in Monocots to 11 members in soybean (Figure 22). Noteworthy, a high number of isoforms is often observed in organisms having undergone recent whole-genome duplication or polyploidisation events (G. max, N. benthamiana or B. rapa).

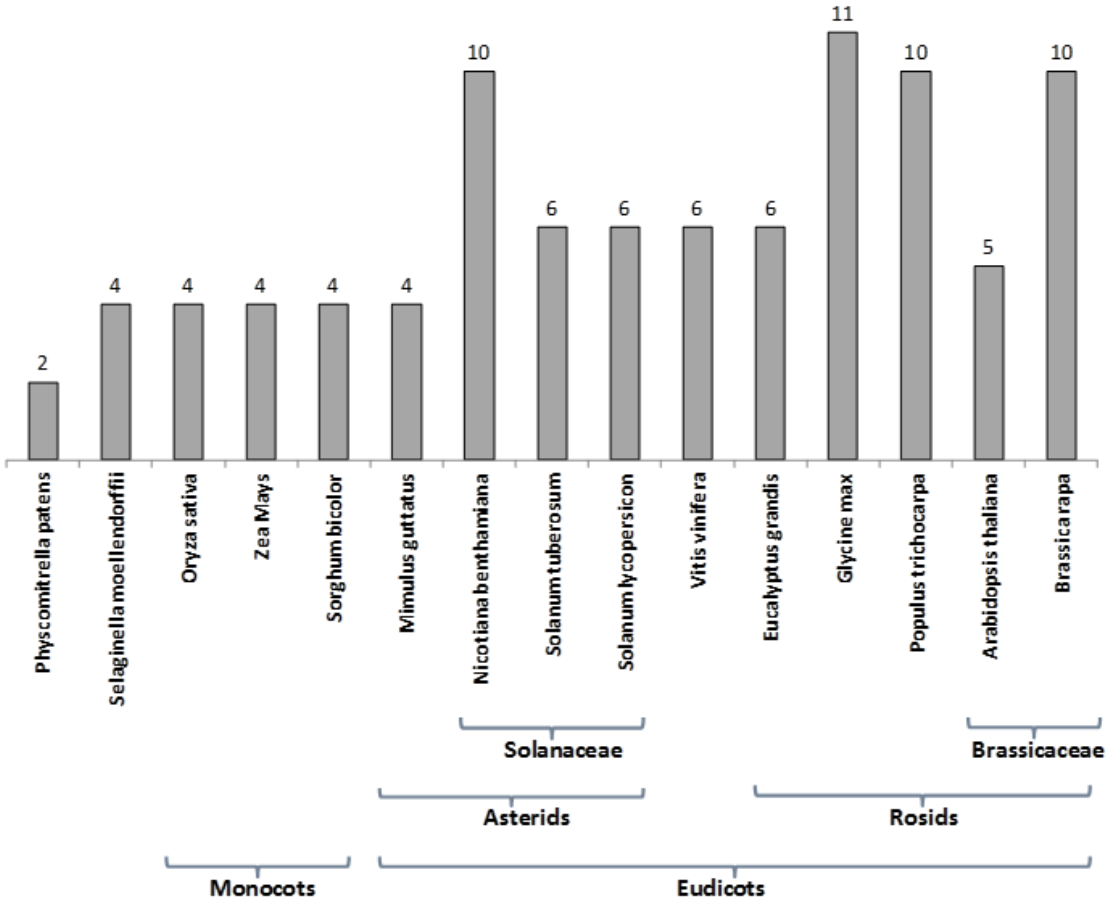
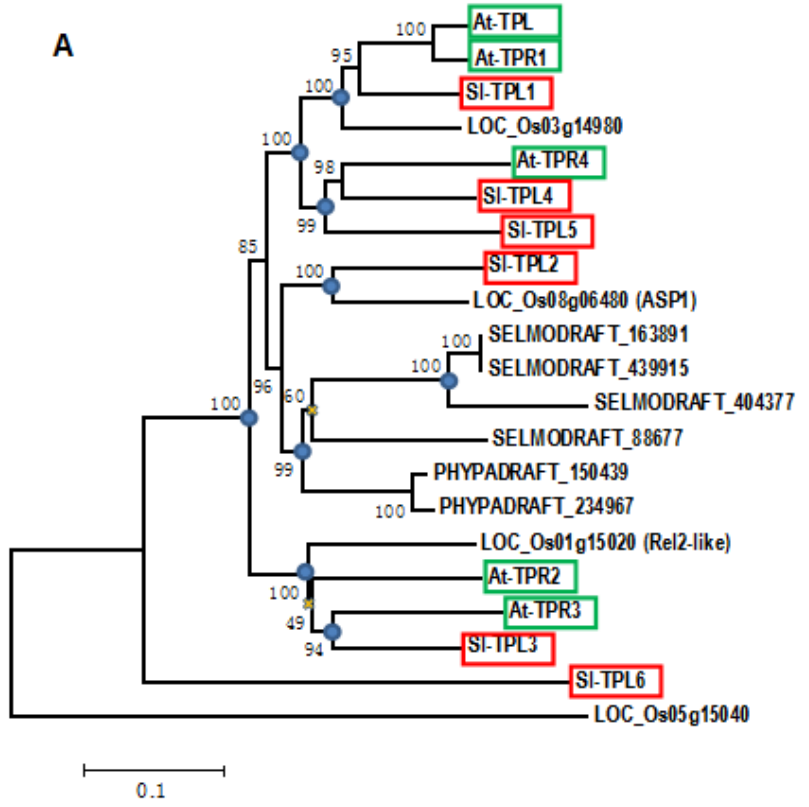


Figure 22. Inventory of *TOPLESS* genes in different plant genomes. Only TPL genes containing the four canonical domains (LisH, CTLH and two WD40 repeats) were considered. Major taxons are reported below.

2.3.2 Sl-TPLs nomenclature and phylogenetic analyses

To adopt a nomenclature consensual with that of Arabidopsis TPL and TPL-related proteins, we carried phylogenetic analyses on different TPL-like proteins or cDNAs from different plant sequenced genomes comprising moss, fern and various angiosperm sequences (see Methods). The phylogenetic trees (Fig. 23A) allowed the individualization of 4 branches. Three branches looked well defined in all Dicot plants: a first branch containing At-TPL, At-TPR1, At-TPR4, Solyc3g117360.2.1 (named Sl-TPL1), Solyc03g117360.2.1 (named Sl-TPL4) and Solyc07g008040.2.1 (named Sl-TPL5); a second branch, absent in Arabidopsis thaliana yet present in Eucalyptus (Eucgr.K00093.1|PACid:23601479) or grapes (GSVIVT01024440001) containing Solyc08g076030 (named Sl-TPL2), rice ASP1 protein and moss or lycophyte TPL-like proteins; and a third branch containing At-TPR2, At-TPR3, and Solyc01g100050.2.1 (named Sl-TPL3). At last, Solyc08g029050.2.1 (named Sl-TPL6) appeared as an out-group branch in the phylogenetic tree (Fig. 23A). The robustness of the tree topology was assessed either with the bootstrap test (Fig. 23A) or by changing the number of genomes used in phylogeny and the portion of the aligned sequence (N-terminal, C-terminal or conserved domains) or the clustering method (Neighbor-Joining or Maximum Likelihood). The vast majority of the nodes presented in Fig. 23A remained unchanged.

To further understand the TPL phylogeny, and notably characterize the Sl-TPL6 out-group, the presence of TPL „orthologous" was investigated in Asteroid genomes belonging either to the Solanaceae family (Solanum tuberosum and Nicotiana benthamiana) or to the Lamiales order (Mimulus guttatus). A Sl-TPL6 homologue was found in all Asteroids, supporting the view that Sl-TPL6 homologues form a distinct clade (Fig. 23B). Within this Sl-TPL6 clade, the length of the branches suggests that these isoforms evolve faster than other TPLs. This observation is supported by sequence divergences: the amino acid substitution rates calculated within Solanaceae orthology groups varying from 2.6 to 6.3% for Sl-TPL1-5 and reaching 22.7% for the Sl-TPL6 (Table 8). Moreover, a neutrality test (dS-dN values) calculated on Solanaceae orthologues suggests that the purifying selection exerted by evolution on Sl-TPL6 family is much weaker than the selection pressure exerted on other TPL genes.


A
At-TPL
At-TPR1
Sl-TPL1
LOC_Os03g14980
At-TPR4
Sl-TPL4
Sl-TPL5
Sl-TPL2
LOC_Os08g06480 (ASP1)
SELMODRAFT_163891
SELMODRAFT_439915
SELMODRAFT_404377
SELMODRAFT_88677
PHYPADRAFT_150439
PHYPADRAFT_234967
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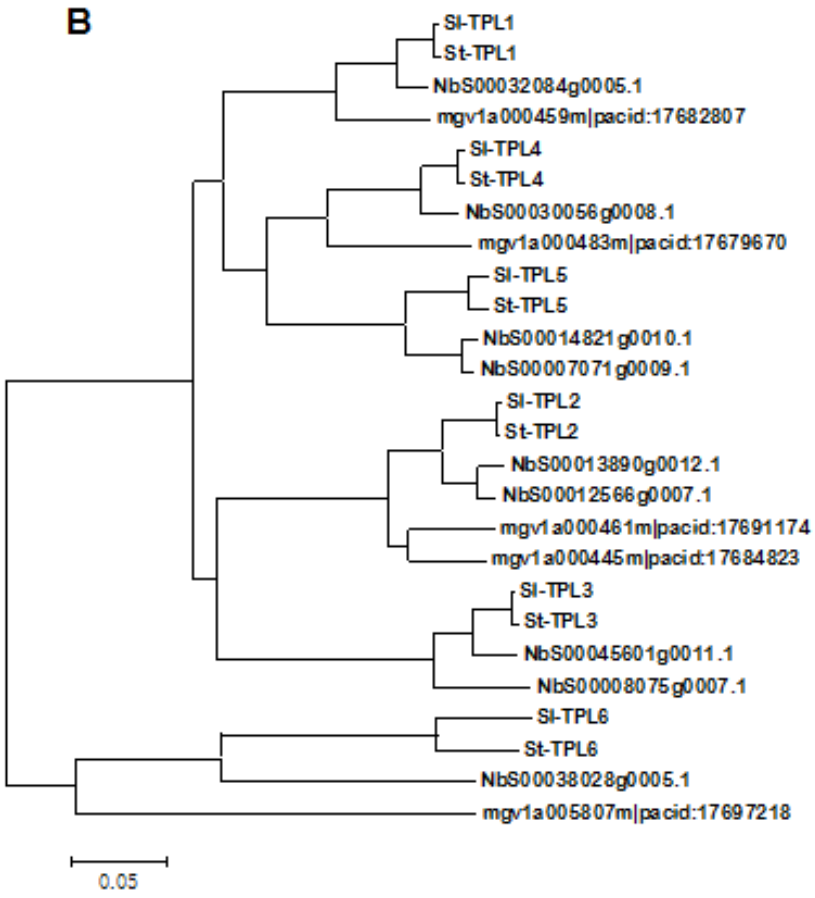
At-TPR3
Sl-TPL3
Sl-TPL6
LOC_Os05g15040
0.1


B
Sl-TPL1
St-TPL1
NbS00032084g0005.1
mgv1a000459m|pacid:17682807
Sl-TPL4
St-TPL4
NbS00030056g0008.1
mgv1a000483m|pacid:17679670
Sl-TPL5
St-TPL5
NbS00014821g0010.1
NbS00007071g0009.1
Sl-TPL2
St-TPL2
NbS00013890g0012.1
NbS00012566g0007.1
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mgv1a000445m|pacid:17684823
Sl-TPL3
St-TPL3
NbS00045601g0011.1
NbS00008075g0007.1
Sl-TPL6
St-TPL6
NbS00038028g0005.1
mgv1a005807m|pacid:17697218
0.05

Figure 23. Phylogenetic trees of some plant and tomato TOPLESS. (A) Representative phylogenetic tree of TPL proteins from land plants: moss *P. patens* (PHYPADRAFT_xxx), lycophyte *S. moellendorffii* (SELMODRAFT_xxx), rice (LOC_Os-xxx), tomato (red characters) and Arabidopsis (green characters). The colored brackets emphasize the main branches conserved among Angiosperms. The present tree was obtained after alignment of full-length TPL sequences using ClustalW and clustering with the Neighbour-Joining Method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Phylogenetic analyses including additional genomes sets (Z. mays, S. bicolor, P. trichocarpa, G. max, V. vinifera, M. guttatus) or using the Maximum-Likelihood clustering method displayed similar topologies, the majority of the nodes being conserved (blue circles) while only few nodes (yellow crosses) were unstable. (B) Phylogenetic tree of TPL proteins among Asteroid and Solanaceaous species. The tree was built using sequences from four genomes: S. lycopersicon, S. tuberosum, N. benthamiana and M. guttatus.

Table 8. Evolutive features of TOPLESS-related genes in Solanaceous species. Mean distance was expressed as the proportion of amino acid or nucleic acids positions different after pairwise alignment. dS-dN values were calculated using the Codon-based Test of Purifying Selection performed on each pair of orthologous sequences from S. lycopersicon and S. tuberosum. The variance of the difference was computed using the bootstrap method (500 replicates). Analyses were conducted using the Nei and Gojobori (1986) method.

		Sl-TPL 1	Sl-TPL 2	Sl-TPL 3	Sl-TPL 4	Sl-TPL 5	Sl-TPL 6
Mean Distance (Solanum/Nicotiana)	Amino Acids	0.026	0.041	0.032	0.029	0.063	0.227
	Nucleic acids	0.055	0.050	0.054	0.054	0.067	0.154
Neutrality test (Solanum)	dS-dN	7.08	6.66	6.98	7.62	6.19	3.645

2.3.3 Subcellular localization of Sl-TPLs

The subcellular localization of the Sl-TPL proteins was assessed by transient expression assay in tobacco protoplasts using a translational fusion between each of the Sl-TPL proteins and the Yellow Fluorescent Protein (YFP). Microscopy analysis showed that Sl-TPL1-5-YFP fusion proteins exclusively localized to the nucleus (Fig. 24) whereas Sl-TPL6 was localized at the cytoplasm and excluded from the nucleus. This result is in agreement with the in silico prediction of a conserved Nuclear Localization Signal (NLS) for the five nuclear Sl-TPL1-5 while Sl-TPL6 NLS scores were below the 5.0 threshold value (Table 9). Altogether, the nuclear localization of the majority of Sl-TPLs is consistent with their putative role in transcriptional regulation activity.

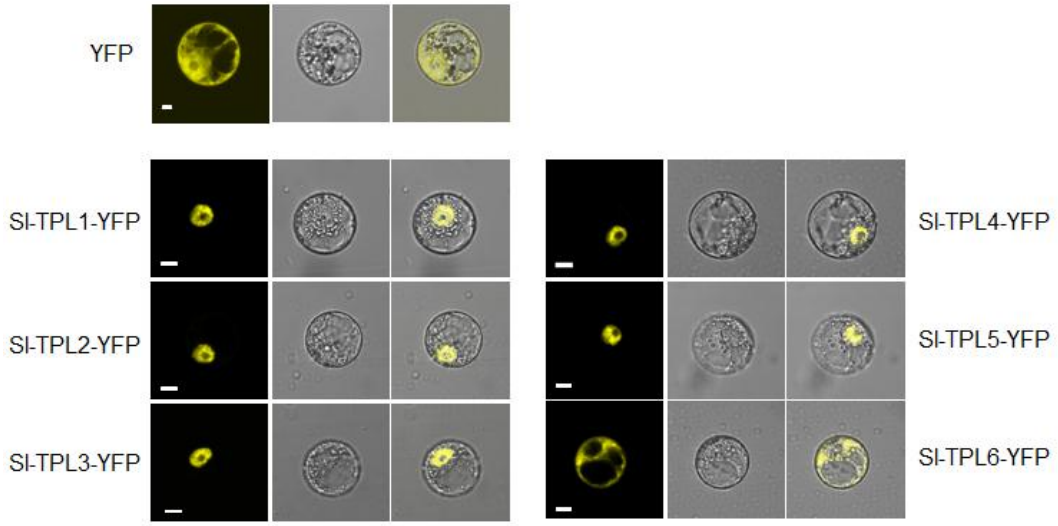


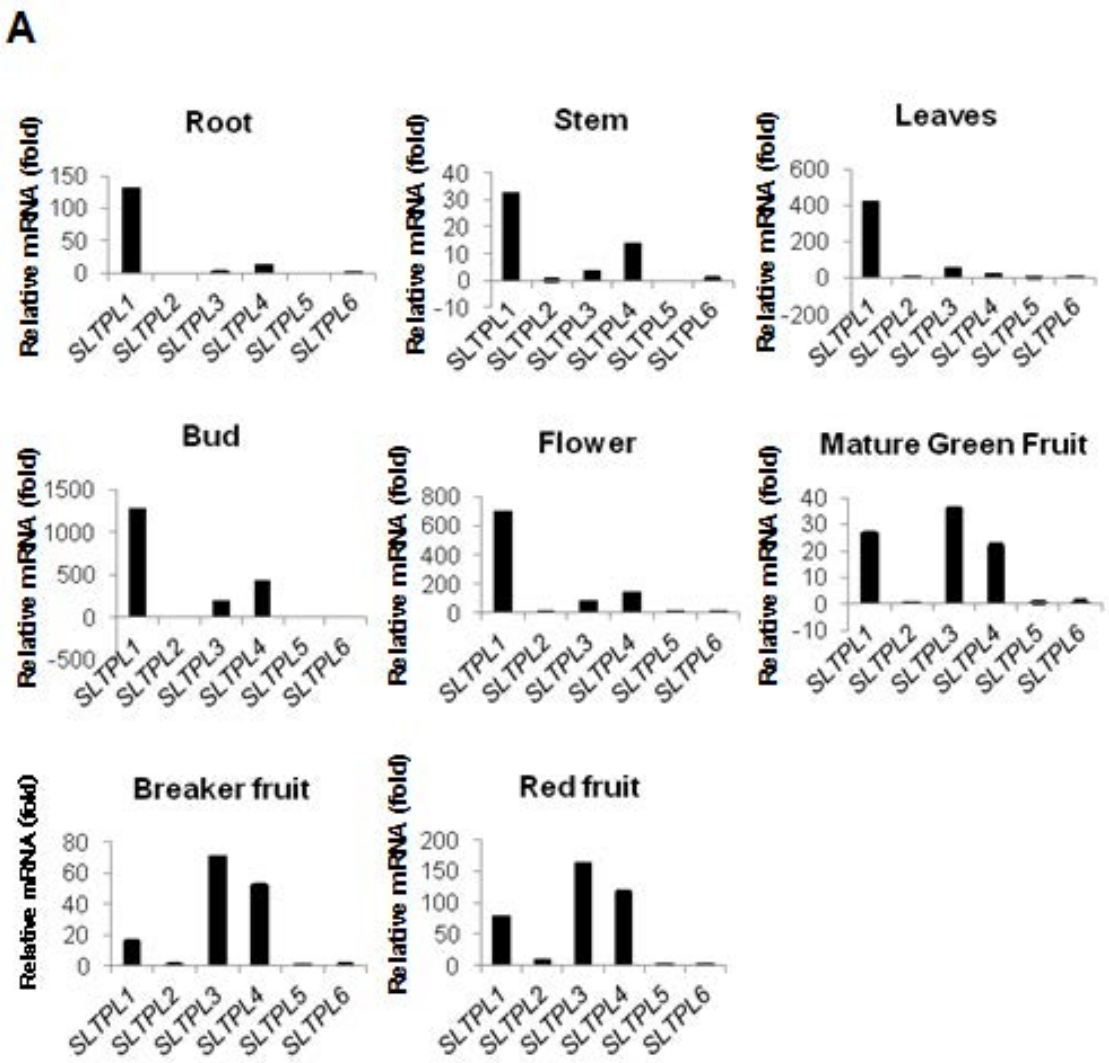
Figure 24. Subcellular localization of tomato TOPLESS proteins. Sl-TPL-YFP fusion proteins were transiently expressed in BY-2 tobacco protoplasts and subcellular localization was analyzed by confocal laser scanning microscopy. The merged pictures of yellow fluorescence channel (left panels) and the corresponding bright field (middle panels) are shown (right panels). The empty vector pEarleyGate104 was used as control. The scale bar indicates 10 μm.

Table 9 : NLS prediction scores computed with cNLS Mapper (Kosugi et al, 2009)

Sl-TPL	Score	Position	NLS sequence	Structure
Sl-TPL1	6	588	rtyqgfrkrslgvvqfdttknrfl	Bipartite
Sl-TPL2	5	304	hlmkrmrag	Monopartite
Sl-TPL3	5.8	283	ilkrpltppatlgmldyqsadheqlmkrl	Bipartite
Sl-TPL4	5.3/ 5	74	feirkqkylealdrhdqakaveilvkdlkv	Bipartite
		163	fpslknsrlrtlinqslnwqhqlcknpkp	
Sl-TPL5	5.2/5.7	-37	feirkqkylealdrndrpkaveilvkdlkv	Bipartite
		-249	mlkrprtptnnsavdyqtadsehmlkrsrp	
Sl-TPL6	4.1/ 4.2	-139	rnrimkilrvvietnpqlngklhfpeltksrl	Bipartite
		-271	mpkpskaisaatpaqlvkqmpgpskaisa	

2.3.4 Expression analyses

In order to study the spatio-temporal expression pattern of the six Sl-TPL genes, quantitative reverse transcription PCR (qRT–PCR) was performed on eight different plant tissues and organs. Three *Sl-TPL* members (*Sl-TPL1*, *Sl-TPL3* and *Sl-TPL4*) displayed significantly higher level of expression than the three remaining paralogs. *Sl-TPL1* and *Sl-TPL4* were found to be highly expressed in flowers and vegetative tissues (roots, stems, leaves) and in developing flowers (bud and during anthesis) but with a reduced expression in ripening fruit while *Sl-TPL3* expression remained constant and high during fruit ripening (Fig. 25). This preferential expression of *Sl-TPL1*, *Sl-TPL3* and *Sl-TPL4* is coherent with their estimated expression in two public databases (RNAseq database: http://ted.bti.cornell.edu and ESTs database: http://solgenomics.net/). Although less expressed, *Sl-TPL2* was found preferentially in leaves and developing flowers; the levels of *Sl-TPL5* transcripts were low in all tissues; Sl-TPL6 expression was restricted to roots and stems (Fig. 25b).



(Figure 25. To be continued)

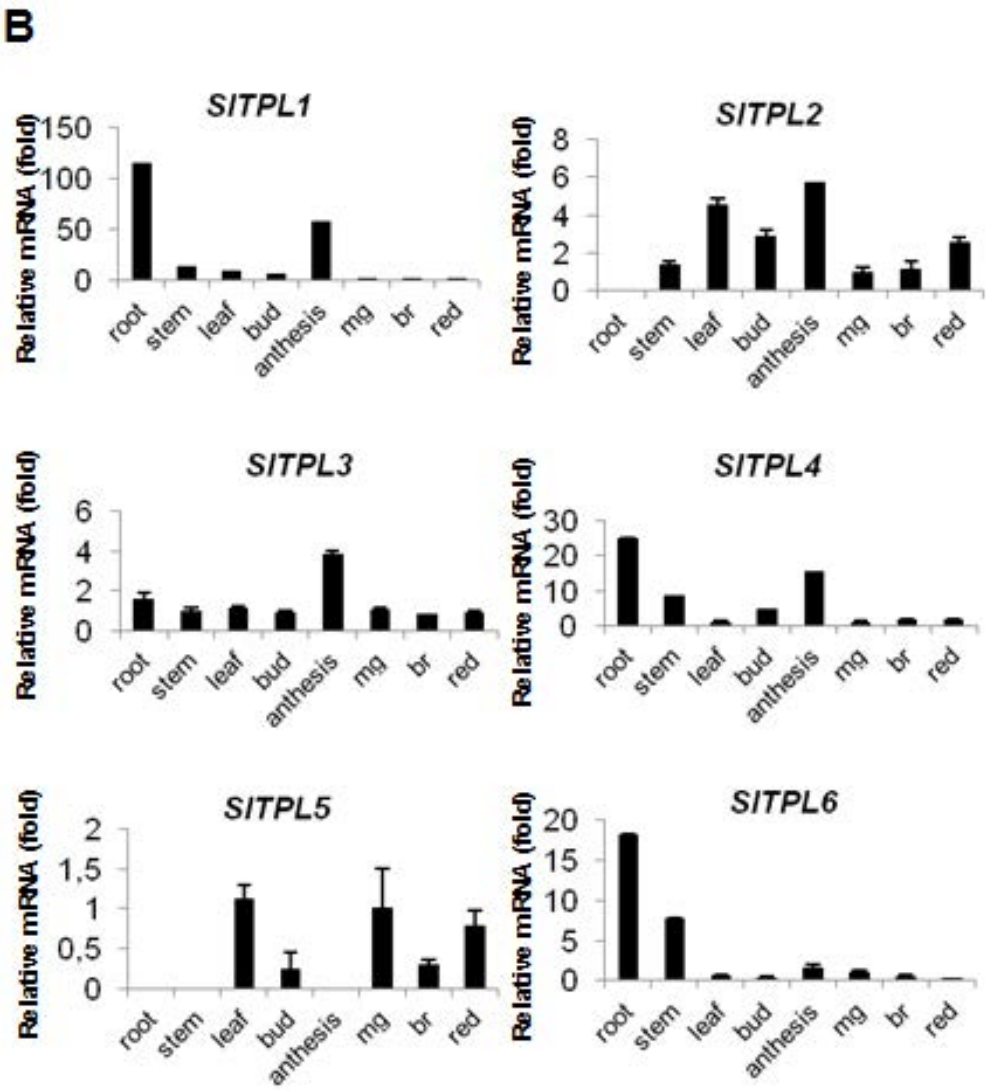


Figure 25. Real-time PCR expression profiles of 6 tomato *TOPLESS* genes. (A): Expression patterns of Sl-TPL genes in each tomato tissues. Relative mRNA levels of each Sl-TPL gene in different tissues were normalized with actin. The results were expressed using the Sl-TPL6 as a reference (relative mRNA level 1). Values represent the best experiment among three independent biological repetitions. Bars indicate the standard deviation of three experimental repetitions. (B): Expression patterns in different tomato tissues of each *Sl-TPL* genes. The relative mRNA level of each *Sl-TPL* gene was normalized with actin. mg: mature green fruit, br: breaker fruit, red: red fruit. The results were expressed using the mg as a reference (relative mRNA level 1). Values represent the best experiment among three independent biological repetitions. Bars indicate the standard deviation of three experimental repetitions.

2.3.5 Examination of protein-protein interactions (PPIs) in the frame of the auxin mediation

The differential expression of *Sl-TPL* genes evokes the critical question of functional redundancy within the TPL family. In a recent paper, Causier et al., (2012) have compared the protein-protein interactions patterns of different Arabidopsis TPL proteins using a high-throughput yeast two-hybrid screen both on a whole-plant and on a transcription factor library. In the present work, we focused the interaction study on the Aux/IAA family by performing an exhaustive targeted analysis of Aux/IAA-TPL interactions. The six Sl-TPL proteins were fused to a binding domain (BD) and used as baits in a yeast two-hybrid test with 17 different Sl-IAA fused to an activating domain (AD). After monitoring the yeast growth on two auxotroph selective media, two patterns of TPL could clearly be defined (Fig. 31A and B): Sl-TPL1, Sl-TPL2, Sl-TPL4, and Sl-TPL5 interacting with the majority of Sl-IAAs and growing in all the selective media and Sl-TPL3 and Sl-TPL6 exhibiting only a limited growth when co-expressed with Aux/IAA-AD fusion proteins. Contrary to other Sl-IAAs, Sl-IAA29 failed to show interaction with any of the Sl-TPLs. With exception of Sl-IAA12 and Sl-IAA15, the Aux/IAAs do not harbor any obvious specificity towards the „TPL" clade (Sl-TPL1, Sl-TPL4 and Sl-TPL5) sharing high similarity with At-TPL. In addition, Sl-TPL2, which belongs to a distinct clade of Sl-TPLs (1, 4 and 5), also exhibits a broad capacity to interact with the majority of Sl-IAAs. As a control, we performed a yeast two-hybrid test with truncated Sl-TPL1 or Sl-TPL5 (ΔLisH-TPL) (Fig. 26C) lacking the LisH domain previously shown to be essential for TPL-WUS or TPL-Aux/IAA interactions (Kieffer et al., 2006, Szemenyei et al., 2008). Contrary to all Sl-TPLs BD-fusions assayed, a complete lack of growth was observed when co-expressing BD-ΔLisH-TPL proteins with AD-Aux/IAAs (Fig. 26B). In addition, the permutation of bait and prey was performed using Sl-TPL-AD fusions and Sl-IAA-BD fusions; results are recapitulated on appendix A9. Only Sl-TPL5 showed a different behavior that remained unexplained.

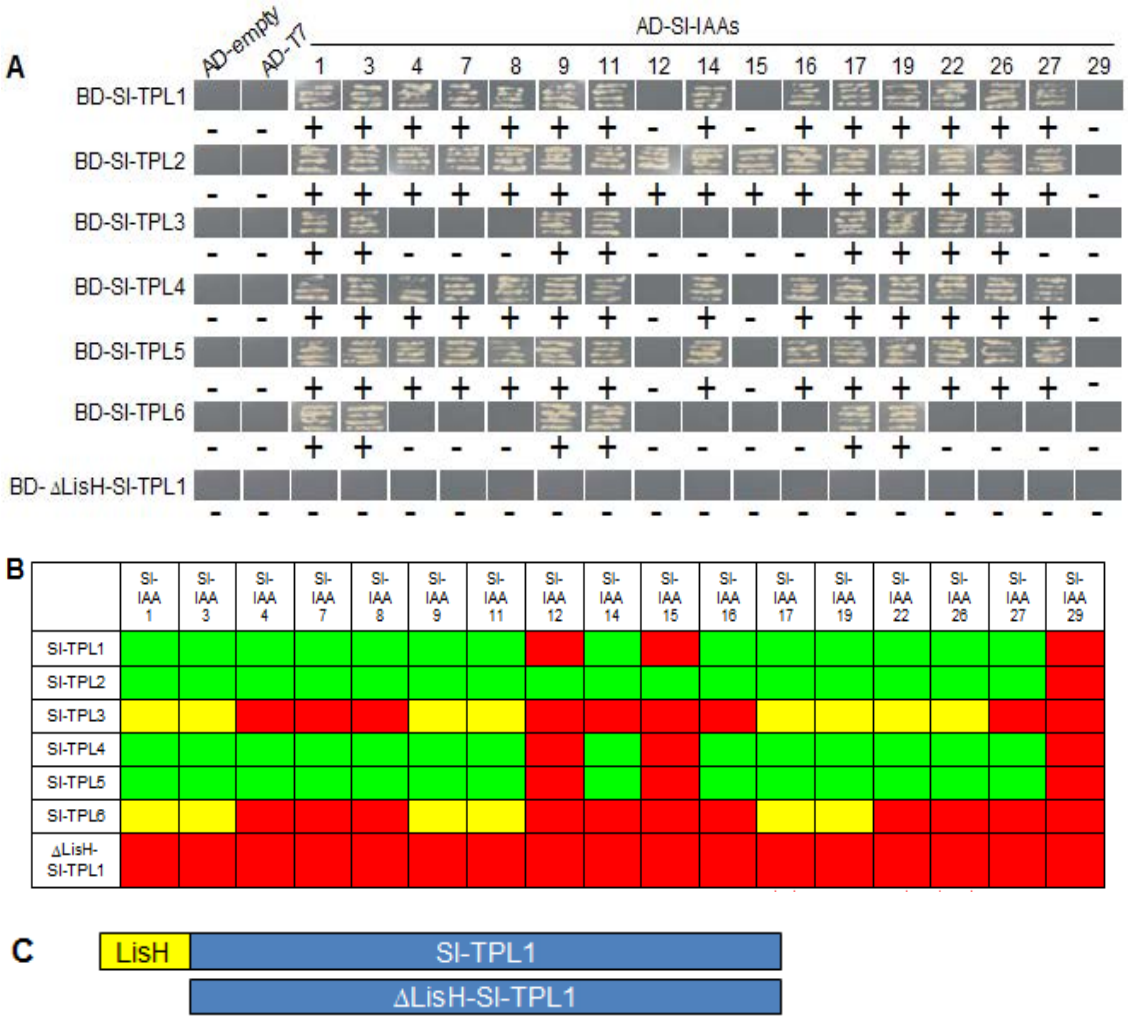


Figure 26. Protein-protein interaction map between Sl-TPLs and Sl-IAAs established by Y2H screen. (A) Yeast growing of co-transformed BD-TPLs and AD-IAAs. The yeast clones grown on selected medium lacking Trp, Leu, His, and Ade (TLHA) were scratched again on the TLHA plate. After 3-4 days, the growth of the yeast strains confirms the positive interaction, as shown in the upper panel picture. AD-empty vector and AD-T7 vector were used as negative controls. (B) Schematic representation of the interaction map between Sl-TPLs and Sl-IAAs. Green color means yeast grew quickly less than 4 or 5 days after co-transformation, indicating a strong interaction between Sl-TPLs and Sl-IAAs partners. Yellow shows yeast grew slowly in 7-8 days after co-transformation indicating a weak interaction between the tested Sl-TPL and Sl-IAA. Red means there is no interaction detected between the tested Sl-TPLs and Sl-IAAs. (C) Truncated form of Sl-TPL1 protein lacking the N-terminal LisH domain N-terminal used as a negative control.

2.4 Conclusion and discussion

The present study addresses the structural, evolutionary and functional features of tomato TPL family. TPL proteins were primarily defined as a major component of the auxin transduction and response pathway, but the present data sustain the hypothesis of a functional diversification of these regulatory proteins. While mainly focusing on the TPL family in tomato, a plant model for Solanaceae and fleshy fruit research, the data also address the comparative features of this gene family within plant kingdom at the evolutionary level, shedding new light on their functional diversification.

The structure of the Sl-TPL family is representative of that found in Angiosperms where these proteins belong to a small multigenic family comprising 5 to 11 members. In the tomato, 6 full-length Sl-TPL genes were identified regardless of the additional 3 pseudogenes with incomplete coding sequences. Among the six Sl-TPL genes, five were highly conserved (Sl-TPL1-5), while the last gene (Sl-TPL6) was more distant. With the exception of poplar genomes and genomes having undergone recent polyploidisation (i.e. *Glycine max*, *Brassica rapa* and *Nicotiana benthamiana*), the number of TPL isoforms ranges from 4 to 6 members, suggesting that the number of genes remains stable in this family and that usually, after a whole-genome duplication event, duplicated copies of TPL genes are not retained. The phylogenetic analysis of TPL genes enabled the distinction of three major clades gathering homologues in the majority of Angiosperm genomes. A last clade, containing the distant Sl-TPL6, displays only clear homologues in closely-related taxa (Asteroids). Interestingly, highly-diverging sequences of TPL-related proteins were also found in other genomes such as the At-TPR-like gene (At2g25420, Causier et al., 2012b) or in poplar but no clear relationship could be established with Sl-TPL6. Contrary to Angiosperms TPL proteins, TPL from *P. patens* and *S. moellendorffii* clustered in a same branch, indicating the existence of ancestral divergences occurring before Angiosperm radiation.

The functionality of Sl-TPL genes was addressed through three approaches: expression analysis, subcellular localization and establishment of an interaction map between Sl-TPL and Sl-IAA proteins. The expression patterns of different Sl-TPLs reveal the tissue-specificity of various isoforms and suggest a functional specialization of Sl-TPL isoforms. For example,

Sl-TPL1 is highly expressed in vegetative organs (stems, roots) and flowers, while the expression of Sl-TPL3 and Sl-TPL4 is prevailing in fruit. Moreover, the overall intensity of gene expression evaluated by quantitative PCR evidenced a distinction between a group of three isoforms (Sl-TPL1, Sl-TPL3 and Sl-TPL4) that are highly expressed, Sl-TPL2 which is moderately expressed in the leaves and flowers, and third group made of two isoforms (Sl-TPL5 and Sl-TPL6) that displays very low level of expression. In agreement with our data, the prevalence of Sl-TPL1, Sl-TPL3 and Sl-TPL4 transcripts was also observed in EST and RNAseq expression databases (http://ted.bti.cornell.edu) whereas the expression of Sl-TPL6 was again found to be very low (no EST and few RNAseq reads). Interestingly, the overall expression level negatively correlates with the amino acid substitution rate. Indeed, after defining orthology groups among Solanaceous TPLs, we found that the highly expressed isoforms (Sl-TPL1, Sl-TPL3 and Sl-TPL4) show the highest amino-acid sequence conservation (<3.2% difference within Solanaceous sequences) while sequences were less conserved within the Sl-TPL6 orthology group (22.7% difference within Solanaceous sequences). The moderately expressed Sl-TPL2 and Sl-TPL5 displayed intermediate substitution rates (4% and 6% difference respectively). This correlation was also supported by a neutrality test (dS-dN values) performed between potato and tomato pairs of othologues. The high substitution rate within Sl-TPL6 orthology group was interpreted as an indication that Sl-TPL6 subfamily undergoes a reduced purifying selection. By contrast, broadly expressed Sl-TPL isoforms are under a stronger purifying selection. Such a correlation between gene expression level and amino acid substitution rate has already been observed in genome-wide comparisons of expression patterns and protein evolution in Arabidopsis-related plants or in the Poaceae family (Wright et al., 2004, Slotte et al., 2011 and Davidson et al., 2012). Indeed, this correlation is consistent with Arabidopsis thaliana expression data (AtGenExpress), At-TPL being more expressed than other At-TPRs and At-TPL orthologs remaining highly conserved either in *Arabidopsis lyrata* or in *Brassica rapa*.

The subcellular localization establishes a second discrimination criterion between Sl-TPLs. GFP fusion proteins of Sl-TPL1 to 5 isoforms all migrated exclusively to the nucleus, as observed with other TPL proteins from Arabidopsis (Long et al., 2006), maize

(Gallavotti et al., 2010) or rice (Yoshida et al., 2012). By contrast, Sl-TPL6-GFP fusion protein displayed a divergent subcellular targeting, this isoform being addressed to the cytosol. This divergent localization is in line with the lower scores calculated by the NLS prediction tool for Sl-TPL6. This observation, in addition to the low expression level and the high substitution rate, supports the view of either a partial loss of functionality or divergent functionality regarding Sl-TPL6.

The first established function of TPL proteins is related to their role in auxin signaling via interaction with Aux/IAA partners (Szemenyei et al., 2008). To check whether this role is conserved among all Sl-TPLs isoforms and gain insight on either functional redundancy or potential functional diversification among family members in tomato, a comprehensive protein/protein interaction study was carried out between all Sl-TPLs and Sl-IAA members using a yeast two-hybrid screen. This targeted interactome study revealed two distinct patterns of interaction for tomato TPLs: four isoforms (Sl-TPL1, Sl-TPL2, Sl-TPL4 and Sl-TPL5) displaying a broad capacity of interaction with the majority of Sl-IAAs and the remaining two isoforms (Sl-TPL3 and Sl-TPL6) showing a more restricted interaction capacity. Noteworthy, a large number of Sl-IAAs show positive interaction with Sl-TPLs, consistent with the outcome of yeast two-hybrid screens performed in Arabidopsis where 20 out of the 29 At-Aux/IAAs were able to interact with At-TPLs (Szemenyei et al., 2008; Arabidopsis Interactome Mapping Consortium, 2011; Causier et al., 2012a). Interestingly, neither Sl-IAA29 nor its arabidopsis homologue At- IAA29 (AT4G32280.1) interacts with TPL proteins although Sl-IAA29 exhibits a repressor activity (Audran-Delalande et al., 2012). On the other hand, the limited interaction capacity displayed by Sl-TPL6 adds another distinctive feature to this isoform which already diverged from other family members by its low expression level, high amino acid substitution rate and different subcellular localization. Altogether, the cumulative distinctive features support the idea that Sl-TPL6 has partially lost its ancestral function and may have gained new functionality.

To compare within Arabidopsis, the selectivity in Aux/IAAs-TPL/TPRs interaction has been demonstrated by the Y2H assay in two studies (Szemenyei et al. 2008; Causier et al. 2012a). There are seven (IAA2, 3, 4, 11, 13, 26, and 28) out of fifteen Aux/IAAs were shown

to interact with TPL in both studies, and two Aux/IAAs (IAA7 and 16) did not interact with TPL. However, four Aux/IAAs-TPLs interactions showed inconsistent result between two studies, indicating that more confirmatory experiments are required. Their data also interestingly showed a high selectivity in the interaction between Aux/IAAs and TPL/TPRs. This leads to the possibility that different Aux/IAAs, recruiting different TPL/ TPRs, regulate the target genes in a diverse manner. Although TPL/TPRs have recently been shown to recruit histone deacetylase to the target gene (Krogan et al. 2012; Wang et al. 2013), more detailed molecular mechanism for the TPL/TPR function and functional diversity of TPL/TPR members remain to be characterized. In previous yeast two-hybrid screen performed in Arabidopsis by Causier et al., (2012a), AtTPR3 and At-TPR2, closely related to Sl-TPL3, both displayed the capacity to interact with various Aux/IAA proteins. However, a closer look to the interaction map published by Causier et al could also suggest differences of specificity between At-TPL and At-TPR2 or At-TPR3 with At-TPR2 and At-TPR3 notably interacting with partners displaying partial repression domains. Such hypothesis opens the possibility that At-TPR2, At-TPR3 and the closely related Sl-TPL3 display a specialization alternative to auxin signaling.

Functional redundancy among Arabidopsis TPL family members is supported by the absence of obvious phenotypes in single loss-of-function mutants of At-TPL/TPR genes and by the requirement for the down-regulation of all five At-TPL-TPRs in order to phenocopy the dominant mutation tpl-1 (Long et al., 2006). However, this assumption is contrasting with the situation prevailing in rice and maize where genetic evidences seem to support a more specialized functionality for TPL genes. That is, in rice (Yoshida et al., 2012), a single recessive mutation in Asp1, a TPL-like gene close to Sl-TPL2, exhibited several pleiotropic phenotypes, such as altered phyllotaxy and spikelet morphology. While these phenotypes suggest a close association of Asp1 with auxin action, they clearly reveal that the specialization of TPL-related proteins in some organisms can differ from that in Arabidopsis. Further evidence sustaining a diversified function for TPL proteins is provided by maize rel2 mutants affected in a TPL-like gene closely related to Sl-TPL3 and At-TPR3 (Gallavotti et al., 2010).

Altogether, the data shed new light on structural, evolutionary and some functional features of tomato TPL gene family in tomato that suggest functional diversification of these regulatory proteins. Of particular interest, the setup of a comprehensive TPL-Aux/IAA interactions map and the differential subcellular targeting of some Sl-TPLs proteins, provide important clues towards designing appropriate strategies for the elucidation of both redundant and specific roles of TPL genes.

3. Complementary results

Since 6 TOPLES members have been identified in tomato in our previous work, the interactions map between Sl-TPL, Sl-IAA and Sl-ARF families could be constructed by Y2H assay. In this part of complementary results, interactions between Sl-TPL and Sl-ARF families will be investigated. Also, the influence of the different TPL domains on these specific interaction patterns will be analyzed.

3.1 PPIs between whole Sl-TPLs and Sl-ARFs family

Y2H was used to test the interaction relationship between the whole family members of tomato TPLs and ARFs. Y2H protocol refers to Appendix A.1.

Tomato *ARF*-1, 2a, 2b, 3, 4, 5, 6, 7, 8a, 9a, 9b, 10a, 10b, 16a, 16b and 17 were cloned into Y2H pGAD-vector respectively as AD-prey protein. The interactions between BD-TPLs and AD-ARFs were tested with these different partners respectively:

BD-TPL1, 2, 3, 4, 5 and 6

AD-ARF1, 2a, 2b, 3, 4, 5, 6, 7, 8a, 9a, 9b, 10a, 10b, 16a, 16b and 17

Figure 27 showed the interaction result between all BD-TPLs and AD-ARFs. Comparing with the interaction result of IAAs/ARFs, none of the ARFs activators (ARF5, 6, 7 and 8a) showed interaction with any TPLs. But 10 ARFs repressors showed at least one interaction with Sl-TPL 1, 2, and 4. For Sl-TPL3, 5 and 6, there were no interactions discovered with any ARFs by Y2H system. The permutation of bait and prey displayed a similar result (Appendix

A9).

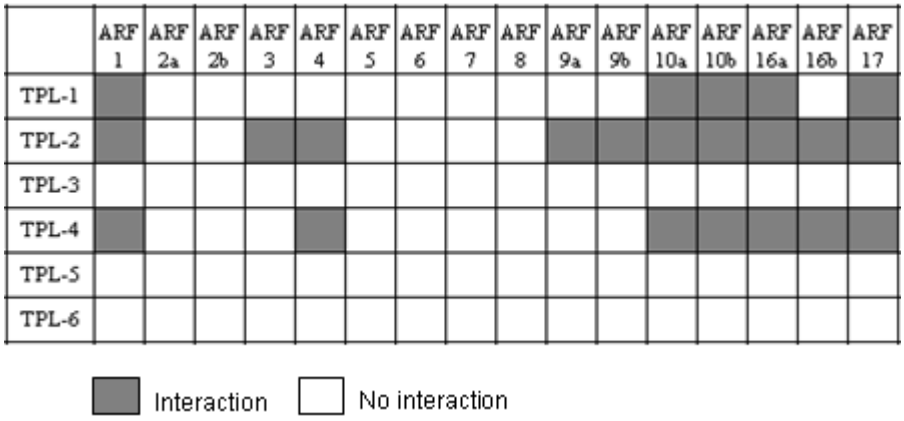


Fig. 27 The interaction map between BD-TPL and AD-ARF families. Black grid means there is an interaction between the two partners. Blank grid shows absence of interaction.

3.2 Involvement of the ARF4 N-terminal region in the TPLs/ARF4 interaction

N-terminal region (+1 ~ +1311 bp) of Sl-ARF4 without domain III and IV (ARF4-del; Fig. 28) was cloned into Y2H pGAD-vector to test if there is an interaction between TPLs and ARF4 N-terminal region. BD-TPL1, 2, 3, 4, 5 and 6 were used as bait proteins. AD-ARF4 and AD-ARF4-del were used as prey protein. BD-p53 and AD-T7 interaction was used to be positive control. Y2H was performed to test the interaction between these proteins.

Fig. 28 The construction of ARF4-del without domain III and IV.

The Yeast two-hybrid result shows that AD-ARF4-del interacted exclusively with BD-TPL2 and BD-TPL4 as well as AD-ARF4 full length (Fig. 29). That means the N-terminal of ARF4 is sufficient for the interaction between TPLs and ARF4. The ARFs possess on their N-terminal region, a DNA-binding domain (DBD) which is used to bind to TGTCTC auxin response elements within the promoter of target genes. Thus, it may indicate the middle SPL-rich RD region of some ARFs is necessary for the interaction between TPLs and ARFs.

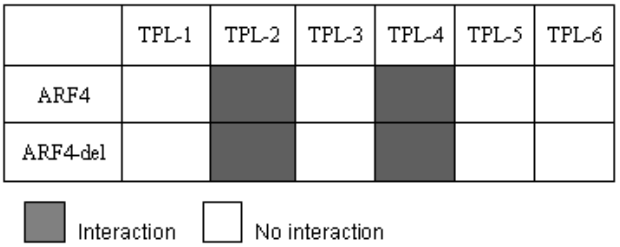


Fig. 29 The interaction result between BD-TPLs and AD-ARF4-del. Black grid means there is an interaction between the two partners. Blank grid shows there is no interaction.

3.3 PPIs Results of mutated Sl-TPL1

Protein sequence and phylogenetic relationship analysis showed that tomato TPL1, 2, 3, 4, and 5 are strongly conserved and we observed that all of them were expressed in nucleus. But the interaction results of TPLs/IAAs showed that Sl-TPL3 has a totally different behavior compared with other Sl-TPLs. As the Lish and CTLH domains are believed to drive the Aux/IAA -TPL interactions, we examined difference in the primary sequence. On the first 200 amino acids, only three sites exhibit a Sl-TPL3-specific signature consisting of three amino acids at positions 44, 99 and 162. Then we performed a mutagenesis experiment on these three amino acids on TPL1 to see if any of these specific amino acids would affect the interaction between Sl-TPLs and Sl-IAAs.

The three mutated forms of *Sl-TPL1* were amplified by PCR. Special "mutated" primers for PCR were listed on Table 10. For each mutation, we substituted one amino acid of Sl-TPL1 to the corresponding amino acid of Sl-TPL3: position 44: TCG (Ser) → AAA (Lys); position 99: AAA (Lys) → AGT (Ser) or position 162: CAG (Glu) → GTC (Val) (Fig. 30). All mutations were cloned into the Y2H BD-vector (BD-TPL1-mut-44, 99 and 162). Then we tested the interaction relationship between each Sl-TPL1 mutant and AD-IAA3, 7 and 9 by Y2H respectively. The full length native sequences of Sl-TPL1 and Sl-TPL3 was used as controls.

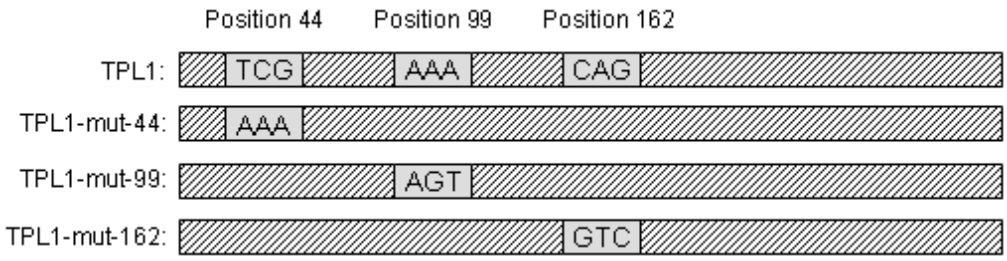


Fig. 30 Targeted mutagenesis sites of *Sl-TPL1*: position 44: TCG (Ser) → AAA (Lys); position 99: AAA (Lys) → AGT (Ser) or position 162: CAG (Glu) → GTC (Val)

Table 10 Primers for TPL1 mutations clone.

Name	Primers
TPL1-mut-44-F	GATAAAGTTACTAATGGAGAGTGGGAT
TPL1-mut-44-R	ATCCCACTCTCCATTAGTAACTTTATC
TPL1-mut-99-F	GTGGAGATTCTAGTGAGTGATCTG
TPL1-mut-99-R	CAGATCACTCACTAGAATCTCCAC
TPL1-mut-162-F	CGCGAGAAGCTTGTCTTTCCTA
TPL1-mut-162-R	TAGGAAAGACAAGCTTCTCGCG

The interactions between BD-TPL1-mut-44, 99, 162, BD-TPL1 full length and AD-IAA3, 7 and 9 were tested respectively. As the results showed (Fig. 31), TPL1 and all mutated forms interacted with IAAs very well. The comparison of the interaction results between TPL1/IAAs and TPL1-mut/IAAs indicated that the mutated amino acids did not affect the interaction of TPL1/IAAs. However, as TPL3 did not interact with these IAAs, it means these three amino acids are not responsible for the absence of interaction between TPL3 and Sl-IAA-3, -7 and -9. There is another mechanism that could suppress the interaction between TPL3 and IAAs. So, in next experiment, we would like to check if the WD40-repeat domain could affect these interactions between Sl-TPL3 and Aux/IAAs.

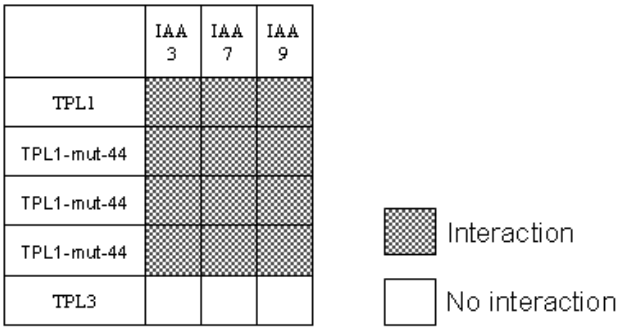


Fig. 31 The interaction result between BD-TPL1-mut and AD-IAAs. Black grid means there is an interaction between the two partners. Blank grid shows there is no interaction.

3.4 PPIs effect of the removal of WD40-repeat domain

In the mutagenesis experiment above, we tried to understand why Sl-TPL3 had a totally different TPLs/IAAs-interaction pattern compared to other TPLs (Sl-TPL1, 2 and 4). The target mutagenesis experiment presented in paragraph 3.3 showed the three mutated amino acids in N-terminal of TPL3 did not affect the interaction between TPL3 and IAAs. The absence of obvious additional Sl-TPL3 specific amino-acid at the N-terminal extremity of the primary sequence prompted us to test whether the WD40-repeat domains on the C-terminal of TPLs may also influence some specific interaction between TPLs/IAAs. We removed WD40-repeat domains from Sl-TPL3 and Sl-TPL4 (TPLs-WD-del; Fig. 32), and tested the interaction of these TPLs-WD-del proteins with some Sl-IAAs. Both TPL3-WD-del and TPL4-WD-del were cloned into the Y2H BD-vector (BD-TPL3-WD-del and BD-TPL4-WD-del). Y2H assay was used to test BD-TPL3-WD-del and BD-TPL4-WD-del interacting with AD-IAA3, 7 and 9 respectively. The full length of BD-TPL3 and BD-TPL4 interacting with AD-IAA3, 7 and 9 respectively were used as comparison.

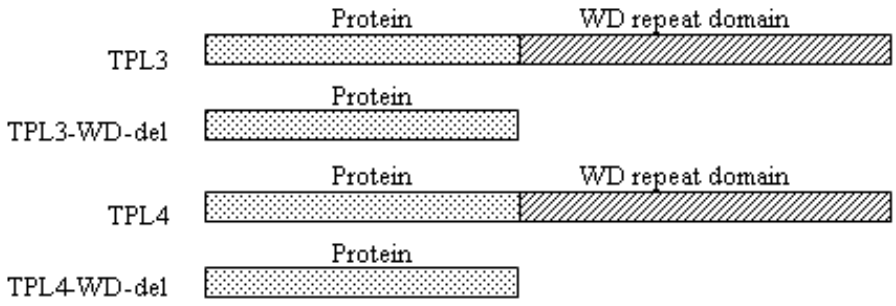


Fig. 32 The constructions of the TPL3 and TPL4 without WD repeat domain.

In this experiment, the interaction between BD-TPL3-WD-del and BD-TPL4-WD-del were positive with AD-IAA3, 7 and 9. The results (Fig. 33) showed that Sl-TPL4 full length and BD-TPL4-WD-del have the same behavior on interacting with Aux/IAAs. This means deleting WD40-repeat region does not affect the interaction of TPL4/IAAs. The results also showed that Sl-TPL3 full length did not interact with IAAs, but TPL3-WD-del interacted strongly with some Aux/IAAs. These results indicate clearly that, for Sl-TPL3, WD-repeat region is crucial for its interaction with Aux/IAAs. The presence of WD40-repeats region apparently suppresses the interaction between Sl-TPL3 and Aux/IAAs. This may also affects the interaction between Sl-TPL3 and ARFs.

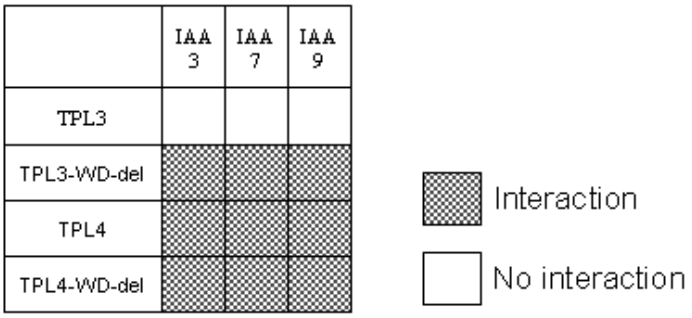


Fig. 33 The interaction result between BD-TPL1-mut and AD-IAAs. Black grid means there is an interaction between the two partners. Blank grid shows there is no interaction.

4. Complementary Discussion

TOPLESS as a corepresor in TIR1-auxin-dependent and independent regulation of ARFs

In order to better understand the mechanisms underlying Sl-TPL involvement in the regulation of auxin signaling pathway, the interactions within Sl-TPL and Sl-ARF family were tested. Sl-TPL1, Sl-TPL2 and Sl-TPL4 displayed interactions with the majority of repressor-ARFs. Moreover, there were no any Sl-TPLs interact with Sl-ARFs activator. That is in sharp contrast with the interaction result of the Sl-IAAs -Vs- Sl-ARFs screen, which indicated that Sl-IAAs interact strongly with all Sl-ARFs activators. In Arabidopsis, it was also mentioned that repressor ARF proteins, such as ARF2 and ARF9, interact directly with TPL/TPR proteins (Causier et al. 2012a), suggesting that a mechanisms for repression implicating TPL/TPR co-repressors may occur in both TIR1-auxin-dependent and TIR1-independent ARF-mediated repression (Fig. 6). Sl-TPLs interacting with some Sl-ARFs repressors in tomato were firstly revealed in our study, such as the Sl-TPL2 + Sl-ARF4 and Sl-TPL4 + Sl-ARF4. Moreover, the deletion of the C-terminal extremity of Sl-ARF4 indicates that the N-terminal part of the ARF4 protein, without the CTD domain, is essential for these interactions. There are two conserved domains on the N-terminal part of canonical ARFs: DBD domain and the middle SPL-rich region (MRs). The DBD of ARFs functioned to recognize and interact with the target DNA of auxin response gene. Possibly the MRs of ARFs might play a key role in the PPIs between TPLs and ARFs. This also could explain why the Sl-TPLs do not interact with the activator ARFs since there is a big difference in the MRs protein sequences between activators and repressors. The ARF activator MRs are rich in glutamine (Q), serine (S), and leucine (L) residues and this might prevent direct interactios with TPL proteins

Using a high-throughput Y2H approach, Causier et al. (2012a) sorted out a list of transcription factors potentially interacting with TPL/TPRs. Various transcription factors belonging to at least 17 distinct families were represented. APETALA2 (AP2)/ETHYLENE RESPONSE FACTOR (ERF), Aux/IAA, TCP and JASMONATE-ZIM-DOMAIN (JAZ) proteins were found to interact with TPL/TPRs. Most of these TPL/TPR-interacting factors

include one or more repressor domains. Causier et al. (2012b) further conducted a Y2H assay using Physcomitrella patens TPL/TPRs, Aux/IAAs, and ARFs to demonstrate an evolutionary conservation of the EAR motif for the interaction between TPL/TPRs and other components. The Aux/IAA with the mutated LxLxL motif failed to interact with TPL/TPRs. Two repressor-like ARFs, containing the LxLxL or similar motif, showed specific interaction with different TPL/TPR members whereas an activator ARF with no LxLxL motif did not interact with TPL/TPRs, indicating that ARF repressors are also able to directly suppress the target genes by recruiting TPL/TPR co-repressors. This study obviously shows that the molecular function of nuclear auxin signaling components and the diversity in interaction between components have been conserved in land plants.

Questioning the molecular bases of Sl-TPL3 specificities

The mutation and deletion of Sl-TPLs also gave some interesting results. According to phylogenetic analyses and TPLs/IAAs interaction map, Sl-TPL3 has a totally different behavior compared with other Sl-TPLs. Firstly we noticed it displays three specific amino acids at positions 44, 99 and 162 compared to TPL1, 2, 4 and 5 on the conserved domain. We made three mutations to substitute each one amino acid of TPL1 to the corresponding amino acid of TPL3. But the Y2H experiment on this mutated Sl-TPL1 did not show any alteration of Sl-TPL1 capacity to interact with Aux/IAAs. Then we deleted the WD40-repeat domains from Sl-TPL3, and tested the interactions again with Sl-IAAs. The Sl-TPL3-WD-del gave us a surprising result since it appeared to interact with all the Sl-IAAs assessed. This result suggests either that the WD40-repeat domains in TPLs may influence the specificity of interactions with other proteins, such as weakening or suppressing the interaction between Sl-TPL3 and Sl-ARF4 or that the presence of some WD40-repeats may affect the expression of reporter gene expression in yeast thus generating false negative in yeast two-hybrid by affecting the expression of reporter genes. The application of alternative PPI methods should help us to elucidate the question.

Chapter IV: Purification of Aux/IAA and ARF partners from tobacco protoplast

Chapter IV: Purification of Aux/IAA and ARF partners from tobacco protoplast

1. Introduction

1.1 General introduction of Chapter IV

By using Y2H, pull-down and BiFC protocols, protein-protein interactions between Sl-IAA, Sl-ARF and Sl-TPL families were assayed in our study (Chapter II and III). Some strong interactions between specific Sl-IAA, Sl-ARF and Sl-TPL were confirmed. Part of our results could match the classical model of auxin signaling pathway: the TPL family members are usually recruited by Aux/IAA, and then these Aux/IAAs may interact with ARF-activators to form a protein complex which can switch off the activation of auxin regulated genes (Vanneste and Friml 2009).

Moreover, the differences between PPIs analyses also highlighted some contradictory results between these three protocols (chapter II). In our study, Sl-IAA3 showed no interaction with Sl-ARF4 by Y2H assay. But on the contrary, several Aux/IAAs seemed to interact with Sl-ARF4 in the BIFC assay. Unfortunately, we could not produce recombinant Sl-ARF4 proteins to solve the contradiction by pull-down.

If we hypothesize that the results given by BiFC and yeast two-hybrid are not the result of a false positive, then the contradiction might find two explanations:

- Existence of post-translational modifications that mask the interaction in yeast;
- Or the involvement of intermediary partners that may establish a link between Sl-IAA9 and Sl-ARF4 and explain the nuclear fluorescence observed in BiFC.

The second hypothesis implies the existence of alternative partners for Aux/IAAs. As transcription complex imply numerous proteins, the list of alternative interactants may be long. Interestingly, a large Y2H screen in Arabidopsis was recently published by the

Arabidopsis Interactome Mapping Consortium (2011). In this work, the interactants of several Aux/IAAs was documented. In addition to Aux/IAA, ARF and TOPLESS-related proteins that have been studied in chapter II and III, the Arabidopsis interactome revealed the potential existence of several alternative partners such as subunits of the COP complex, HomeoBox proteins (HB32), transcription factors (AGL20), other proteins with WD40 domains (see Table 11).

The existence of these alternative partners that escapes the canonical Aux/IAA-ARF-TPL model led us to develop a new approach based on copurification. We hoped to reveal and fish specific protein or proteins complex from the plant cell directly by non-target in-plant approach. For that purpose, specific Sl-IAAs and Sl-ARFs proteins were fused with GFP-tag in our study: IAA3-GFP, IAA9-GFP and ARF8a-GFP. All these fused proteins were expressed in BY-2 tobacco protoplast and to be extracted and analyzed by western blot. We wanted to establish a feasible protein expression and extraction protocol for Sl-IAAs, Sl-ARFs and Sl-TPLs to utilize to copurify prey proteins in plant cells suitably. This would also give us more useful information to understand better other PPIs mechanism in plant in the future.

Table 11. Arabidopsis proteins interacting with several Aux/IAA according to the Arabidopsis Interactome Mapping Consortium (2011). Interactants corresponding to Aux/IAA, ARF and TPL-TPR proteins were removed.

Gene accession	Annotation	Aux/IAA interacting
AT1G01910	P-loop containing nucleoside triphosphate hydrolases superfamily protein	IAA2, IAA10, IAA16, IAA27
AT1G14687	homeobox protein 32 (HB32)	IAA8, IAA10, IAA27
AT1G71230	Encodes a subunit of the COP9 complex	IAA1, IAA2, IAA7, IAA8, IAA10, IAA27, IAA28
AT2G39760	BPM3; protein binding	IAA2, IAA6, IAA8, IAA10
AT2G43790	ATMPK6 (ARABIDOPSIS THALIANA MAP KINASE 6)	IAA10, IAA31
AT2G45660	AGL20 (AGAMOUS-LIKE 20); transcription factor	IAA10, IAA16, IAA31
AT4G25660	hypothetical protein	IAA10, IAA16
AT5G08080	SYP132 (SYNTAXIN OF PLANTS 132); SNAP receptor	IAA10, IAA11
AT5G02870	60S ribosomal protein L4/L1 (RPL4D)	IAA3, IAA11
AT3G06430	EMB2750 (embryo defective 2750)	IAA11, IAA18
AT3G18140	transducin family protein / WD-40 repeat family protein	IAA1, IAA18
AT3G06720	IMPA-1 (IMPORTIN ALPHA ISOFORM 1); binding / protein transporter	IAA2, IAA11, IAA19
AT4G02150	MOS6 (MODIFIER OF SNC1, 6); binding / protein transporter	IAA1, IAA19
AT4G16143	IMPA-2 (IMPORTIN ALPHA ISOFORM 2); binding / protein transporter	IAA2, IAA19
AT1G16890	ubiquitin-conjugating enzyme, putative	IAA2, IAA7
AT2G34090	MEE18 (maternal effect embryo arrest 18)	IAA1, IAA2
AT4G35000	APX3 (ASCORBATE PEROXIDASE 3)	IIAA1, IAA2
AT5G52547	hypothetical protein	IAA2, IAA3, IAA4, IAA11
AT1G12840	DET3 (DE-ETIOLATED 3); proton-transporting ATPase, rotational mechanism	IAA1, IAA3
AT2G38270	CXIP2 (CAX-INTERACTING PROTEIN 2); electron carrier/ protein disulfide	IAA3, IAA11
AT5G58720	PRLI-interacting factor, putative	IAA27, IAA31
AT1G22920	CSN5A (COP9 SIGNALOSOME 5A)	IAA2, IAA7, IAA8, IAA28, IAA31
AT1G70410	Putative / carbonate dehydratase, putative	IAA8, IAA19

1.2 The aim of the work in Chapter IV

To better understand the complexity of the protein interaction network involved in auxin regulation in plant, a non-targeted approach was initiated. Our aims in this Chapter are: (1) To establish a highly efficient expression system for tagged-protein by using transient expression in tobacco protoplasts. (2) To succeed extracting and purifying the tagged-proteins from protoplast. (3) Try to pull down (capture) new potential protein partner or protein complex in plant cell. Ultimately, the idea of this work was to analyze the co-purified proteins using mass spectrometry, but we could not reach that point.

2. Materials and Methods

In Chapter II, the protoplast system was used in BiFC to confirm that both Sl-IAA3 and Sl-IAA9 interact with Sl-ARF8. In this Chapter, GFP-tagged protein (Sl-IAA3, Sl-IAA9 and Sl-ARF8) were expressed using a transient expression system on tobacco protoplasts. Trying to extract these GFP-tagged proteins as bait was the first mandatory step before discovering any new potential protein partner in plant system.

2.1 BY-2 tobacco cells and growth condition

Refers to Appendix A5.

2.2 Plasmids for protoplast transformation

The coding sequence of Sl-IAA3, Sl-IAA9 and the Sl-ARF8a proteins were cloned in frame with GFP into the pGreen vector (Hellens et al. 2000) by Smal I enzymatic digestion and expressed under the control of the 35S CaMV promoter. Plasmids of pGreen-IAA3-GFP, pGreen-IAA9-GFP and pGreen-ARF8a-GFP were used for transient expression in tobacco protoplasts. The empty pGreen-GFP vector plasmid was used as a control.

2.3 Transient expression in tobacco protoplasts

pGreen-GFP, pGreen-IAA3-GFP, pGreen-IAA9-GFP and pGreen-ARF8a-GFP were used for transient expression in tobacco protoplasts respectively. Materials and methods refer to Appendix (A5).

2.4 Preliminary detection of GFP-tagged proteins expressed in protoplast

Before extracting GFP-tagged protein from protoplast, we performed a simple and quick pre-test by using western blot to check if the GFP-tagged proteins were expressed in transformed protoplasts. pGreen-IAA3-GFP, -IAA9-GFP and -ARF8a-GFP were introduced into protoplast with PEG. Intact protoplasts were vortexed sufficiently and centrifuged at 13000 rpm to separate the soluble fraction (Sol) from the pellet fraction (Pel). The soluble fraction (Sol) and the pellet fraction (Pel) were loaded directly onto a SDS-PAGE gel. Signals were detected with GFP-antibody (Invitrogen) by western blot. Materials and methods of western blot refer to Appendix A3. The dilution ratio of GFP antibody (Mouse monoclonal anti-GFP from Sigma-Aldrich, G6539) was: 1:5000, and the dilution ratio of second antibody (Anti-mouse IgG, Sigma-Aldrich) was: 1:20000.

2.5 Extraction of GFP-tagged protein from tobacco protoplast

GFP alone, IAA3-GFP, IAA9-GFP and ARF8a-GFP proteins were extracted from transformed protoplast after washing the protoplast on a HEPES-sorbitol-Ficoll buffer (protocol refers to Appendix A7). Protoplasts were then lysed by sonication or through a needle. Proteins extracted from protoplast were detected with GFP-antibody (Invitrogen) by western blot assay.

2.6 Nucleus extraction from tobacco protoplast

pGreen-IAA9-GFP was used for transient expression assay in tobacco protoplasts. After protoplast purification (see above), the nucleus was purified after disrupting protoplasts through a hypodermic needle. A detailed protocol of nucleus extraction is presented in Appendix A8. Intact Sl-IAA9-GFP from purified intact nucleus was detected with

GFP-antibody (Invitrogen) by using western blot assay. The dilution ratio of GFP antibody is: 1:5000, and the dilution ratio of second antibody is: 1:20000.

3. Results

The tobacco protoplast system was chosen for in-plant PPIs assay in our study. Before bound- proteins were fished by our GFP-tagged bait proteins, the first step was to establish an approach for expressing and extracting the GFP-tagged proteins from the protoplast system.

3.1 IAAs-GFP and ARFs-GFP proteins were expressed in Tobacco protoplast nucleus

The observation, under confocal microscopy or under direct fluorescence-microscopy, revealed that protoplasts transformed could produce with high efficiency GFP-tagged proteins in tobacco protoplast. Nearly 5~10% of total cells were transformed successfully each time. Undoubtedly, a high efficiency of transformation is important for the next experiment of proteins extraction from protoplast. As expected, GFP-IAA3, GFP-IAA9 and GFP-ARF8a all localized in tobacco protoplast nucleus (Fig. 34).

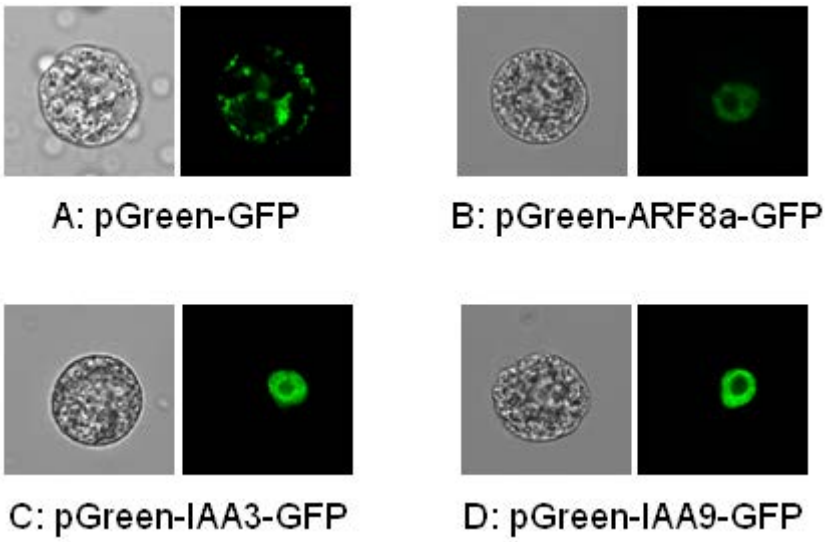


Fig. 34 GFP-IAA3, GFP-IAA9 and GFP-ARF8a all localized in tobacco protoplast nucleus.

3.2 Visualization of GFP-tagged proteins expressed in protoplast by Western-blot

Before extracting GFP-tagged protein from protoplast, we performed a simple and quick pre-test using western blot to check if the GFP-tagged proteins were abundant in transformed protoplasts. All protoplast samples were separated by centrifugation into two parts: soluble fraction (sol) and pellet fraction (pel). The western-blot result (Fig. 35) showed that for IAA3-GFP, IAA9-GFP and ARF8a-GFP, a band of the expected size could be visualized after ECL revelation. But all proteins except GFP alone remained associated to the pellet although none is predicted as a hydrophobic molecule. The observation under microscopy of the cells showed that even after sonication we could still observe „nucleus"-like structure that may trap the GFP-tagged proteins and complicate its extraction.

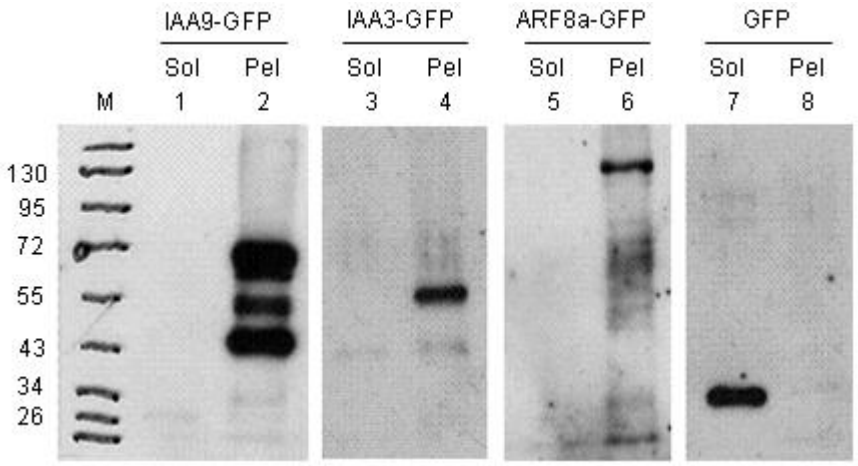


Fig. 35 Monitoring of GFP tagged proteins in protoplasts. IAA3-GFP, IAA9-GFP and ARF8a-GFP proteins display a strong and specific signal. All proteins except GFP-alone were stayed in pellet fraction (Pel).

3.3 Western-blot result of IAAs-GFP proteins extracted from protoplast

We further improved the extraction method by washing and purifying the intact protoplasts in a HEPES-sorbitol-Ficoll buffer. Following this new method of protein extraction from protoplast, the revelation of proteins by western-blot showed that (Fig. 36) IAA3-GFP and IAA9-GFP proteins could be extracted from protoplast, but they were degraded very quickly. For ARF8a-GFP, up to now, we have had great difficulty to get the protein in the soluble fraction.

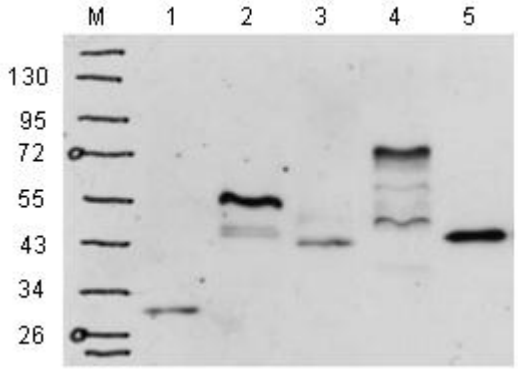


Fig. 36 Evolution of AuxIAA-GFP size after protein extraction. Western blot result showed the proteins of IAA3-GFP and IAA9-GFP extracted from protoplast. 1: GFP protein alone. 2: IAA3-GFP before extraction. 3: IAA3-GFP after extraction. 4: IAA9-GFP before extraction. 5: IAA9-GFP after extraction.

3.4 IAA9-GFP full length stayed in purified tobacco protoplast nucleus

In order to prevent the degradation of the IAA9-GFP protein, we purified nucleus from transformed tobacco protoplast. After purification, we controlled nucleus integrity and purity with DAPI staining (Fig. 37). In parallel, a minority of nucleus displaying GFP fluorescence could still be observed. The western-blot results showed that IAA9-GFP protein could still conserve its full length in intact nucleus compared with the protein extracted from broken protoplast (Fig. 38).

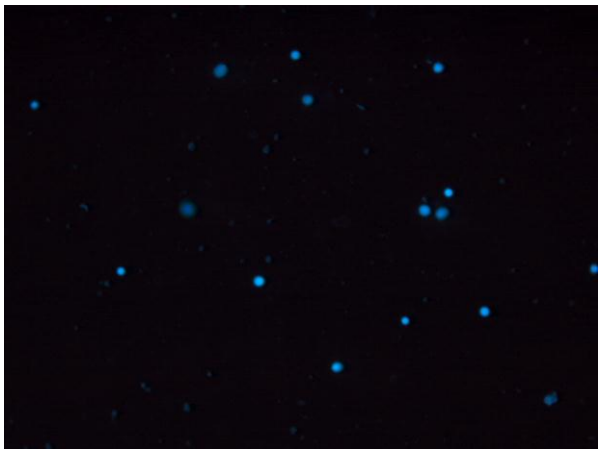


Fig. 37 Visualization of nucleus extracted from tobacco protoplasts with DAPI. Fluorescence was excited at 488 nm wavelength and emission was captured at 530 to 570 nm wavelength.

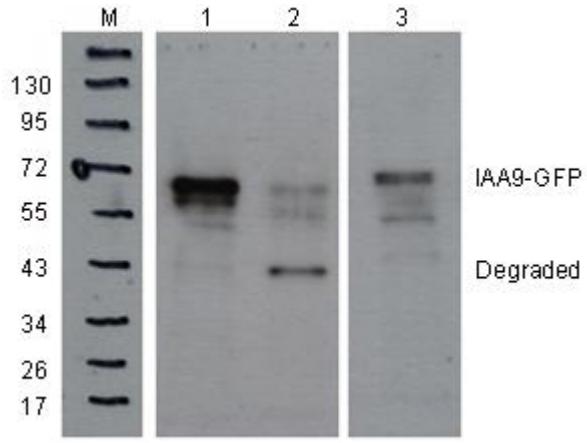


Fig. 38 Integrity of IAA9-GFP in purified nuclei. The western result showed IAA9-GFP protein still conserve its full length in intact nucleus compared with the protein extracted from broken protoplast. 1: IAA9-GFP in intact protoplast (before extracting). 2: IAA9-GFP in broken protoplast (after extracting). 3: IAA9-GFP in intact nucleus.

3.5 Degradation analysis of IAAs-GFP proteins

As we know, Aux/IAA proteins are protein which could be degraded very quickly (present a high turn-over) both in in-vivo and in-vitro environments, even in absence of auxin and TIR1 (Dreher et al. 2006). Interestingly, when we extracted IAAs-GFP protein from protoplast and performed the PAGE-gel electrophoresis for western, we always found that IAA proteins are degraded very quickly (Fig. 39).

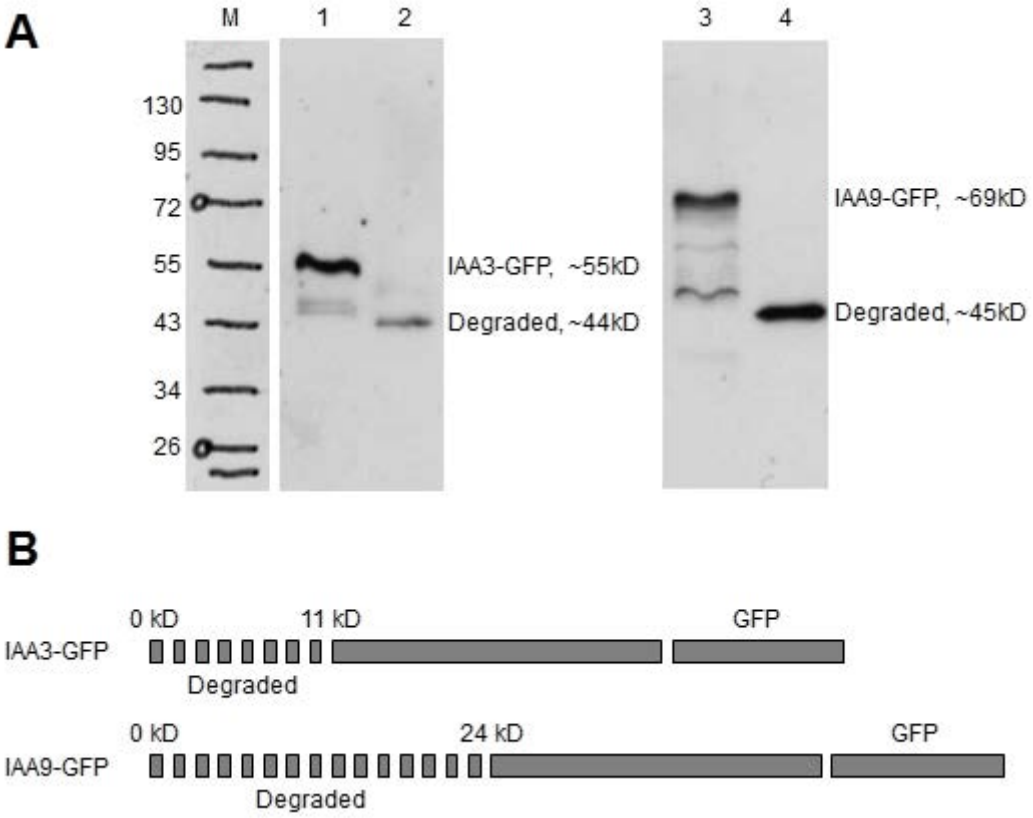


Fig. 39 **Estimation of cleavage zone during IAA degradation** (A): Western blot result showed the IAA3-GFP and IAA9-GFP proteins were degraded. 1: IAA3-GFP before extracting; 2: IAA3-GFP after extracting; 3: IAA9-GFP before extracting; 4: IAA9-GFP after extracting. (B): The image of the degraded structure of IAA3-GFP and IAA9-GFP.

After repeating the experiment more than 3 times, we could confirm and analyze the reproducible degradation of IAA3-GFP and IAA9-GFP by PAGE-gel electrophoresis. We determined the size of these protein fragments. As shown on Fig. 39, the protein size of full-length IAA3-GFP is approximate 55 kDa, and the degraded IAA3-GFP is about 44 kDa. The protein size of full-length IAA9-GFP is approximate 69 KD, and the degraded IAA9-GFP is about 45 KD. Therefore, the size of degraded sequence could be calculated approximately, and the cleavage site was deduced from the full-length protein sequence (Fig. 40). Approximately 11 kDa of IAA3 N-terminal and 24 kDa of IAA9 N-terminal were degraded during protein extraction form protoplast. For both tagged proteins, such a position is located between domain II and III (Fig. 40).

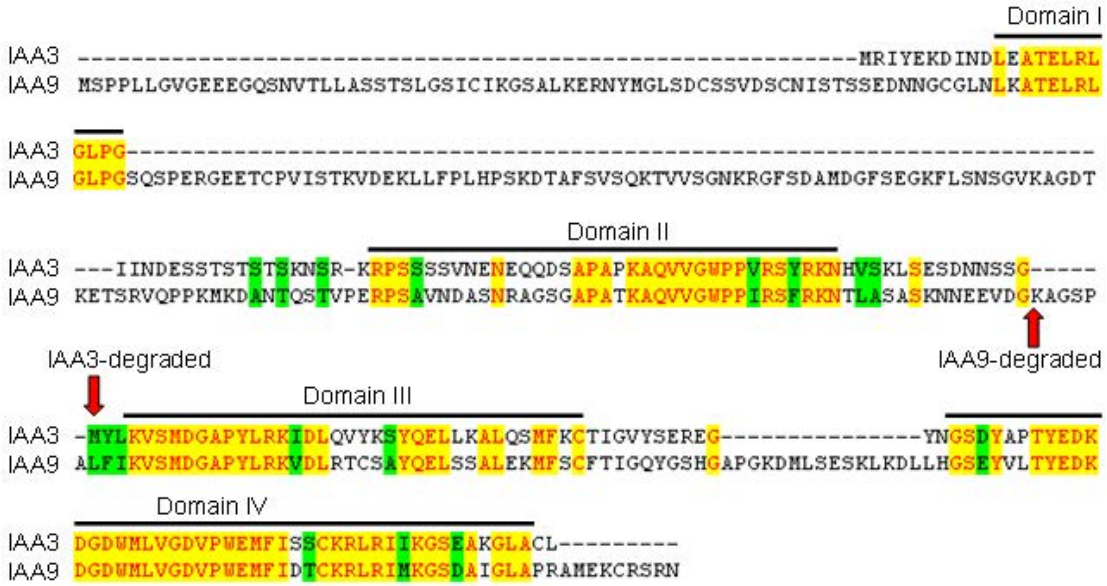


Fig. 40 Prediction of the IAA degradation zone. The full length sequences of IAA3 and IAA9 proteins. Red arrows indicate the digestion site was always regularly between domain II and III.

The Aux/IAA protein degraded very quickly and regularly. Before starting the protein extraction experiment, the Aux/IAAs could keep their full-length in the intact cells or nucleus. But when we performed the extraction experiment, protein was degraded quickly and regularly even though we tried to add some different protease inhibitors into the extraction buffer. It is still not clear what exactly happened to explain this quick and regular degradation.

4. Conclusion and discussion

We tried to establish a non-targeted approach in this study. Three GFP-tagged proteins: Sl-IAA3-GFP, Sl-IAA9-GFP and Sl-ARF8a-GFP were expressed and extracted from protoplast. Our aim was to find some potential protein partners or complex depending on these GFP-tagged bait proteins. Alternatively, the system could also have been used to produce tagged-protein in an *in planta* environment and provided us alternative conditions to perform pull-down assays. Although we could not finish all planed experiment during the

PhD thesis, we still got some useful and interesting information.

pGreen-IAA3-GFP, pGreen-IAA9-GFP and pGreen-ARF8a-GFP were transformed to BY-2 tobacco protoplast and these GFP-tagged proteins were expressed successfully. Confocal microscopy images confirmed that IAA3-GFP, IAA9-GFP and ARF8a-GFP were all localized in tobacco protoplast nucleus.

The soluble fraction of IAA3-GFP and IAA9-GFP were extracted from protoplast and verified by western blot. So far, we could not extract soluble ARF8a-GFP from protoplast. The size of proteins extracted from protoplast deduced from a western-blot showed that IAA3-GFP and IAA9-GFP proteins were degraded very quickly. As soon as we started the extraction experiment and broke the protoplast to release cell contains, the degradation of IAAs-GFP happened quickly. There might be an enzymatic or spontaneous mechanism causing the degradation for IAAs-GFP when proteins are released or are in contact with the cytoplasm. By contrast, IAAs-GFP still kept its full length in the intact nucleus, even after a four hours subcellular fractionation protocol, thus illustrating the importance of the nuclear structure to preserve Aux/IAA integrity. We tried to perform a lysis of the nucleus to see if we could obtain the soluble protein fraction of IAA9-GFP. But unfortunately, we did not succeed up to now. There are at least two possible reasons: first, there is too small quantity of transformed nucleus; second, the lysis buffer needed to be optimized before extracting nuclear proteins more efficiently. In that view, the work described in this chapter can be viewed as unsuccessful. For ARF proteins, the obtention of significant quantities of soluble proteins represents the main bottleneck for further purification. For Aux/IAA, although the quantity of protein is significantly higher, the rapid cleavage of the protein prevents us from using it as a bait to capture other interactants.

However, the analysis of the degraded IAA3-GFP and IAA9-GFP sequence revealed at least one interesting feature. SDS-PAGE-gel electrophoresis and western blot results indicated that the degraded size of IAA3-GFP and IAA9-GFP protein fragments were very regular. For both IAA3-GFP and IAA9-GFP, the digestion site located always between domain II and III. Earlier study indicates Aux/IAA domain II is responsible for the stability of Aux/IAA proteins (Dreher et al. 2006; Worley et al. 2000). The interaction between Aux/IAAs domain

II and the SCF complex leads Aux/IAAs become ubiquitinated and are targeted for proteolysis (Dharmasiri et al. 2005a; Dharmasiri et al. 2005b; Kepinski and Leyser 2005; Leyser 2006; Tan et al. 2007; Chapman and Estelle 2009). Now it is still not clear when and how this degradation happens exactly in the tobacco protoplast for IAA3-GFP and IAA9-GFP. But we can think that when IAA3-GFP or IAA9-GFP is released into the cytoplasm, some enzyme recognized and digested these proteins very quickly via the domain II. This result also showed us there is a big difficulty to obtain the full length functional IAAs proteins and to utilize them to capture other potential protein partners.

General conclusions

General conclusions

1. Scientific perspectives

TOPLESS family in tomato was characterized in our study

TOPLESS family of tomato was firstly reported in details in this thesis. Nine ORFs coding for topless-like proteins (Sl-TPLs) were identified in the genome. Six corresponded to full-length proteins (Sl-TPL1, 2, 3, 4, 5 and 6) containing the TOPLESS canonical domains (LisH, CTLH and two WD40 repeats) and were further analyzed. Combining different approaches (phylogeny, nuclear localization, expression analysis and exhaustive Y2H screen of Aux/IAA-TPL interactions), we could categorize the TOPLESS family into four branches:

- A first branch containing three isoforms (Sl-TPL-1, -4, -5) very similar to At-TPL, displaying a wide capacity of interactions with Aux/IAA proteins and comprising two members (*Sl-TPL1* and *Sl-TPL4*) highly expressed in vegetative tissues. These two genes may be seen as some kind of functional homologues of At-TOPLESS protein.
- A second branch (Sl-TPL2) absent in Arabidopsis but sharing high homology with distant TPL-like proteins in moss or lycophyte or with a rice TPL-like protein (ASP2) that has also conserved a broad capacity to interact with Aux/IAAs and ARFs.
- A third branch (Sl-TPL3) found in all Angiosperms, with a distinct PPI pattern but highly expressed even in reproductive organs. This last isoform represents possibly an interesting object of study to assay the specific contribution of TPL-like proteins in fruit development and ripening.
- A last branch (Sl-TPL6), more distant but evolving fast and being poorly expressed. This isoform may be considered as a near-pseudogene.

Protein-protein interactions and a new model of auxin signaling pathway

Up to now, in the auxin signaling pathway, it is widely accepted that Aux/IAAs interact

with activator-ARFs to prevent the target gene expression. The TPLs are considered as co-repressor which could interact with IAAs to form the TPL-IAA-ARF protein complex. By building exhaustive PPi maps within the Aux/IAA, ARF and TPL family proteins, we could provide a complete picture (Fig. 41) that comforts and completes the current auxin perception model. Indeed, the Aux/IAA-activator-ARF interaction appears as a general trait and at least four TPL isoforms show a broad capacity to bind the majority of Aux/IAAs (with exception of SlIAA29). The interactions between TPL and ARFs gives also some hints about the mechanism of repression exerted by repressor ARF since some have a clear capacity to interact with TPL corepressor (Fig. 42A, B). Such result was also observed by Causier et al. (2012). Thus, the TPL protein seems to play a central role in both auxin-dependent and independent repression of auxin-response genes. The TPL- inhibition mechanism is still unclear, however some authors supposed that TPL acted through the recruitment of histone deacetylases (HDACs) into transcription complexes, thus changing the chromatin state from active to inactive (Long et al., 2006; Liu and Karmarkar, 2008; Krogan and Long, 2009; Krogan et al., 2012). At last, in presence of auxin, the Aux/IAA proteins are removed and targeted to the proteasome, thus releasing the activator-ARFs (Fig. 42C). In that case, some authors (Vernoux et al. 2011, Guilfoyle & Hagen, 2012) suggested the existence of a competition between both types of ARFs and that activator ARF binds with high affinity to the promoters of auxin-response genes.

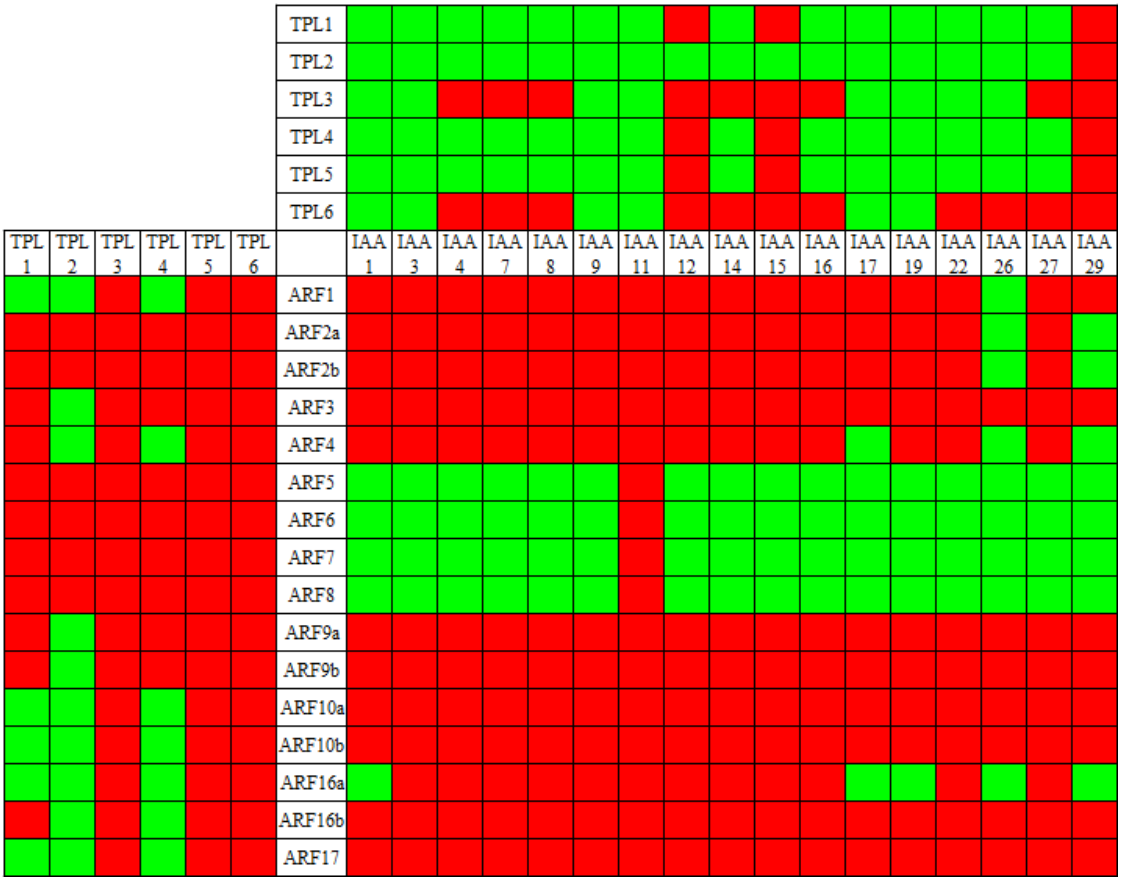


Fig. 41 Exhaustive Protein-protein interactions map within the Aux/IAA, ARF and TPL family proteins (Yeast two-hybrid result). Green box indicates there is an intraction between the tested proteins. Red box means there is no interaction.

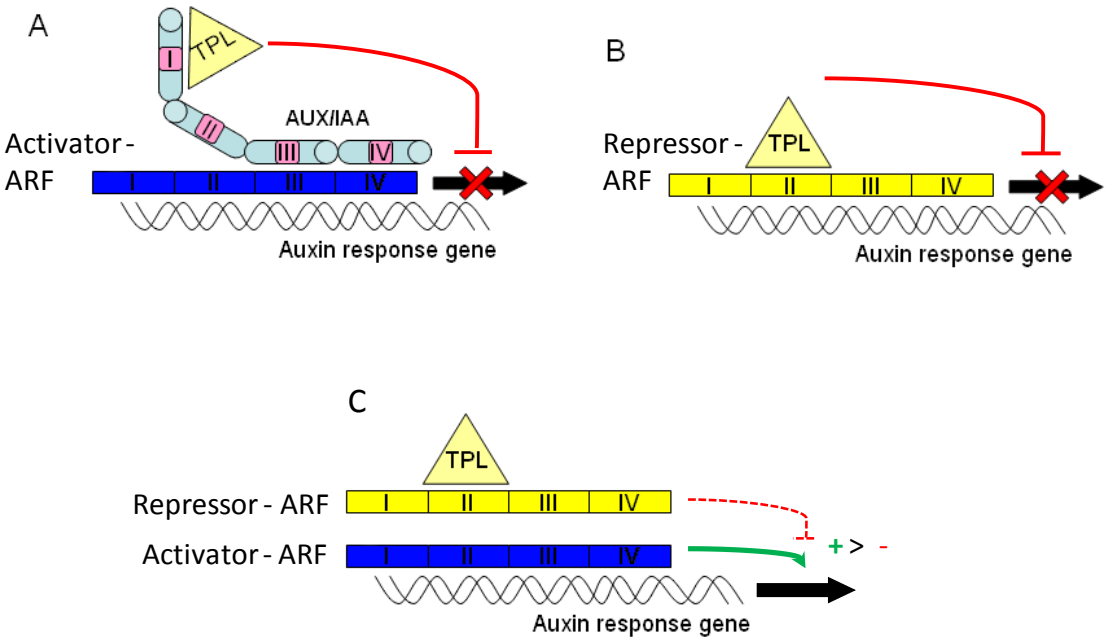


Fig. 42 Possible model of the TPL/IAA/ARF PPIs involved in the auxin signaling pathway. (A): Some Aux/IAA interacts directly with ARF activator domain III and IV, and also these IAA recruit some co-repressors TPL in the same time. This repression occurs in absence of auxin. (B): For some repressor ARFs, the TPL can bind directly the transcription factor, thus exerting a repression effect. This repression is auxin-independent. (C): In presence of auxin, the Aux/IAA is removed. The activation of auxin-response gene depends only of the balance between activator and repressor ARFs.

2. Methodological perspectives

Three protocols for PPIs assay were established in our lab

General experiment protocols for PPIs assay were established for the first time in our lab including Y2H, pull-down and BiFC. Using these protocols, we analyzed the affinity between tomato Aux/IAA, TOPLESS and the Auxin Response Factor (ARF). All these PPIs protocols are available for routine tests and even for some mutated or deleted proteins tested to learn the function of specific domains involved in their PPIs. Y2H provides a high throughput PPIs method while pull-down looks more like a complement experiment to confirm the directly

interaction between two protein partners. The BiFC is also used as a high throughput PPIs method, but the reproducibility of results still represents a huge challenge, thus questioning the reliability of the method. Hence, it will still be helpful to confirm and find some potential partners involved in tomato cell‟s auxin signaling pathway, for example to examine in further detail the specificity of interactants observed on Sl-TPL3 (contrary to Sl-TPL1, 2, 4, 5).

Non-targeted approach for PPIs assay should be investigated in the future

In order to know better the interaction relationships of TPL/IAA/ARF in plant or even their interactions with some unknown proteins and target genes, the protein expression and extraction systems from nature plant cell will be investigated in the future. In my PhD study, we tried to establish a protocol which could extract specific tagged protein from tobacco protoplasts. Thus, we hoped to use these tagged bait proteins to capture the potential partners or protein complex and take advantage of the sequence conservation between Nicotiana and tomato, two Solanaceae species. Until now, we were not completely successful and the way to analysis of bound proteins by mass spectrometry or Surface Plasmon Resonance (BIAcore) looks quite unsure. However, we may also question the use of the protoplast system which displays several limitations (low quantity of material, use of transient expression instead of stable transgenic transformation). A future work on these non-targeted approaches probably needs strategy-refining.

3. Future experiments

Transgenic plant study

In the GBF laboratory, several Sl-IAAs and Sl-ARFs have already been targeted for both over-expression and down-regulation studies after transformation of tomato *via Agrobacterium tumefaciens*. For the Sl-TPL family, we could also design a genetic engineering strategy. As both Sl-TPL1 and Sl-TPL4 are highly expressed in vegetative tissues and display an interaction pattern similar to At-TPL, we could, by RNA-interference, down

regulate both genes (some regions of the genes share 90% identity between the two nucleic acid sequences). Hopefully, the phenotype would be very consistent with rice ASP2-down-regulated lines (Yoshida et al. 2011) or with semi-dominant *tpl-1* mutant (Long et al. 2006). For fruit biology, Sl-TPL3 represents a target of choice. In that case, if we design a construct for RNA interference, we would better target a very specific region of the gene, so that only this isoform will be affected.

Analysis of mutated or deleted forms of Sl-IAA or Sl-TPLs

The study on the TPL multigenic family revealed some differences between isoforms. For the Sl-IAAs, a non-targeted approach or a large Y2H screens with several partners (perhaps on a cDNA library) might highlight differences between isoforms and yield a PPI signature for some members. In that case, a complementary approach would be to design deletions or targeted mutagenesis to understand the structural bases of these differences. Analysis of mutated or deleted Sl-IAA/ARF/TPL PPIs will thus help us to understand better how these potential amino acid works in the interaction, and how could these potential protein complex evolve during the radiation of these multigenic families.

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Appendix

Appendix

A.1 Protocol of yeast two-hybrid (Y2H)

The Y2H experiment protocol mainly refers to the Y2H User Manual (Clontech). If there are any optimizations and/or changes about the protocol in our experiment, we would give a "Note" after each step of the protocol.

Preparation before experiment

--- Medium YEPD for yeast growth (1 L)

Yeast extraction	10g
Bacto-peptone	20g
Adenine hemisulfate salt	50mg
Glucose	2%

Before autoclave, adjust pH to 5.8 with HCl. For solid YEPD, add 15g Agar to the solution.

--- Selection medium TL (without Trp and Leu) (1 L)

Yeast Nitrogen base	6.7g
Drop out - TL (Clontech, ref. 630417)	X mg (Depend on the product standard)
Adenine hemisulfate salt	50mg
Glucose	20g
Agar	20g

Need autoclave, no need to adjust pH.

--- Selection medium TLHA (without Trp, Leu, His and Ade) (1 L)

Yeast Nitrogen base	6.7g
Drop out - TLHA (Clontech, ref. 630428)	X mg (Depend on the product standard)
Glucose	20g

Agar 20g

Need autoclave, no need to adjust pH.

--- Selection medium TLH (without Trp, Leu and His) (1 L)

Yeast Nitrogen base 6.7g

Drop out - TLH (Clontech, ref. 630419) X mg (Depend on the product standard)

Adenine hemisulfate salt 50mg

Glucose 20g

Agar 20g

Need autoclave, no need to adjust pH.

--- Clone of isolated yeast AH109 grew 2-3 days, 28 °C, on the YEPD plate, after that, it could be kept on -4 °C for 2 month.

--- Sterilized H_2O.

--- Sterilized eppendorfs tube (1.5mL) and tips (10uL, 200uL and 1mL).

--- 100mM and 1M Lithium Acetic (LiAc), adjust pH to 7.5, sterilize by pass through the 0.22 μM filter. Keep in room temperature (RT).

--- 50% PEG 3350, completely dissolve in 50 °C, Keep in room temperature (RT).

Note: If the yeast transformation is in low efficiency, try to increase the concentration to 60%.

--- 2 mg/ml salmon sperm DNA (SS-DNA). Before use, boil it for 5 min, and after keep in ice for at least 5 min.

Yeast transformation protocol

1. Scratch the stock yeast AH109 on the YEPD plate, culture on 28 ℃ for 2-3 days.

2. Pick up a huge amount of isolated AH109 clone on the YEPD plate and culture with 5mL liquid YEPD on 28 ℃ , 250 rpm, ~24h.

Note: Normally we did this step on the morning of the day. Then the next day morning we could perform the Y2H experiment.

3. Transfer over-night-yeast culture (step 2, ~3.5mL) to 100mL fresh YEPD, measure the OD600 ≈ 0.15, and then culture on 28 ℃ , 250 rpm, to the OD600 ≈ 0.4-0.5.

Note: Normally, it will takes 4h that yeast culture OD600 increase from 0.15 to 0.4.

4. Separate the yeast culture to two 50mL tubes and centrifuge at 4500rpm, 5min.

5. Resuspend the pellet in 25mL sterilized water, and mix completely. Then centrifuge again at 4500rpm, 5min.

6. Resuspend the pellet in 1mL LiAc (100mM), mix well and transfer the mixture to two 1.5mL tubes. Then centrifuge at 13000rpm, 15sec.

7. Resuspend the pellet again in 1mL LiAc (100mM), mix well and waiting for use (Yeast-LiAc-solution).

8. Prepare the transformation mixture and mix well by vortex.

--- 665uL 50%PEG3350

--- 100uL 1M LiAc

--- 140uL SS-DNA (Before use, boil it 5min firstly, and then keep it in ice at least 5min)

--- 95uL H2O

Note: Total mixture volume is 1mL, it could be used for 4 transformations

9. Prepare a 1.5mL tube, add the followed things:

--- 100uL Yeast-LiAc-solution

--- 500ng BD-bait plasmid

--- 500ng AD-prey plasmid

--- 250uL transformation mixture

Then mix well by short vortex (3-5sec, two times).

Note: For positive control Y2H experiments, yeasts were co-transformed with the pBD-p53 (BD-p53) and the pAD-SV40T (AD-T7) vectors (Clontech).

10. Put the tube on 28 ℃ , 250 rpm, 30 min. After that, transfer the tube to 42 ℃ , 30min.

11. Centrifuge the tube at 7000 rpm, 15 sec. Resuspend the pellet in 200uL sterilized water.

12. Plating the yeast suspension on selection medium: 100uL for TL plate, another 100uL for TLHA plate or TLH+3AT plate. Keep the plates on 28 ℃ for 3-7days.

Note: An interaction between bait and prey in the Y2H system results in the induction of

HIS3, which enables yeast to grow in the absence of histidine in the media. Unfortunately, some bait clones exhibit auto activation (i.e., they are able to turn on reporter genes in the absence of an interaction). In most cases, this auto activation can be controlled by the addition of 3-Amino-1,2,4-Triazol (3AT), which acts as a quantitative inhibitor of the HIS3 reporter gene. Thus determining the optimal concentration of 3AT for each clone is critical. Bait and prey clones (along with control plasmids) will be transformed into his- yeast and replica plated onto plates containing 0, 10, 20, 50, 75, and 100mM 3AT. The lowest concentration of 3AT that inhibits growth will be used for library screening and/or pairwise interactions. If auto activation cannot be suppressed with the addition of 3AT, the bait clone is unsuitable for Y2H analysis and will need to be redesigned.

Note: Normally, all transformed yeast will grow on TL plate after 3-4 days. If there is a strong interaction between bait and prey proteins, the yeast will grow in 3-4 days on the selection medium TLHA or TLH+3AT, and the color of yeast clone is white, and the clone size could be more than 2 mm. If there is a weak interaction between bait and prey proteins, yeast will grow after 6-7 days on TLHA or TLH+3AT, and the color looks a little bit white and brown, the clone size is approximate 1 mm. If there is no interaction, yeast will only grow on the TL plate, not on the TLHA or TLH+3AT plate.

A.2 Protocol of protein expression and extraction in *E. coli* strain

The *E. coli*-express system was used in our study to yield the GST or His tagged proteins for pull-down assay.

Preparation before experiment

--- Extraction buffer for GST-tagged protein (pH 7.5, keep in 4 ℃)

Tris 50 mM

NaCl 50 mM

Glycerol 10%

--- Extraction buffer for His-tagged protein (pH 7.8, keep in 4 ℃)

1M KH_2PO_4 stock solution (A)

1M K_2HPO_4 stock solution (B)

For 100mL His extraction buffer:

Potassium phosphate	50 mM (0.3 mL A + 4.7 mL B)
NaCl	400 mM (2.3 g)
KCl	100 mM (0.75 g)
Glycerol	10% (10 mL)
Triton X-100	0.5% (0.5 mL)
Imidazole	5 mM (34 mg)

Preculture of *E. coli* strains

1. Scratch the stock bacteria containing the plasmid of interest on LB plate with 50 ug/ml CBC, culture on 37°C, overnight (O/N).

2. Take a huge amount of bacteria in 5 ml LB with 50ug/ml CBC. Shake at 250 rpm, 37°C, O/N.

3. Transfer 2 ml of culture in 50 ml LB with 50ug/ml CBC. Shake at 250 rpm, 37 °C, till OD600≈0.5.

4. Keep 1-2 ml of non-induced cells, centrifuge at 5000 rpm for 2 min. Resuspend pellet in 0.1-0.2 ml (10% of initial volume) extraction buffer, and then sonication 15 sec. This non-induced sample could be kept at -20 °C.

Expressing induction

5. For BL.21 strains: When OD600≈0.5, add 1 ml arabinose (0.2% final) and keep shaking at 250 rpm, 24 °C, 3-4h. For Rosetta: When OD600≈0.5, directly transfer the flask to shake at 250 rpm, 24 °C, 3-4h.

Note: The expressing conditions for different proteins are different. So it is necessary to optimize the induction temperature and/or the induction time.

6. Centrifuge the culture at 5000 rpm for 10 min. Discard the supernatant, and the pellet can be kept at -80 °C for a long time use.

Break the bacteria

8. Resuspend the pellet in 5mL extraction buffer.

Note: If the protein expressed in a low level, try to use less buffer (~2mL) to resuspend the pellet, and then mix the pellet together.

9. The plasma membrane of the cells are disrupted using **French pressure cell press** (LRSV, Université Paul Sabatier, Toulouse). The samples are kept in ice.

10. Keep the solution on ice surrounding and perform sonication 30 sec, 2 times.

Protein extraction

11. After sonication, remove 60ul aliquot (Total fraction).

12. Centrifuge remaining fraction at 13000rpm, 4 °C, 10 min. Remove carefully the supernatant (Soluble fraction).

13. Resuspend pellet in 600ul extraction buffer, and mix well (Insoluble fraction).

Note: All proteins samples could be kept in -20 °C for about 1-3 months (depending on the protein), and should be analyzed by western blot.

A.3 Protocol of western blot

Buffer preparation

--- Migration buffer: Glycine 192 mM; Tris 25 mM; SDS 0.1%.

--- Transfer buffer: Glycine 192 mM; Tris 25 mM; Methanol 20%.

--- TBS (Washing buffer): Tris 20 mM; NaCl 0.5 M; Tween 0.1%.

--- Blocking buffer: 2% blocking powder (ECL kit, GE Healthcare) dissolve in TBS.

Western blot protocol

1. Different protein fractions (20μL) are loaded on SDS-PAGE gel (10% polyacrylamide) for electrophoresis. After running the gel, proteins were transferred to membrane (Nitrocellulose, 0.45 μm) at 150 V for 1 h.

2. Shake the membrane in blocking buffer at 50 rpm, room temperature for 1 h or 4 ℃ over night.

3. Briefly rinse the membrane with two changes of washing buffer.

4. Primary antibody is diluted with blocking buffer. The dilution ratio needs to be optimized for specific antibody and protein: anti-GST: 1:10000, or anti-His: 1:5000 (Mouse monoclonal anti-GST/His, Sigma).

5. Incubate the membrane with Primary antibody at 50 rpm, room temperature for 1 h.

6. Briefly rinse the membrane with two changes of wash buffer.

7. Wash the membrane in wash buffer (25-30mL) at 50 rpm, room temperature for 15 min.

8. Wash the membrane for 5 min with fresh changes of wash buffer (25-30mL).

9. Repeat Steps 7 two more times for a total of four washes. And then discard the wash buffer.

10. Secondary antibody (Anti-mouse IgG, Sigma) is diluted 1:20000 with blocking buffer. The dilution ratio needs to be optimized for specific antibody and protein.

11. Incubate the membrane with secondary antibody at 50 rpm, room temperature for 1h.

12. Wash as step 7-9

13. Drain off excess of washing buffer. Then expose the membrane with ECL kit (GE Healthcare) by following the instruction book.

Note: For each membrane containing 10 lanes of samples, 2-3 ml of the solution from ECL kit is enough for exposure.

A.4 Protocol of pull-down assay

The pull-down experiment protocol mainly refers to the MagneGST Pull-Down System User Manual (Promega). If there are any optimization and/or changes about the protocol in our experiment, we would give a "Note" after each step of the protocol.

Preparation before experiment

MagneGST Binding/Wash Buffer (pH 7.2)

4.2mM	Na_2HPO_4
2mM	KH_2PO_4
140mM	NaCl
10mM	KCl

2X SDS gel-loading buffer

100mM	Tris-HCl (pH 6.8)
4%	SDS
0.2%	bromophenol blue
20%	glycerol
20mM	dithiothreitol

Note: SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before use.

Particle Equilibration

1. Thoroughly resuspend the MagneGST Particles by inverting the bottle several times to obtain a uniform suspension.

2. Pipet 30µl of MagneGST™ Particles into a 1.5ml tube. Do not allow the MagneGST Particles to settle for more than a few minutes during capture of the bait protein as this will reduce binding efficiency.

3. Place the tube in the magnetic stand and allow the MagneGST Particles to be captured by the magnet. Magnetic capture will typically occur within a few seconds.

4. Carefully remove and discard the supernatant.

5. Remove the tube from the magnetic stand. Add 250µl of MagneGST Binding/Wash Buffer to the particles and resuspend by pipetting or inverting.

6. Repeat Steps 3-5 two more times for a total of three washes.

Binding

1. After the final wash, resuspend the particles in 100µl of MagneGST Binding/Wash Buffer.

Note: Addition of up to 1% BSA may reduce nonspecific binding and potential problems with background. The amount of BSA used may need to be optimized for your particular protein.

2. Add 50µl GST-fusion bait protein or the GST control to the MagneGST Particles.

3. Incubate (with constant gentle mixing) for 30 minutes at room temperature on a rotating platform.

Washing

1. Place the tube in the magnetic stand and allow the MagneGST Particles to be captured by the magnet. Carefully remove the supernatant and save for gel analysis (optional).

2. Add 250µl of MagneGST Binding/Wash Buffer to the particles and gently mix. Incubate at room temperature for 5 minutes while mixing occasionally by tapping or inverting the tube.

3. Place the tube in the magnetic stand and allow MagneGST Particles to be captured by the magnet. Carefully remove the supernatant and discard (or save if analysis of wash is desired).

4. Add 250µl of MagneGST Binding/Wash Buffer to the particles and mix gently by inverting the tube. (The 5-minute incubation is not required at this wash step.)

5. Place the tube in the magnetic stand and allow the MagneGST Particles to be captured

by the magnet. Carefully remove the supernatant and discard (or save if analysis of wash is desired).

6. Repeat Steps 4-5 for a total of three washes.

7. After the last wash, resuspend the particles in 50µl of MagneGST Binding/ Wash Buffer.

Optional: Remove a 10µl aliquot for analysis of the efficiency of immobilization of the GST-fusion protein or GST control onto the particles. To this aliquot, add 10µl 2X SDS loading buffer and elute proteins by boiling for 5 minutes. Analyze by SDS-PAGE.

Capture

1. To 40µl of particles carrying GST-fusion protein (or GST control) add 100µl of the prey protein.

2. Add 400µl MagneGST Binding/Wash Buffer and 60µl 10% BSA (or 460µl MagneGST Binding/Wash Buffer if BSA is omitted) to a final volume of 600µl for each pull-down reaction. (Addition of 1% BSA may reduce nonspecific binding and potential problems with background. The amount of BSA used may require optimization for your particular protein-protein interaction.)

Note: MagneGST Binding/Wash Buffer is a neutral PBS buffer, allowing the user to optimize buffer conditions for each specific protein-protein interaction. Some protein interactions will require the presence of various cofactors, salts and detergents.

3. Incubate for 1 hour (with gentle mixing) at room temperature on a rotating platform.

Note: Briefly vortexing at the end of this incubation period may help remove non-specific adherent proteins and reduce background. A different incubation temperature and time may be required for each specific protein-protein interaction.

4. Place the tube in a magnetic stand and allow the MagneGST Particles to be captured by the magnet. Carefully remove the supernatant and save for analysis (optional).

Washing

The stability of different protein-protein interactions is protein pair-specific and depends

on the Kd of the interaction. Optimization of washing conditions may be required for less stable interactions. For example, the number of washes and the volume of each wash may need to be changed.

1. Add 400µl of MagneGST Binding/Wash Buffer, and mix gently by inverting the tube.

2. Incubate at room temperature for 5 minutes while mixing occasionally by tapping or inverting the tube.

3. Place the tube in the magnetic stand and allow the MagneGST Particles to be captured by the magnet. Remove the supernatant and save for analysis (it is especially important to keep this fraction during initial optimization).

4. Add 400µl of MagneGST Binding/Wash Buffer and mix gently by inverting the tube. (The 5-minute incubation is not required at this wash step.)

5. Place the tube in the magnetic stand and allow the MagneGST Particles to be captured by the magnet. Carefully remove the supernatant and save for analysis (optional).

6. Repeat Steps 4 and 5 two more times for a total of four washes.

Elution

1. Add 40µl of 2X SDS loading buffer.

2. Incubate for 5 minutes at room temperature with mixing.

3. Place the tube in the magnetic stand and allow the MagneGST Particles to be captured by the magnet. Remove the eluate for analysis.

Note: After adding 2X SDS loading buffer, this SDS solution sample containing MagneGST Particles can be used directly for SDS-gel.

Prepare samples for SDS-PAGE analysis. For Western blot analysis, boil 20ul eluted sample (or SDS solution containing MagneGST Particles) for 5 minutes then load onto an SDS-PAGE gel. Stronger signals in the experimental samples compared to the GST control lanes indicate that the prey is specifically pulled down by the bait protein.

A.5 Protocol of transient expression in tobacco protoplasts

Culture of BY-2 tobacco cells

Cells culture is under rotative agitation, in the dark at 25 °C. 7-day-old cells are subcultured under sterile conditions once a week: 2 ml cell suspension in 50 ml medium. For 1 liter of medium: MS Medium 4,3 g, KH_2PO_4 200 mg, sucrose 30 g, myoinositol 100 mg. Adjust pH to 5.8 with KOH 1 M. Autoclave and then add: Thiamine 10 mg (1 ml of 10 mg/ml stock solution), 2,4-D: 180 µg (180 µl of 1 mg/ml stock solution in H_2O stored at 4°C).

Preparation of cells for transformation

Twenty mL of tobacco BY-2 cell suspension were taken from one week old culture and were centrifuged at 3500 rpm for 5 min, the supernatant was discarded ant the pellet was rinsed by centrifugation by 40 mL of Tris-MES 25 mM buffer and mannitol 0.6 M maintained at pH5,5. The supernatant was discarded and 2 g of cell culture was placed on a Petri dish.

Enzymatic digestion

20 mL enzymatic solution which contained the following: 1 % Caylase 345 (*CAYLA*, Toulouse, France), 0.2% pectolyase Y-23, 1 % BSA, in Tris-MES 25 mM Mannitol 0,6M, pH 5.5. The solution was then filtered on 0.45 µm filter and 2 g of culture cells were taken and poured into the tube containing 20 mL of enzymatic solution. Then the cells were incubated for 30 min at 37°C in an agitative water bath (30-40 rpm) in obscurity. Finally, the digestion was verified by microscopy.

Purification of protoplast

Protoplasts were then filtered through 30 µm Nylon cloth in a 30 mL tube and washed two times with W5 solution (NaCl 154 mM, $CaCl_2$ 125 mM, KCl 5mM, glucose 5 mM and MES 0.1 %, pH 5.6) by centrifugation 5 min at 1000 rpm (100 g).

The supernatant is removed by aspiration, the pellet resuspended in 10 mL W5 medium

and the protoplasts counted under the microscope.

The protoplast suspension is centrifuged 5 min at 10000 rpm and the pellet resuspended in a volume of mannitol/Mg buffer (0,55 M mannitol, 15 mM $MgCl_2$, 0,1% MES, pH 5,6) to obtain a final protoplast concentration of 1.10^6 protoplasts /ml. The protoplasts were then incubated 30 min on ice.

Transient transformation

Protoplasts were transfected by a modified polyethylene glycol method as described by Abel and Theologis, 1994. Typically 0.2 ml of protoplast suspension (1.10^6 / ml) were transfected with 25 µg of sheared salmon sperm carrier DNA (Clontech) and 10 -20 µg of the appropriated plasmid DNA. Then 200 µl of protoplasts suspension were added together with the same volume of PEG solution (40 % PEG 4000, 0,1 M $Ca(NO_3)_2, 4\ H_2O$, 0,5 M mannitol, 0,1 % MES, pH 6,5).

The protoplasts were incubated for 60 min at room temperature, mixing slowly the tubes by inversion several times. Then, 0.8 ml solution W5 was added, and the protoplast suspension homogenized by inversion and centrifuged 10 min at 100 g. The supernatant was removed by aspiration and the pellet resuspended in 1 ml W5 solution. The tubes were incubated at 25 °C, in the dark during 16 hours at least.

A.6 Protocol of bimolecular fluorescence complementation (BiFC)

BiFC fusion constructs

The coding sequence of the potentially interacting protein A and B have been cloned into the BiFC vector pAM-35SS-YFPc-GW and pAM-35SS-YFPn-GW by the gateway cloning technology (Invitrogen). The N terminal of protein-A was fused to the N terminal half of YFP (nYFP) to yield nYFP-A. The N terminal of protein-B was fused to the C-terminal half of YFP (cYFP) to yield cYFP-B.

BiFC experiment protocol

1. The tobacco protoplasts were transformed by the PEG transient method described above.

2. Protoplasts co-transfected with 10 µg of nYFP- and cYFP-fusion plasmids were assayed for fluorescence 16 hours after transformation using a Leica TCS SP2 confocal laser scanning microscope (FR AIB, FR 3450 CNRS, 24 chemin de Borde Rouge, 31326 Castanet Tolosan). Images were obtained with a 40x 1.25 numerical aperture water-immersion objective.

3. YFP was excited at 488 nm wavelength and emission was captured at 530 to 570 nm wavelength.

4. To quantify more precisely the fluorescence intensity, the YFP fluorescence was also analyzed by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA) on the Cytometry and cell sorting platform (INSERM UPS UMR 1048, Toulouse RIO imaging platform).

5. Data were analyzed using Cell Quest software. For each sample, 5000 protoplasts were gated on forward light scatter and the GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the cells population after subtraction of auto-fluorescence determined with non-transformed BY-2 protoplasts.

A.7 Protocol of protein extraction from tobacco protoplast

In our study, GFP, IAA3-GFP, IAA9-GFP and ARF8a-GFP proteins were extracted from transformed tobacco protoplasts.

Buffer preparation

--- Protoplast Washing Buffer, pH 6.7

HEPES-KOH	25 mM
Sorbitol	0.6 M

--- 10% and 20% Ficoll dissolved in Protoplast Washing Buffer

Protein extraction protocol

1. Protoplasts (~4 mL transfected protoplast suspension) are centrifuged at 400 rcf, 4℃ for 5 min.

Note: The starting volume of transfected protoplast suspension can be optimized depending on the cells quality and transformation efficiency.

Note: Keep ~50ul protoplast suspension as a control (Sample before extraction) for western blot assay.

2. The pellet is resuspended slowly and mixed softly in 2 mL 20% Ficoll.

Note: All buffers and materials needed to be pre-cold at 4°C. Keep everything on ice or at 4°C during experiment till the end.

3. Slowly add 2 mL 10% Ficoll on the top of the 20% Ficoll solution, and again, slowly add 2 mL washing buffer on the top of 10% Ficoll.

4. Centrifuge the three-layer solution at 200 rcf, 4℃ for 30 min.

5. Purified protoplasts are recovered from the interface between the Washing Buffer and the 10% Ficoll layer.

6. Carefully remove the purified protoplast, and wash with the Washing Buffer by

mixing well and then centrifuge at 800 rcf, 4℃ for 5 min.

7. Discard the supernatant. The pellet is resuspended in 1 mL Washing Buffer.

8. Pass six times through a 0.45×12-mm needle to completely lyse the cells, and then sonication 30s×3 times.

9. Centrifuge the solution at 13000 rcf, 4℃ for 10 min.

10. Keep supernatant as the soluble fraction of proteins (Sample after extraction).

Note: If needed, pellet can be resuspended in 400ul Washing Buffer (Pellet fraction or Non-soluble fraction) for western analysis.

Note: Normally, samples can be kept in -20℃ at least 2 months.

Western blot assay

GFP, IAA3-GFP, IAA9-GFP and ARF8a-GFP proteins extracted from protoplasts were detected with anti-GFP (Mouse monoclonal anti-GFP, Sigma) by western blot assay. The materials and methods of western blot refer to the Part-2.3 of Chapter II. The dilution ratio of GFP antibody is: 1:5000, and the dilution ratio of secondary antibody is: 1:20000 (Anti-mouse IgG, Sigma).

A.8 Protocol of nucleus extraction from tobacco protoplast

pGreen-IAA9-GFP was used for transient expression assay in tobacco protoplasts. The tobacco protoplast nuclei were extracted from transformed protoplast to analyze the IAA9-GFP protein expression pattern. Materials and methods for nucleus extraction (Xiong TC, 2005) as follow:

Buffer preparation

--- Protoplast Washing Buffer, pH 6.7

HEPES-KOH	25 mM
Sorbitol	0.6 M

--- Protoplast Lysis Buffer, pH5.3

MES-KOH	10 mM
Sucrose	0.4 M
NaCl	10 mM
$MgCl_2$	5mM
DTT	0.1 mM
EDTA (pH8)	5 mM
PMSF	0.5 mM

Note: PMSF could be replaced by other Protease inhibitor.

--- 10% and 20% Ficoll dissolved in Protoplast Washing Buffer

--- 20%, 25% and 36% Iodixanol dissolved in sterilized water

Nucleus extraction protocol

1. Protoplasts (~4 mL transfected protoplast suspension) are centrifuged at 400 rcf, 4℃ for 5 min.

Note: The starting volume of transfected protoplast suspension can be optimized depending on the cells quality and transformation efficiency.

Note: Keep ~50ul protoplast suspension as a control (Sample before extraction) for western blot assay.

2. The pellet is resuspended slowly and mixed softly in 2 mL 20% Ficoll.

Note: All buffers and materials needed to be pre-cold at 4°C. Keep everything on ice or in 4°C surrounding during experiment till the end.

3. Slowly add 2 mL 10% Ficoll on the top of the 20% Ficoll solution, and again, slowly add 2 mL washing buffer on the top of 10% Ficoll.

4. Centrifuge the three-layer solution at 200 rcf, 4℃ for 30 min.

5. Purified protoplasts are recovered from the interface between the Washing Buffer and the 10% Ficoll layer.

6. Carefully remove the purified protoplast, and wash with 6mL Washing Buffer by mixing well and then centrifuge at 800 rcf, at 4℃ for 5 min.

7. Discard the supernatant. The pellet is resuspended in 2 mL Lysis Buffer.

8. Pass six times through a 0.45×12-mm needle to completely lyse the cells.

9. Add 2 mL 20% iodixanol and mix well to have a final concentration of 10% iodixanol (4 mL of total sample).

10. Prepare an empty tube and carefully add solutions as followed order: 2 mL 36% iodixanol (bottom) --- 2 mL 25% iodixanol (middle) --- 4 mL total sample (top).

Note: Each solution should be added very slowly to avoid mixing between the different iodixanol solutions. Then three distinct layers will be seen after this step.

11. Centrifuge the three-layer solution at 3000 rcf, 4℃ for 30 min.

12. Take the colorless interface between 25% and 36% iodixanol. This fraction contains the crude extract of protoplast nuclei.

13. Add 2mL of Lysis Buffer and mix well for washing, then centrifugation at 3000 rcf, 4℃ for 10 min.

14. Pellet (nucleus) are resuspended in 100 ul Lysis Buffer, and kept at -20℃ .

Western blot assay

Purified intact nuclei from pGreen-IAA9-GFP-transformed protoplasts were detected with anti-GFP-antibody (Mouse monoclonal anti-GFP, Sigma) by using western blot assay. For a control, IAA9-GFP protein before extraction and IAA9-GFP protein after extraction (Samples to see part-2.6 this Chapter) were also loaded on the gel for western blot. The dilution ratio of GFP antibody is: 1:5000, and the dilution ratio of secondary antibody is: 1:20000 (Anti-mouse IgG, Sigma).

A.9 Additional result in Y2H

	IAA 1	IAA 3	IAA 4	IAA 7	IAA 8	IAA 9	IAA 11	IAA 12	IAA 14	IAA 15	IAA 16	IAA 17	IAA 19	IAA 22	IAA 26	IAA 27	IAA 29
TPL1																	
TPL2																	
TPL3																	
TPL4																	
TPL5																	
TPL6																	

Y2H result of BD-TPL + AD-IAA. Green color means yeast grew quickly less than 4 or 5 days after co-transformation, indicating a strong interaction between BD-TPLs and AD-IAAs partners. Yellow shows yeast grew slowly in 7-8 days after co-transformation indicating a weak interaction between the tested BD-TPL and AD-IAA. Red means there is no interaction.

	IAA 1	IAA 3	IAA 4	IAA 7	IAA 8	IAA 9	IAA 11	IAA 12	IAA 14	IAA 15	IAA 16	IAA 17	IAA 19	IAA 22	IAA 26	IAA 27	IAA 29
TPL1																	
TPL2																	
TPL3																	
TPL4																	
TPL5																	
TPL6																	

Y2H result of BD-IAA + AD-TPL. Green color means yeast grew quickly less than 4 or 5 days after co-transformation, indicating a strong interaction between BD-IAAs and AD-TPLs partners. Yellow shows yeast grew slowly in 7-8 days after co-transformation indicating a weak interaction between the tested BD-IAA and AD-TPL. Red means there is no interaction.

	ARF 1	ARF 2a	ARF 2b	ARF 3	ARF 4	ARF 5	ARF 6	ARF 7	ARF 8	ARF 9a	ARF 9b	ARF 10a	ARF 10b	ARF 16a	ARF 16b	ARF 17
TPL1																
TPL2																
TPL3																
TPL4																
TPL5																
TPL6																

Y2H result of BD-TPL + AD-ARF. Green color means yeast grew in 4 or 5 days after co-transformation, indicating an interaction between BD-TPLs and AD-ARFs partners. Red means there is no interaction.

	ARF 1	ARF 2a	ARF 2b	ARF 3	ARF 4	ARF 5	ARF 6	ARF 7	ARF 8	ARF 9a	ARF 9b	ARF 10a	ARF 10b	ARF 16a	ARF 16b	ARF 17
TPL1																
TPL2																
TPL3																
TPL4																
TPL5																
TPL6																

Y2H result of BD-ARF + AD-TPL. Green color means yeast grew in 4 or 5 days after co-transformation, indicating an interaction between BD-ARFs and AD-TPLs partners. Red means there is no interaction. (Concentration of 3-AT added in TLH or TLHA plate: 20mM 3-AT for BD-ARF6, 7 and 8; 100mM 3-AT for BD-ARF5)