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Possible interactions between TFL2 and proteins in the AUX/IAA family

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Summary

Auxins regulate important process during the plant development and growth. In a recent study an interaction between two important proteins involved in regulation of the gene expression during the plant development was found. These proteins are the TERMINAL FLOWER2 (TFL2), a gene repressor of important genes involved in the transition from vegetative to reproductive stage in plants; and the AUX/IAA5 protein, belonging to a protein family of repressors over the transcription factors that regulate the expression of primary auxin response genes. In this study we checked the interaction between 24 of the 29 members of the AUX/IAA family with TFL2 and we found one new interaction between AUX/IAA20 and TFL2, not known until this study.

Introduction

Auxins, mainly indole-3-acetic acid (IAA), are vital phytohormones involved in plant development and growth. Auxins are mainly synthesized in the apical meristem and in young leaves. They are transported basipetally from the shoot to the root through the proposed chemiosmotic model based on proton motive force and the membrane potential. Auxin are involved in different processes during plant development such as the formation of lateral and adventitious roots, in apical dominance, vascular differentiation, floral development and phyllotaxy, fruit development, cell elongation in stems and coleoptiles or in phototropism and gravitropism. (Taiz & Zeiger, 2006)

One protein involved in the interaction is the TFL2 protein, this is encoded by the *TFL2* gene and belongs to a subset of genes that regulate the function of the shoot meristems during the vegetative to reproductive transition in the plant. The TFL2 protein is also called LIKE HETEROCHROMATIN PROTEIN1 (HP1) due to the similarity in the sequence with the protein HP1 from metazoans and fungi. (Turck et al. 2007) However, TFL2 functions similarly to HP1γ from mammals which is located in euchromatic sites and mainly associates with genes marked by a Histone 3 (H3K27me3) that has been tri-methylated in the lysine 27 by the Polycomb repressive complex 2 (PRC2). HP1 proteins have two conserved domains, one is a monomeric N-terminal chromo domain (CD) that binds to chromatin, and the other is a dimeric C-terminal chromo-shadow domain (CSD) involved in protein interaction with HP1 associating proteins, such as the chromatin assembly factor 1 (CAF1) in mammals (Hiragami et al. 2005) Therefore, the proposed function of TFL2 is to maintain the transcriptional repression of some genes targeted by that PRC2 through the interaction with its chromo domain. (Turck at al. 2007) Some genes repressed by TFL2 are involved in the reproductive stage during the plant development, this is the case of the important floral repressor FLOWERING LOCUS C (FLC) which is active during the vegetative stage and inactivated by TFL2 during flowering. (Mylne et al. 2006) Another important gene is the floral promoter FLOWERING LOCUS T (FT), it is repressed by TFL2 during the vegetative stage and is responsible of the early flowering in *tfl2* mutants (Kotake et al. 2003)

AUX/IAA5 belongs to the auxin/indole-3-acetic acid protein family with 29 different genes and proteins in *Arabidopsis* (Overvoorde et al, 2005). These proteins are a short-lived protein family of transcriptional repressors which expression is induced by auxins and they are responsible of the rapid responses to auxins, such as cell elongation or tropisms(Abel et al, 1994). They have a repression effect over a transcription factor family, the Auxin Response Factor (ARF). The ARF family is encoded by 23 *ARF* genes and they bind specifically to the auxin response elements AuxRE's, the promoters of auxin response genes, activating or repressing the activity of these. The ARF structure contains a N-terminal DNA-binding domain (DBD) that binds to AuxRE's, an activation or repression domain (AD or RD) that repress or activate the transcription and a C-terminal dimerization domain (CTD) that binds to AUX/IAA's (Guilfoyle et al. 2007). The AUX/IAA protein structure consists in four conservated domains separated in the amino acid sequence. The domain I is which binds to the AD/AR of ARF's repressing them, the domain II is involved in the AUX/IAA instability and degradation by polyubiquinitation, and the domains III and IV are which heterodimerizate with the CTD domain of ARF's and merge both proteins (Overvoorde et al., 2005). The AUX/IAA protein synthesis and activity is directly related to

auxins and they regulate the auxin-induced gene expression (Abel, 2007). In high concentrations of auxins, it binds to the ubiquitin E3 ligase of the Skp1/Cullin/F-box protein complex (SCF), a protein abundance regulator complex, which F-box protein domain, called transport inhibitor response 1 (TIR1) in *Arabidopsis,* is an auxin-receptor domain in the SCF that binds to auxin (Woodward et al., 2005; Delker et al., 2008) When TIR1 binds to auxin the SCF complex is stabilized and the ubiquitin transferase activity of the SCF is activated (Gray et al. 2001) Then, the $SCF^{TIR1\text{-}Auxin}$ can bind to AUX/IAA protein, attaching covalently ubiquitin to the domain II of it and then, an ubiquitin-mediated proteolysis is carried out by a 26S proteasome, degrading the AUX/IAA and releasing the ARF (Abel, 2007; Woodward et al., 2005).

Until now, only the interactions between the subfamily of AUX/IAA proteins (Overvoorde et al, 2005), AUX/IAA5, AUX/IAA6 and AUX/IAA19, with TFL2 have been reported (Landberg et al., 2007; Rizzardi, K. 2008, not published) but not the interaction of TFL2 with other proteins of the family. In this project we report a new interaction between TFL2 and AUX/IAA20, for that, we cloned the coding sequence of the AUX/IAA proteins from cDNA of 14 days seedlings into expression vectors and we used the Yeast-2-hybrid system to find interactions. We checked 24 of the 29 AUX/IAA's and also two interactions of the AUX/IAA5, which interaction with TFL2 is known, with a deletion of the domain I in one case and with the deletion of domains II , III and IV in the other.

Results

We got two interactions on selective agar plates of 52 interactions checked. One was TFL2 with TFL2, one of the positive controls, and the other was the interaction IAA20-AD-BD-TFL2 and TFL2-AD-BD-IAA20 on the selection plates SD – Leu/Trp/His and SD - Leu/Trp/His/Ade (**Figure 1**) We didn't get yeast growth in the other three positive controls, the AUX/IAA 5, 6 and 19 with TFL2 as well as in the other interactions checked. We also had growth in some negative controls, not mated yeast, such as AUX/IAA 4, 5A234,15, 17-AD and AUX/IAA 19-BD. They showed growth on SD – Leu / Trp agar plates but they did not grow when they were repeated again and also when they were streaked on SD - Leu/Trp/His and SD - Leu/Trp/His/Ade agar plates. We could not check the AUX/IAA 12, 13, 30, 32 and 33 because we had problems to amplify the coding sequence of these AUX/IAAs by PCR and we had to delay them with respect the other ones. Now, they are ready to send for sequencing after the BP reaction.

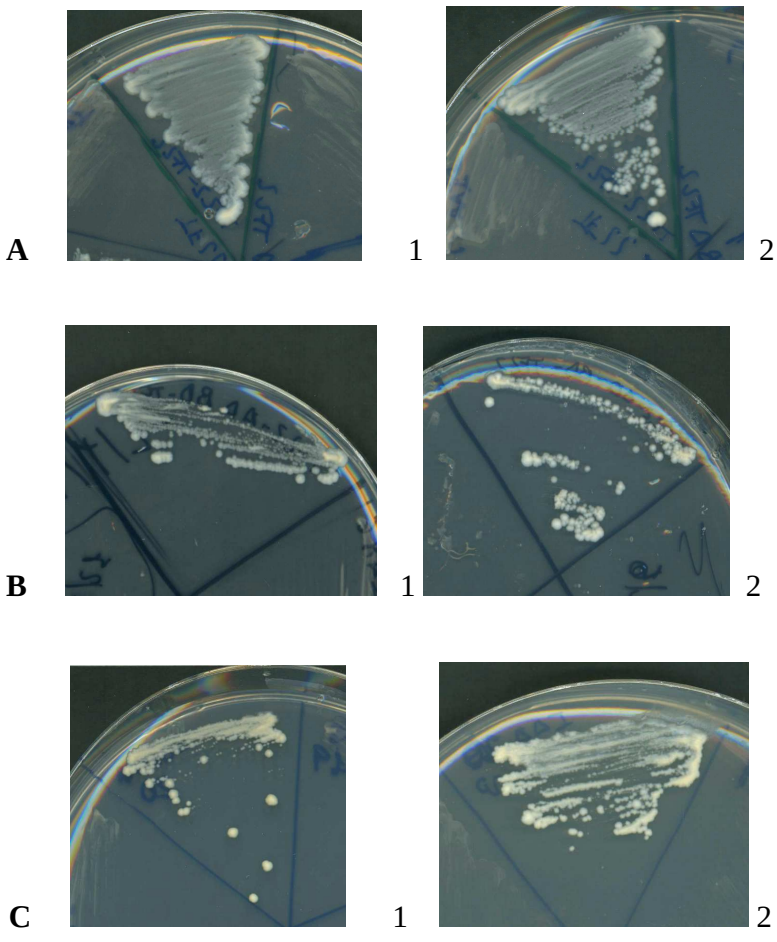

Figure 1. **Interactions found on plates. A**: TFL2-TFL2; **B:** IAA20-AD-BD-TFL2; **C**: TFL2-AD-BD-IAA20. **1**: SD-Leu/Trp/His agar plates ; **2:** SD-Leu/Trp/His/Ade agar plates.

Discussion

The Yeast-2-hybrid system is a very common method to check protein interactions in vitro. This method was designed by Fields & Song in 1989 as a new and relatively quick system to find interactions between proteins. Since that time, this method has been improved and developed to avoid false positives and negatives and increase its reliability. In this project we have used the yeast strain PJ69-4A/α, this was created by James et al. (1996) to reduced the false positives using three different reporter genes, the *HIS3* gene, the *ADE2* gene and the *lacZ* gene, merged to GAL promoters located in different sites along the yeast genome. We used for selection of the protein interaction selective SD agar plates based in the absence of His and Ade. This selection method is, presumably, a good selective system with accurate results or at least with almost any false positives and, by this reason, we can believe in the interaction between AUX/IAA20 with TFL2. Otherwise, we repeated the interaction in tree occasions and we got, in all of them, yeast growth in both selective plates that we used, SD - Leu/Trp/His and SD - Leu/Trp/His/Ade, and also in both interactions, IAA20-AD-BD-TFL2 and TFL2-AD-BD-IAA20, fact that support our result. The absence of interaction in three of the four positive controls we took, could limit the accuracy of our results but the interaction between TFL2 with TFL2 may be enough because we got this interaction in every repetition of the mating and, however, any growth of the other positive interactions. In the case of the last interactions checked, the AUX/IAA 2, 3, 4, 5 Del. 234, 9, 11 15 26, 27, 31 and 34, we did not get yeast growth even in the TFL2-TFL2 interactions, we think that these results are because the plates with the transformed yeast containing TFL2 clones are more than one month old and the yeast survival and efficiency is very low for the mating.

Until now, just the interactions between TFL2 with AUX/IAA5, 6 and 19 have been checked (Rizzardi, 2008) but anyone else. We do not know how strong are the different interactions of the TFL2 with AUX/IAA proteins, perhaps there are protein interactions much weaker than the TFL2-TFL2 interaction and also between other AUX/IAA with TFL2 such as AUX/IAA20 with TFL2. Consequently, it is possible that yeast with weak interactions could not grow in very selective media or takes much more time than yeast with stronger interactions. So, to detect growth in yeast with weak interactions would be interesting in future interaction screening using the third reporter gene, the *lacZ* gene or β-galactosidase gene, that we have not used in this project. It could be very useful for measure the growth of yeast with weak interactions, through growing it in liquid SD-media with X-gal and determining the growth by spectrophotometry, such as it is mentioned in James et al. (1996). Apart of that, other reasons for absence of interactions may be in the protein interaction itself. For example, the protein folding could be affected by the phusion to *GAL4*-AD and *GAL4*-DBD used in Y2H, or furthermore, in protein interaction usually participates more than two proteins as well as ligands or inorganic ions very important for the union. This is the case of the protein TOPLESS, recently it has been reported in auxin regulation that the repressor effect of the IAA12/BDL, a transcription repressor, over the Ethylene Response Factor (ERF), a transcription factor, needs of the interactions with the protein TOPLESS to take place (Szemenye et al. 2008).

Overall, the repetition of the last interaction with fresh transformed yeast containing TFL2 and AUX/IAA 5, 6 and 19 streaked on new plates, would be necessary to check those interactions more accurately. As well as search for weak interaction, quantifying the yeast growth in SD

liquid media with X-gal. Also, it would be important to check the found interaction between AUX/IAA 20 with TFL2 using others methods, such as the interaction directly *in planta* by BiFC (Bracha-Drori et al. 2004) to confirm this result. In conclusion, further experiments are necessary to study the interaction between TFL2 and AUX/IAA proteins and to understand this new connection between these previously unconnected proteins

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Materials and Methods

Cloning of AUX/IAA's.

We started amplifying by PCR the open reading frame (ORF's) of every AUX/IAA from cDNA of 14 day old seedlings using the Phusion™ High-Fidelity DNA Polymerase from Finnzymes. For cloning the AUX/IAA's we used the Gateway® Technology with Clonase™ II from Invitrogen™, by this reason, the primers were designed with attB sites incorporated in the sequence of the forward and reverse primer. The primers used were TFL2 F 5'-GGG GAC AAG TTT GTA C AAA AAA GCA GGC TTC ATG AAA GGG GCA AGT GGT GC-3', TFL2 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA AGG CGT TCG ATT GTA CTT GAG-3'-3', IAA1 F 5'- GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAA GTC ACC AAT GGG C-3', IAA1 R 5'- GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TAA GGC AGT AGG AGC TTC-3', IAA2 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GCG TAC GAG AAA GTC AAC-3', IAA2 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TAA GGA AGA GTC TAG AGC AGG-3', IAA3 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAT GAG TTT GTT AAC CTC-3', IAA3 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TAC ACC ACA GCC TAA ACC-3', IAA4 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAA AAA GTT GAT GTT TAT G-3', IAA4 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA AAG ACC ACC ACA ACC TAA-3', IAA5 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GCG AAT GAG AGT AAT AAT C-3', IAA5 R 5'- GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TCC TCT GTT ACA TGA TCT C-3', IAA6 F 5'.-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GCA AAG GAA GGT CTA GCA C-3', IAA6 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA ATC TTG CTG GAG ACC AAA ACC-3', IAA7 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG ATC GGC CAA CTT ATG AAC C-3', IAA7 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA AGA TCT GTT CTT GCA GTA C-3', IAA8 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG TCT TAT CGA TTG CTA AGT G-3', IAA8 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA AAC CCG CTC TTT GTT C-3', IAA9 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG TCC CCG GAA GAG GAG CTA C-3', IAA9 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA AGC TCT CAT CTT CGA TTT-3', IAA10 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG AAT GGT TTG CAA GAA GTT TG-3', IAA10 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA CTT ACC TAC TCC AGC TCC-3', IAA11 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAA GGC GGT TCC GCT AG-3', IAA11 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA CAA AGA GAA CAT ATA ACT-3', IAA12 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG CGT GGT GTG TCA GAA TTG-3', IAA12 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA TAG TGT ACG CAT TTG AAA AC-3', IAA13 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG ATT ACT GAA CTT GAG ATG-3', IAA13 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA AAC CGG CTG CTT TCG CTG-3', IAA14 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG AAC CTT AAG GAG ACG GAG C-3', IAA14 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TGA TCT GTT CTT GAA C-3', IAA15 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG TCA CCG GAG GAA TAC G-3', IAA15 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC

TGG GTA CTA TAA TCC AAT AGC ATC TCC GG-3', IAA16 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG ATT AAT TTT GAG GCC ACG-3', IAA16 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA ACT TCT GTT CTT GCA CTT TTC-3', IAA17 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATGATGGGCA GTGTCGAGCT GAATC-3', IAA17 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA AGC TCT GCT CTT GCA CTT C-3', IAA18 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAG GGT TAT TCA AGA AAC GG-3', IAA18 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TCT TCT CAT TTT CTC TTG-3', IAA19 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAG AAG GAA GGA CTC GGG-3', IAA19 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA CTC GTC TAC TCC TCT AGG C-3', IAA20 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATGGGAAGAG GGAGAAGTTC ATC-3', IAA20 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA GTA GTG GTA ATT AGC TC-3', IAA26 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATGGAAGGTT GTCCAAGAAA CAG-3', IAA26 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA GTG CAT CAT CTT CTC TTG -3', IAA27 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG TCT GTA TCT GTA GCA GC-3', IAA27 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA GTT CCT GCT TCT GCA CTT C -3', IAA28 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAA GAA GAA AAG AGA TTG G-3', IAA28 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTACTA TTC CTT GCC ATG TTT TC-3', IAA29 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAG TTG GAT CTT GGT CTA TC-3', IAA29 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA AAA CAA ACA TCT TGT ATA TG -3', IAA30 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATGGGAAGAG GGAGAAGCTC-3', IAA30 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA GTA GTG ATA AGC TCT TGAG-3', IAA31 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATGGAGGTCT CTAACTCTTG TTC-3', IAA31 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA ATA CCT CTC CGG TCT CGT-3', IAA32 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG GAC CCA AAC ACA CCT GCA G-3', IAA32 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA GTC TGG AAC AAC CTC AAT C-3', IAA33 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG AAT AGT TTC GAG CCA CAA AG-3', IAA33 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA CTC GTT TCT TTT AAC TTG TC-3', IAA34 F 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC ATG TAT TGC AGC GAT CCT CCC-3', IAA34 R 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA AAA GGG AAG TAC AGC ATC -3', IAA5 AI 5'-GGG GAC AAG TTT GTA CAAA AAA GCA GGC TTC AAA TGT GAA CCG GCG AAA AAG-3', IAA5 R 5'- GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TCC TCT GTT ACA TGA TCT C -3', IAA5 A234 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTACTT TTT CGC CGG TTC ACA TTT-3', IAA5 R 5'- GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TCC TCT GTT ACA TGA TCT C-3'. The PCR products were checked on an 0.8% agarose gel. PCR products were purified following the protocol from Invitrogen™ and the DNA concentration was measured using a Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer. We cloned our amplified sequences into a entry vectors through the BP recombination reaction. We used the vector pDONR™/Zeo, the *att*B labeled PCR-products and the Gateway® BP Clonase® II enzyme mix to create the entry clones. The obtained entry clones were transformed in competent E. Coli DH5α that were grown on LB low salt agar plates with 50 µg/ml of Zeocin. To extract the entry clones from E. coli colonies we used the QIAprep Spin Miniprep Kit from Qiagen and we followed the protocol provided by the

manufacturer. We also measured the DNA concentration with a Nanodrop Spectrophotometer and checked the plasmids on a 0.8% agarose gel. Subsequently, the obtained entry clones were sequenced through the sequence service of Macrogen and using the universal primers M13 Forward and M13 Reverse. The sequences we got were analyzed with BLAST from the NCBI webpage to we checked if the inserted sequences in the vector were correct. We followed with the LR recombination reaction using the entry clones with every AUX/IAA coding sequence and two destination vector, the pDEST22 and pDEST32 destination vectors. The pDEST22 is the bait or AD-plasmid with the *GAL*4-AD sequence inserted next to the *att*R1 site and the pDEST32 is the prey or BD-plasmid with the *GAL4*-DBD sequence also inserted next to the *att*R1 site in the vector. The LR reaction was performed using the Gateway® LR Clonase® II enzyme mix and combining every AUX/IAA entry clone with the AD destination vector and BD destination vector. The expression clones obtained were transformed in competent *E. coli* DH5α and grown on LB agar plates with 100 µg/ml Carbenicillin for *E.coli* with AD-expression clones and 12.5 µg/ml Gentamycin for *E.coli* with BD-expression clones. Colonies from the transformation plates were streaked on new plates. The AD and BD expression clones were taken out using a QIAprep Spin Miniprep Kit again. The AD/BD expression clones were checked on an agarose gel and the DNA concentration measured with a Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer.

Yeast-2-hybrid system.

We followed with Y2H system transforming the expression clones into the yeast strain of Saccharomyces cerevisiae PJ69-4A and PJ69-4α mating types (James et al., 1996). The selection of the yeast on agar plates was based in the absence of Tryptophan (Trp), Leucine (Leu), Histidine (His) and Adenine (Ade). The different selective agar plates used were: – Trp SD agar plates for selection of the transformed yeast with AD-clones and – Leu SD agar plates for selection of BD-clones. – Trp/Leu SD agar plates to select mated yeast after mating. And – Trp/Leu/His and – Trp/Leu/His/Ade SD agar plates were used to select yeast with interaction between AUX/IAA and TFL2. The AD-expression clones were transformed into PJ69-4A while the BD-expression clones were into PJ69-4α. The transformed yeast was grown at 30oC overnight on SD agar plates without Trp for AD-expression clones and without Leucine (Leu) for BD-expression clones. Colonies from the transformation plates were streaked again on new plates. We started the mating of the yeast growing yeast colonies with every expression clone in SD liquid medium without Trp for PJ69-4A yeast with the AD expression clone and without Leu for PJ69-4 α yeast with the BD expression clone at 30oC overnight. We also included the AD and BD expression clones with TFL2 inserted in. We made the mating on YAPD agar plates divided in eight portions with one spot everyone. We mix on every spot 5 µl of SD solution with yeast containing the AD-TFL2 expression clone with every SD solution containing yeast with every BD-AUX/IAA plasmid and also the opposite, every yeast solution with AD-AUX/IAA plasmid with yeast containing BD-TFL2 expression clone. The plates were grown overnight and then, we streaked the obtained colonies on selective SD agar plates without Trp and Leu to grow yeast with both AD and BD clones. We did negative controls mating every yeast solution with water on the YAPD agar plates and streaking the colonies on – Trp/Leu SD agar plates. The positive controls we did were based on the known interactions of TFL2 with itself (Nilsson et al. 2007, not published) and the interactions found by Rizzardi, K. (2008) of AUX/IAA 5, 6 and 19 with TFL2. From – Trp/Leu plates we streaked colonies on new SD agar plates without His and also without His and Ade. Plates were left growing at 30^{o}C until growth was clearly visible and every mating was replicated to confirm them.

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