

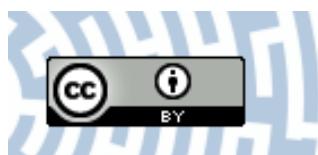


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Extensive Modulation of the Transcription Factor Transcriptome during Somatic Embryogenesis in *Arabidopsis thaliana*

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Abstract

Molecular mechanisms controlling plant totipotency are largely unknown and studies on somatic embryogenesis (SE), the process through which already differentiated cells reverse their developmental program and become embryogenic, provide a unique means for deciphering molecular mechanisms controlling developmental plasticity of somatic cells. Among various factors essential for embryogenic transition of somatic cells transcription factors (TFs), crucial regulators of genetic programs, are believed to play a central role. Herein, we used quantitative real-time polymerase chain reaction (qRT-PCR) to identify TF genes affected during SE induced by *in vitro* culture in *Arabidopsis thaliana*. Expression profiles of 1,880 TFs were evaluated in the highly embryogenic Col-0 accession and the non-embryogenic *tanmei/emb2757* mutant. Our study revealed 729 TFs whose expression changes during the 10-days incubation period of SE; 141 TFs displayed distinct differences in expression patterns in embryogenic versus non-embryogenic cultures. The embryo-induction stage of SE occurring during the first 5 days of culture was associated with a robust and dramatic change of the TF transcriptome characterized by the drastic up-regulation of the expression of a great majority (over 80%) of the TFs active during embryogenic culture. In contrast to SE induction, the advanced stage of embryo formation showed attenuation and stabilization of transcript levels of many TFs. In total, 519 of the SE-modulated TFs were functionally annotated and transcripts related with plant development, phytohormones and stress responses were found to be most abundant. The involvement of selected TFs in SE was verified using T-DNA insertion lines and a significantly reduced embryogenic response was found for the majority of them. This study provides comprehensive data focused on the expression of TF genes during SE and suggests directions for further research on functional genomics of SE.

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Introduction

Most plant cells, in contrast to animal cells, express an amazing developmental plasticity allowing their reprogramming and manifestation of totipotency [1]. Our current understanding of the genetic mechanisms controlling plant totipotency are largely based on studies on somatic embryogenesis (SE), the process through which already differentiated cells reverse their developmental program during *in vitro* culture and become embryogenic giving rise to the formation of somatic embryos which then develop further into entire plants. Thus, deciphering the molecular determinants of SE can directly contribute to revealing the genetic programme underlying the phenomenon of cell totipotency. Moreover, considering similarities between SE and zygotic embryogenesis (ZE), functional genomics of SE became a model for the analysis of the molecular mechanisms of ZE [2,3]. Importantly, knowledge about the molecular mechanisms governing SE has also a practical value in plant biotechnology for the improvement of existing and the establishment of new protocols for plant regeneration.

The control of plant embryogenesis, similar to other developmental processes, occurs through a complex set of intrinsic signals that are involved in providing information to the dividing and differentiating cells. Of them, phytohormones and transcription factors (TFs) are believed to play central roles [4]. TFs constitute sequence-specific DNA-binding proteins that are capable of activating and/or repressing transcription of target genes and thus are responsible for gene expression regulation. TF genes are often expressed in a tissue- or developmental stage-specific mode or in a stimulus-dependent manner, and many have been shown to obey important roles in developmental processes [5,6,7]. Moreover, in adult human somatic cells a specific combination of TFs was found to re-programme differentiated cells into pluripotent embryonic stem cells [8,9]. More specifically, a combination of only four over-expressed TFs was sufficient to induce the formation of pluripotent stem cells from e.g. adult human fibroblasts [10,11].

In contrast to the spectacular progress that has been made with respect to the identification of key genetic factors able to transform differentiated animal cells into totipotent stem cells much less is known about the master regulators of genomic reprogramming in

plant cells. Of note, transcriptional regulation is thought to play a more important role in plants than in animals and accordingly, recent analyses have recognized over 2,000 TFs to be encoded by the *Arabidopsis* genome and revealed a higher ratio of TF genes to the total number of genes in this plant than in several animal model organisms such as *Drosophila melanogaster* or *Caenorhabditis elegans* [12].

In agreement with the model that TFs play fundamental roles in the control of plant cell totipotency, genes encoding TFs are currently overrepresented among the genetic factors reported to be essential for SE. The list of genes affecting SE includes *BABY BOOM (BBM)* [13], *WUSCHEL (WUS)* [14], *AGAMOUS-LIKE15 (AGL15)* [15], *LEAFY COTYLEDON (LEC)* [16], *LECI-LIKE (LIL)* [17] and genes encoding MYB transcription factors, i.e., *AtMYB115*, *AtMYB118* [18] and *EMK (EMBRYOMAKER)* [19]. Several TFs involved in SE have been reported to enhance plant regeneration efficiency when overexpressed [13,14,20].

Various molecular tools have been employed to identify genes essential for embryogenic transition of somatic plant cells. Microarray-based transcriptome analyses were used to discover genes involved in SE induction and somatic embryo development in various plant species including gymnosperms such as *Picea sp.* [21,22], cereals such as maize [23] and rice [24], and eudicots, such as e.g. *Glycine max* [25] and *Solanum tuberosum* [26]. In contrast to commonly used DNA microarrays, transcriptome analysis based on quantitative real-time polymerase chain reaction (qRT-PCR) provides an up to 100 times more sensitive tool for transcript detection [27]. With respect to TFs, which are often expressed at a low level or in a cell-specific manner, the superior sensitivity of multi-parallel qRT-PCR over microarray hybridisations has been reported [28]. Recently, multi-parallel qRT-PCR was employed in a number of biological studies, e.g. to determine the expression levels of ~1,900 TFs in *Arabidopsis* in response to different carbon sources [29] or phosphorus treatment [30]. Similarly, multi-parallel qRT-PCR has been used to study the expression of more than 2,000 TFs in rice [31], of 1,000 TFs in *Medicago truncatula* [32], or of 1,000 TFs during tomato fruit development [33].

In the present study we took advantage of the available *Arabidopsis* TF qRT-PCR platform to indentify TF genes involved in the process of SE induced *in vitro* in *Arabidopsis* cultures. To identify TFs prominently expressed during SE we compared transcriptomes of *Arabidopsis* genotypes exhibiting largely different embryogenic capacities, namely the highly embryogenic accession Col-0 and the embryonal mutant *tanmei/emb2757* entirely lacking an embryogenic response *in vitro* [34]. Expression of 1,880 TFs was profiled at selected time points during SE culture and TFs prominently expressed in Col-0 were identified. The capacity for SE induction was evaluated in mutants carrying T-DNA insertions in 17 TF genes that showed SE-modulated expression; the majority of the mutants displayed a significantly impaired embryogenic response, indicating that our transcriptome screening indeed revealed genes functionally relevant for SE. Our approach constitutes the first comprehensive analysis of the global TF transcriptome involved in the process of SE induced in plant tissue culture and provides the basis for a better understanding of the genetic determinants of plant developmental plasticity.

Results

Experimental Design

To indentify TF genes potentially involved in SE, we employed a well established protocol for the induction of

somatic embryos (see Materials and Methods). In brief, immature zygotic embryos (IZEs) at the late cotyledonary stage of development were carefully excised from siliques 10–12 days after pollination and cultured on solid medium containing the synthetic auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D, 5 µM). Induction of SE in this experimental setup is accompanied by distinct morphological changes of the explant. In *Arabidopsis* (Col-0 accession), a straightening and expansion of previously bent cotyledons and swelling of the cotyledon node are observed during the first week of *in vitro* culture. The first somatic embryos become visible at days 8 to 10, on the adaxial sides of the cotyledons proximal to the cotyledon node, and at around day 15 the cotyledon part of the immature zygotic embryo is covered with somatic embryos at various stages of development [35].

The experiment was designed to monitor the expression of 1,880 TF genes at three distinctive stages of IZE-derived embryogenic culture: (i) freshly isolated explants (0 d), (ii) explants subjected to SE induction for 5 days (5 d), and (iii) explants at an advanced stage of embryogenesis related to somatic embryo formation (10 d). To identify genes exhibiting preferential expression during SE, we compared the TF transcriptomes of the highly embryonic Col-0 accession and the *tanmei* mutant unable to form somatic embryos (**Figure 1**). The *TANMEI/EMB2757 (TAN, At4g29860)* gene encodes a regulatory WD repeat protein involved in early and late phases of zygotic embryo development [36] as well as SE [34]. Its molecular mode of action has not been reported yet, however, the fact that TAN harbours seven WD repeats suggests that it interacts with other proteins to exert its biochemical function. Recently, a regulatory function of TANMEI in cell cycle progression and differentiation was reported [37].

PCA (Principal Component Analysis; **Figure 2**) and HCA (Hierarchical Cluster Analysis; not shown) demonstrated high reproducibility of the three experimental replicates performed, i.e., samples representing biological repeats of the same combination (genotype x culture time point) grouped together. In addition, we observed a clear separation of samples from different combinations indicating that expression profiles of embryogenic Col-0 and non-embryogenic *tanmei* tissues differ significantly. Moreover, the 5 d- and 10 d-Col-0 embryogenic cultures tended to overlap indicating similarities between the TF transcriptomes of the different stages of embryogenic culture.

TF Genes Related to Embryogenic Competency of Explant Tissue

In Col-0, a large number of TFs were expressed at the different time points (0, 5 and 10 d) of the culture (**Figure 3**). The biggest number of TFs was expressed in explants before embryogenic induction (0 d) and 83 of them were repressed thereafter. Of the TFs analysed, 1602 were expressed in all culture stages, whilst SE stage-specific transcripts were rare and limited to two and seven for the 5-d and 10-d culture time points, respectively.

To identify TFs specific for SE-competent tissue we compared the Col-0 and *tanmei* transcriptomes (**Figure 4A**). This revealed expression of 1727 TFs, of which 1690 were commonly expressed in both types of explants. With respect to genes related to embryogenic competency of somatic tissue, transcripts highly enriched in Col-0 versus *tanmei* were of particular interest. Following this criterion, 41 TFs only expressed in Col-0 and TFs highly overexpressed (over 10-fold) in Col-0 versus *tanmei* (108) were inspected further; for 61 TFs a function was predicted, including genes related to stress

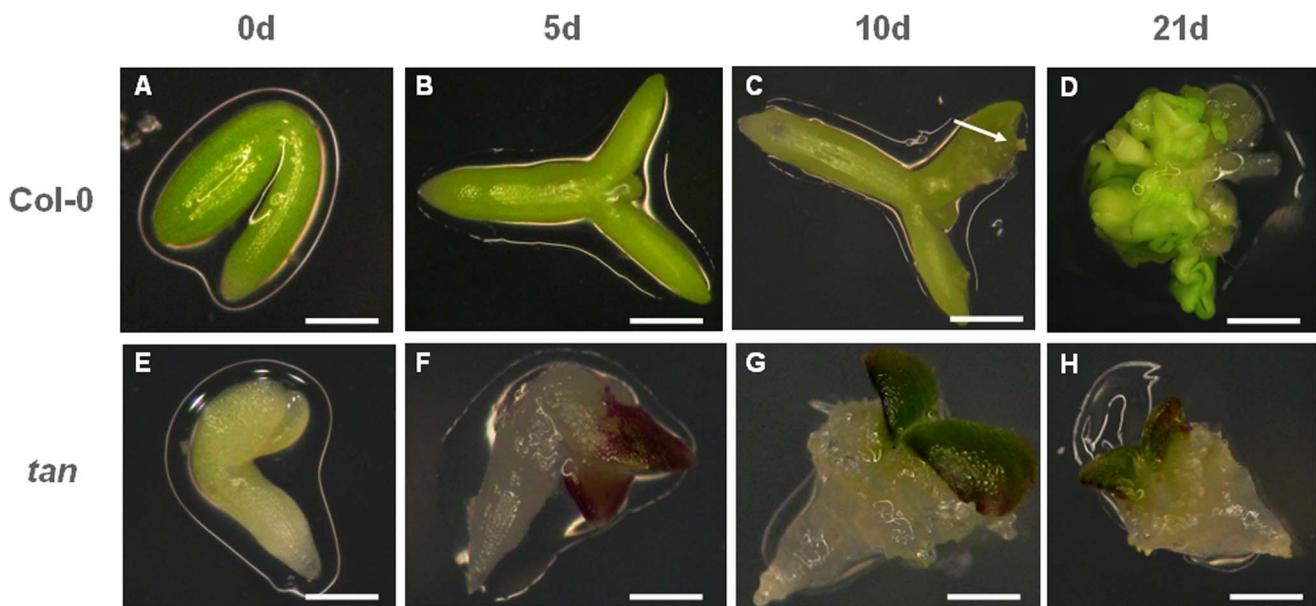


Figure 1. Developmental changes in *Arabidopsis Col-0* and *tanmei* IZE explants induced on auxin-containing medium. A–D) *Col-0* accession. E–H) *tanmei* mutant. Explants were induced on auxin-containing medium (E5) and monitored at days 0 (A, E), 5 (B, F), 10 (C, G) and 15 (D, H) of *in vitro* culture. A, E) Freshly isolated IZE 12 days after pollination (DAP). B) Straightening, enlargement and swelling of IZE cotyledons. C) Tissue proliferation and somatic embryo-like protuberances formed at adaxial side (arrow). D) Numerous somatic embryos at the adaxial side of IZE cotyledons. F) Anthocyanin accumulation in IZE cotyledons and tissue proliferation from IZE hypocotyl. G) Non-embryogenic watery callus. H) Progression of non-embryogenic callus production. Bars: 0.2 mm (A, B, E, F); 0.3 mm (C, G); 0.6 mm (H) and 1.0 mm (D).

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tolerance, zygotic embryogenesis, developmental processes, hormone biology and *in vitro* responses (**Table S1**). We found that one third (44) of the TFs highly enriched in *Col-0* explants were differentially expressed in the embryogenic culture. The set of genes highly up-regulated (at least 10-fold) exclusively in *Col-0* explants and SE-modulated in the derived cultures includes TFs related to stress responses (12) and development of zygotic embryos (10), flowers (4), leaves (2) and roots (1).

The Global TF Transcriptome changes during Somatic Embryogenesis in *Col-0*

Of the 1,880 TFs analysed, 1,768 were found to be expressed in *Col-0* explants in at least one of the three time points, and only 112 TFs were not expressed at any stage (**Figure 4B**). To gain insight into TF expression patterns associated with SE we compared the expression levels observed in explant tissue (0 d) to the expression levels obtained after 5 d (early embryo induction) and 10 d (advanced embryo formation) of culture.

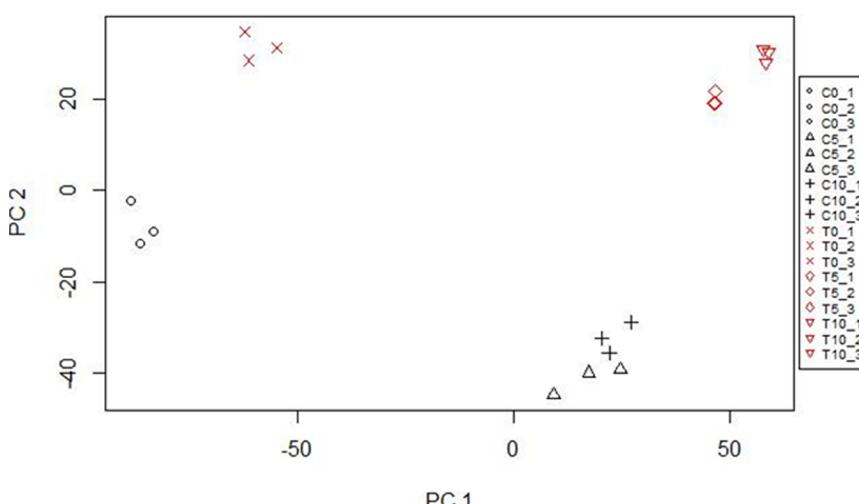


Figure 2. Principal Component Analysis (PCA). The analysis demonstrates a clear separation of TF expression in *Col-0* and *tanmei* (*tan1-2*), both in explants (0 d) and during embryogenic culture (5 d and 10 d). Expression data from three independent biological replicates were analysed each. Samples: C0, *Col-0*, day 0; C5, *Col-0*, day 5; C10, *Col-0*, day 10; T0, *tanmei* mutant, day 0; T5, *tanmei* mutant, day 5; T10, *tanmei* mutant, day 10. Numbers 1 to 3 denote replicates 1 to 3. Approximately 67.6% of the variation is captured by the first two components.

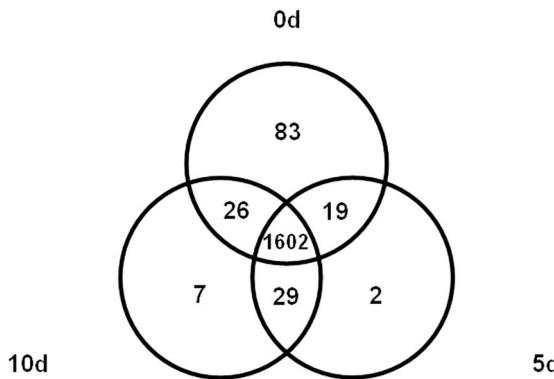


Figure 3. Venn diagram demonstrating the number of genes expressed during SE in the Col-0 accession. Numbers in intersections represent TFs commonly expressed at the different culture time points: 0 d, explant; 5 d, induction phase of SE; 10 d, advanced SE culture.

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Our analysis revealed 729 TFs (representing ~41% of all detected TFs) to be differentially expressed (by at least 2-fold) in embryogenic cultures versus explants (**Figure 5B; Table S2**). A closer inspection of the transcriptomes associated with embryo induction identified 673 and 688 genes, respectively, that were modulated at early (5 d vs. 0 d) and advanced (10 d vs. 0 d) stages of SE. The vast majority (602 TFs; 83%) of the modulated TFs were up-regulated, rather than down-regulated, compared to the initial explant (0 d) transcriptome. Of the TFs modulated during SE, 358 displayed a dramatic change in expression level ($x \geq 10$) and most (312 TFs; ~87%) were found to be up-regulated.

The transcript levels detected in the 0-, 5- and 10-d samples were subjected to *k*-means clustering and four major gene

expression patterns were observed (**Figure 5**). The cluster analysis confirmed that most TFs were up-regulated in embryogenic cultures; the increased expression was either dominant during the early stages of SE induction (**Figure 5C**), or was observed during both SE stages, early and advanced (**Figure 5B,D**).

In summary, global transcriptome analysis identified an extensive expressional reprogramming of TF genes during SE, where an up-regulation of TF expression was predominantly observed.

TF Transcriptomes of Early (Embryo-induction) versus Advanced (Embryo-formation) SE Stages

Given that the early days of an embryogenic culture are critical for embryogenic transition of somatic tissue and decisive for the transcriptional re-programming of the explant, we focused our further analysis on TFs undergoing expression changes during the early embryogenic response. To reveal TFs modulated during SE induction, we compared the transcriptome of the 5-d culture with that of the explant (5 d–0 d) and the 10-d embryo culture (5 d–10 d) (**Table 1**). Our analysis revealed that TF transcriptomes associated with the early and advanced SE stages differed significantly with respect to the level and direction of the expression changes. In contrast to SE induction (5 d vs. 0 d), ~2.5 times fewer genes (284 vs. 673) were differentially expressed between the early and late embryo formation stages (10 d vs. 5 d) and a number of up-regulated genes was distinctly decreased resulting in a similar fraction of up- (154) and down- (130) regulated TFs in the advanced, embryo-formation culture stage. In addition, at the embryo formation stage (10 d vs. 5 d) differentially expressed genes exhibited less drastic changes in transcription and accordingly, the number of genes (32) exhibiting an at least 10-fold change in expression between the 5 d- and 10 d-cultures was over 10 times lower than in a preceding SE induction stage (5 d vs. 0 d).

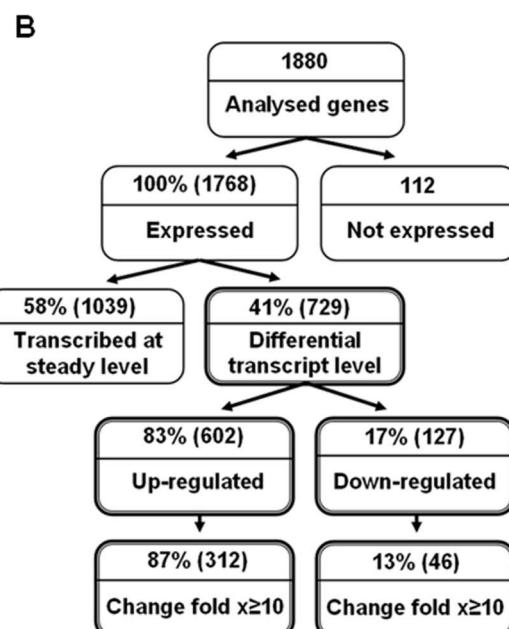
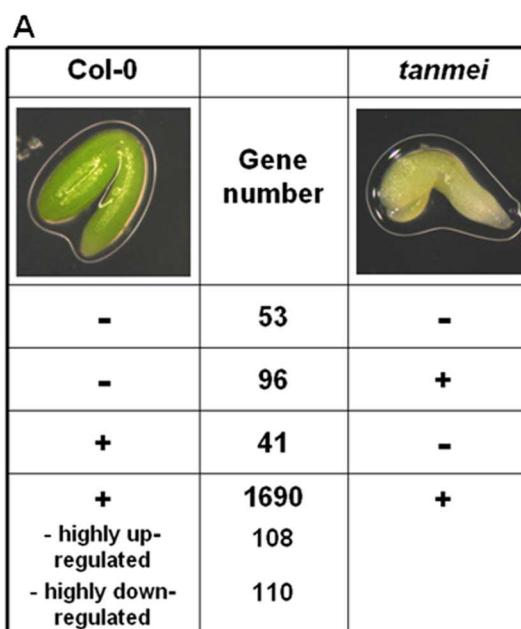


Figure 4. Numbers of TF genes expressed in explants of the highly embryogenic Col-0 genotype and the non-embryogenic *tanmei* mutant (A) and in embryogenic Col-0 culture (B). +, genes for which expression was observed; -, genes for which no expression was observed. Differentially expressed and highly-regulated genes show at least 2- ($x \geq 2$) and 10-fold ($x \geq 10$) change, respectively, in expression level in any of the compared culture points.

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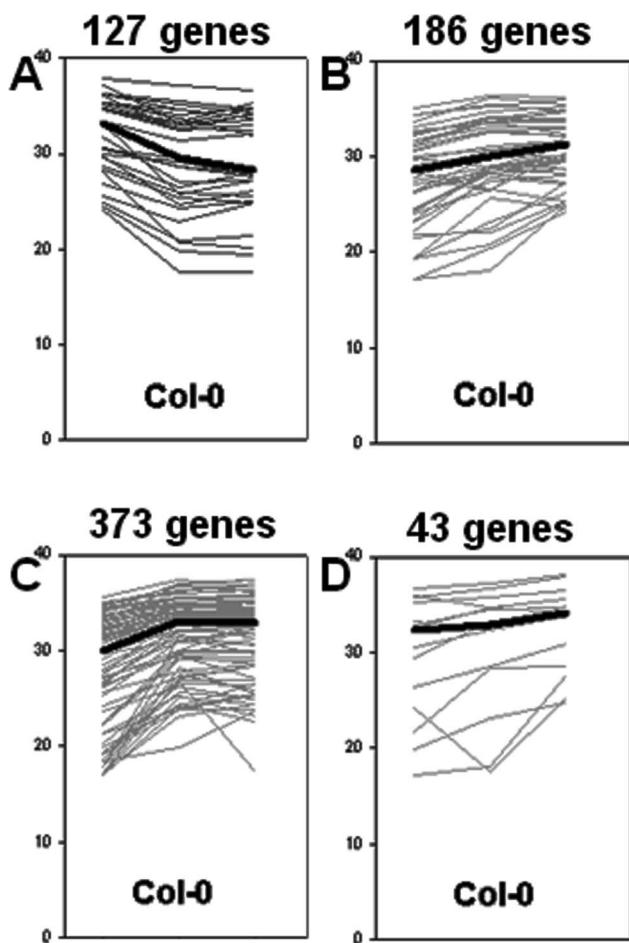


Figure 5. Cluster analysis. K-means clustering revealed four main expression patterns of TF genes in Col-0 embryogenic cultures. The levels of expression changes are given as 40-ddCt. The cluster analysis shows up-regulation of the great majority of TFs (B, C, D), and down-regulation of a small group of TFs (A). Increased TF expression was either restricted to the early stage of SE (C), or was observed during both SE stages, early and advanced (B, D).
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To identify genes modulated at the early culture period, we tracked transcript levels of individual genes during the two successive culture periods (5 d–0 d and 5 d–10 d). To this end,

TFs up- or down-regulated, or remaining unchanged during SE induction, were grouped together according to their expression profiles during the subsequent embryo formation stage (**Table 2**). Scrutiny of the individual gene expression patterns revealed that most TFs (67%) up-regulated during embryo induction (5 d) did not significantly change expression thereafter during embryo formation; only few genes were down- (~15%) or up-regulated (~18%) in the 10-d culture compared to the 5-d culture.

Stabilization of the TF transcriptome in advanced cultures was also observed for genes down-regulated during SE induction (5 d–0 d). We found that almost half of the genes (48%) down-regulated during embryo induction were not further modulated at the later stage of embryo formation, whilst the remaining genes were up- (32%) or further down-regulated (20%). In contrast to the vast number of genes differentially regulated during SE induction, a small set of 38 TFs was found to be modulated exclusively in the advanced SE culture. The transcript levels of these genes remained stable until the embryo formation stage when most of them (~66%) were found to be down-regulated.

To identify TFs specific for SE induction we searched for those that drastically (by at least 10-fold) changed their expression levels during the early culture stages. We identified genes of high and temporal changes in expression specific to SE induction and among them were the key regulators of embryogenic transition induced in cultured cells in response to auxin treatment (**Table S3**).

Collectively, by analyzing TF gene expression profiles across the time points of SE we obtained the following results: (i) The embryo-induction stage of SE is associated with a robust change of the TF transcriptome. (ii) Transcriptome reprogramming during SE induction includes a drastic up-regulation of a great majority (over 80%) of the TFs active in culture. (iii) TF expression patterns of embryo induction and embryo formation stages are largely different. (iv) In contrast to SE induction, attenuation and stabilization of transcript levels of a great fraction of the TFs is observed in the advanced embryo formation stage.

Col-0 versus *tanmei* Transcriptome and SE-associated Genes

To identify candidate TFs of SE-associated functions we compared the transcriptomes of cultures derived from the highly embryogenic Col-0 genotype and the *tanmei* mutant lacking the embryogenic response; genes of distinctly different expression profiles were selected. We identified 141 TF genes with SE-specific expression (**Table 3**) falling into the following groups: (i) genes exclusively expressed in embryogenic culture (2 genes); (ii) genes

Table 1. Number of TF genes whose expression changes during somatic embryo formation.

Compared culture stages	Number of genes showing differential expression	Up-regulated genes	Down-regulated genes
Fold change x≥2			
5 d–0 d	673	546 (81%)	127 (19%)
10 d–0 d	688	542 (79%)	146 (21%)
10 d–5 d	284	154 (60%)	130 (40%)
Fold change x≥10			
5 d–0 d	357	312 (87%)	46 (13%)
10 d–0 d	379	331 (87%)	48 (13%)
10 d–5 d	32	6 (19%)	26 (81%)

x, fold change.

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Table 2. Number of differentially expressed TF genes exhibiting convergent expression profiles across SE culture.

Embryo-induction stage (5 d vs. 0 d) ^a		Embryo-forming stage (10 d vs. 5 d) ^b				
Expression change	Number of genes	Down-regulation		Up-regulation		Steady expression
		x≥2	x≥10	x≥2	x≥10	
Up-regulation	x≥2:546	81	5	100	11	368
	x≥10:307	62	5	58	3	187
Down-regulation	x≥2:125	25	1	40	12	60
	x≥10:45	1	1	25	9	19
Steady expression	x<2:38	25	1	13	2	0

x, fold change of gene expression.

^aExpression behavior of TF genes within the first five days of somatic embryogenesis.

^bExpression change of the genes grouped in column 1 ("Embryo-induction stage") during the second phase of somatic embryogenesis (expression at day 10 compared with expression at day 5).

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differentially expressed in Col-0, but steadily expressed in *tanmei* (72 genes); (iii) genes exhibiting opposite expression patterns in Col-0 and mutant cultures, including genes up-regulated in Col-0 and down-regulated in *tanmei* (33), and genes down-regulated in Col-0 and up-regulated in *tanmei* (10); examples are shown in **Figure 6**; and (iv) genes significantly down-regulated in non-embryogenic *tanmei* culture (24). We found that, similar to the global Col-0 transcriptome, SE-specific transcripts were predominantly up-regulated during SE and for a substantial part of them the changes in expression level were drastic ($x \geq 10$) (**Figure 7**).

Annotation of Differentially Expressed Genes

The TF genes differentially expressed in embryogenic Col-0 culture were annotated to 50 gene families of which 14 included the great majority (541 genes; 74%) of the differentially expressed

transcripts (**Figure S1**). The most frequently represented families were bHLH (75), AP2/EREBP (69), MYB (62), NAC (54), C2H2 (49); WRKY (45), HB (41) and MADS (38), each of which represents 5–11% of the SE-modulated genes.

We next analysed the representation of TF families within the set of SE-associated genes. The SE-associated genes represented 32 TF families and particularly enriched were the MADS (20), MYB (16), AP2/EREBP (15), C2H2 (12), NAC (11), bHLH (11) and ABI3/VPI (4) families. We also noticed that several SE-associated genes belong to the WRKY (7) and DREB (7) families known for their involvement in stress responses.

Functional Categories of Differentially Regulated Genes

To provide an overview of the potential contribution of TF genes to the regulatory mechanisms involved in SE, the genes

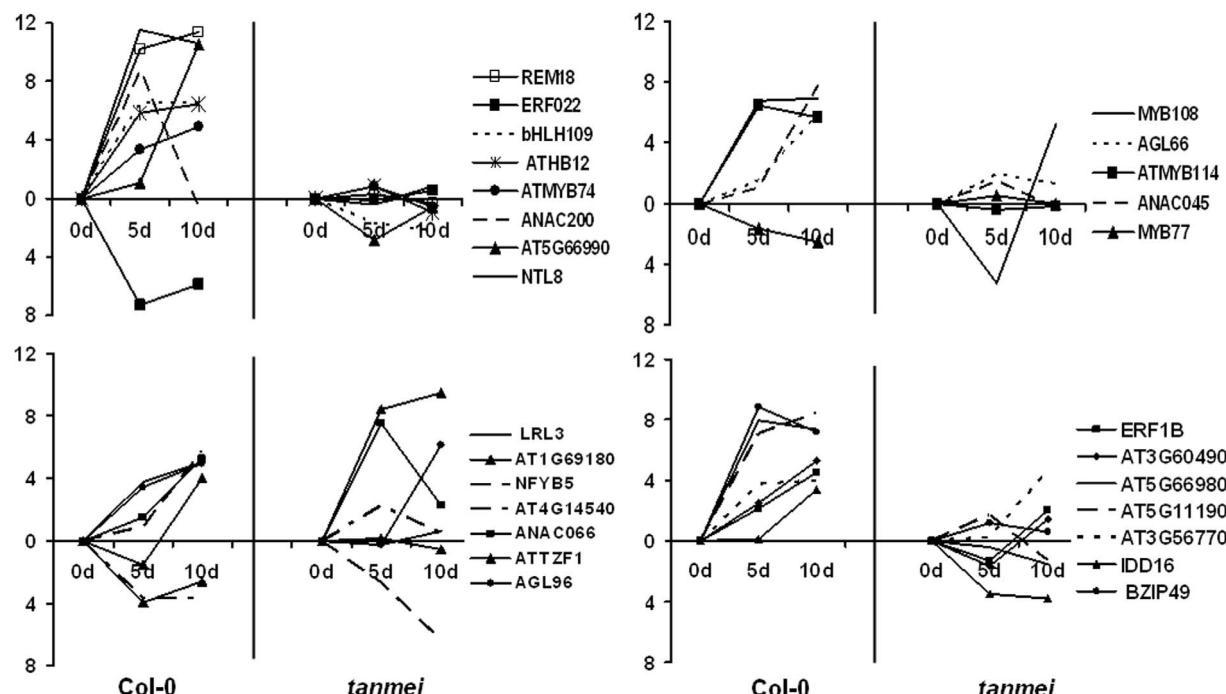


Figure 6. Expression profiles of SE-associated genes. The graph shows contrasting expression levels of TFs in embryogenic (Col-0) and non-embryogenic (*tanmei*) cultures. The relative transcripts levels of the genes are shown as ddCt.

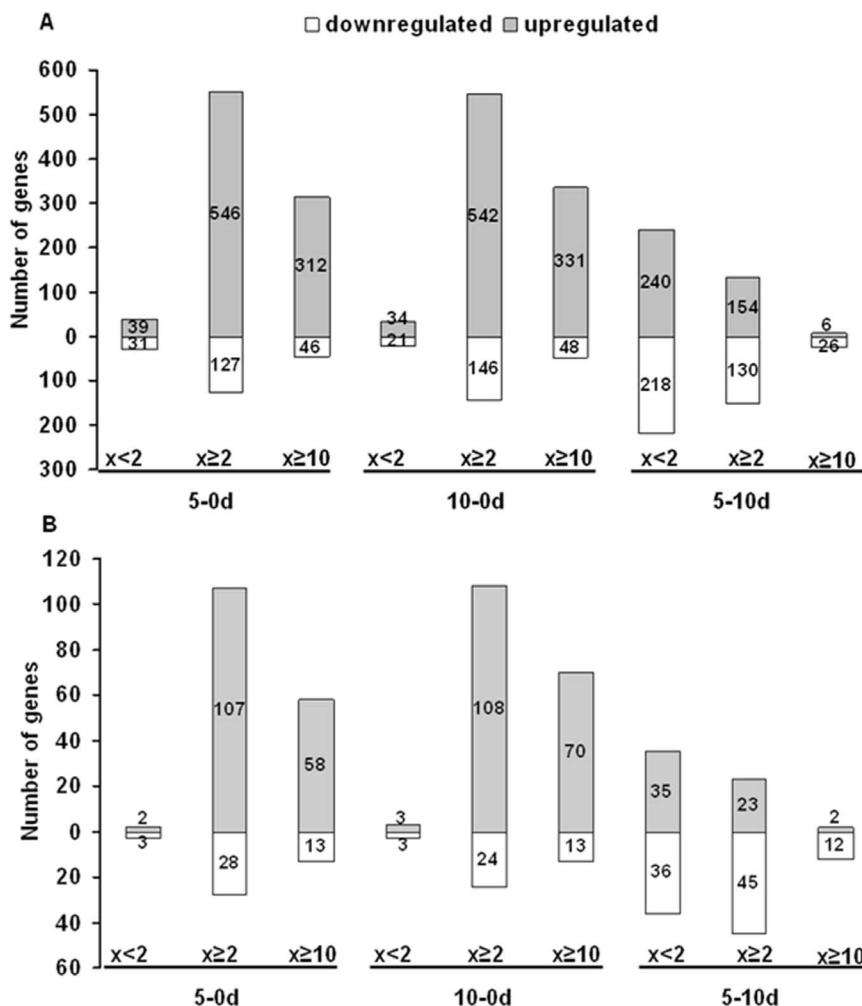


Figure 7. Number of TF genes of modulated expression in embryogenic cultures. A) TFs expressed in Col-0 culture. B) TFs of SE-specific expression pattern, i.e. those displaying distinctly different expression profiles in Col-0 and *tanmei* cultures. Numbers of TFs of steady (fold change <2) and modulated (fold change ≥ 2) expression in embryogenic cultures referenced to the indicated culture time points (i.e. 5 d-0 d; 10 d-0 d, and 5 d-10 d) are given. Genes with up- and down-regulated expression are indicated.
doi:10.1371/journal.pone.0069261.g007

differentially expressed in embryogenic culture were annotated according to their known or predicted functions (**Figure 8A**). In total, 519 genes (71%) were functionally annotated and the analysis revealed that the most abundant transcripts are related to plant development, phytohormone biology and stress responses. A great majority (~78%, 407) of the SE-modulated TFs are related to plant development and in particular TFs involved in flower development were highly abundant (24%; 125). Other numerously represented genes of the plant development category were reported to be involved in embryo and seed development (~22%, 71).

The number of TFs related to phytohormones and stress responses were similar and these functional categories included ~43% and ~39% of the genes, respectively. Within 221 hormone-related, SE-modulated TFs all major classes of phytohormones were represented and the most numerous were genes related to auxin (~24%, 54). Half of the auxin-related genes encoded major auxin signaling molecules: ARF (7) and AUX/IAA (20). Beside auxin-related TFs, many genes related to other phytohormones (e.g. ethylene, ABA, cytokinin, GA) were observed to be prevalently up-regulated during SE (**Figure 9**). Among 201

stress-related TFs modulated during SE, genes responding to different abiotic stress factors (salt, water, temperature, oxidative stress) were represented more frequently than those involved in pathogen responses.

Within the group of functionally annotated SE-modulated TFs, 101 (~20%) represented SE-specific expression, and the number and representation of functional categories were similar to those of globally affected genes (**Figure 8B**). A great majority (~70%) of the SE-specific TFs were annotated to plant developmental processes and predominantly contribute to flower development (~27%).

We observed some notable differences between SE-modulated and SE-associated genes with respect to functional annotations. Strikingly, the number of stress-responsive TFs, especially those related to biotic stress, was higher (~50%) among SE-associated transcripts, whilst the percentage of phytohormone-related genes was lower (~33%), but interestingly the representation of cytokinin- and gibberellin-related genes was increased over auxin-related genes.

Table 3. TF genes showing SE-specific expression.

AGI	Gene name	TF family	Known or predicted function	Fold change $2^{\Delta\Delta Ct}$			
				Col-0		<i>tan</i>	
				5d-0d	5d-10d	5d-0d	5d-10d
AT1G02030		C2H2	Seed germination	30.06	2.10	Steady expression	
AT1G06170	bHLH89/EN24	bHLH	Flower development, ZE [49]	7.89	4.59	Steady expression	
AT1G19790	SRS7	SRS	Flower development	3.92	1.32	Steady expression	
AT1G25250	IDD16	C2H2		1.10	-2.50	Steady expression	
AT1G25560	EDF1/TEM1	AP2/EREBP	Flowering time	2.95	-9.51	Steady expression	
AT1G28160	ERF087	AP2/EREBP	Stress	9.51	-1.88	Steady expression	
AT1G34650	HDG10	HB		25.63	-14.72	Steady expression	
AT1G44830		AP2/EREBP	Biotic stress	6.19	-2.79	Steady expression	
AT1G51220		C2H2		2.50	1.21	Steady expression	
AT1G54330	ANAC020	NAC		16.34	-2.64	Steady expression	
AT1G59640	BIG PETAL	bHLH	Flower development	4.47	-1.29	Steady expression	
AT1G59810	AGL50	MADS	Flower development	32.67	-1.12	Steady expression	
AT1G60920	AGL55	MADS		340.14	3.41	207.94	-1.47
AT1G65300		MADS	Seed/embryo development	64.45	1.66	3.51	-1.56
AT1G66380	MYB114	MYB	ZE [49], cell wall	88.65	1.32	Steady expression	
AT1G67030	ZFP6	C2H2	Cell cycle	89.26	1.78	13.36	-2.03
AT1G68240		bHLH		92.41	-2.00	Steady expression	
AT1G68480		C2H2	Flower development	3.51	-1.01	Steady expression	
AT1G77850	ARF17	ARF	Auxin	3.84	-1.78	2.48	1.56
AT1G77980	AGL66	MADS	Flower development	2.93	-1.47	Steady expression	
AT2G17150		NIN-like		4.53	-19.43	Steady expression	
AT2G23740	SUVR5/SET6	C2H2	Flower development	5.86	-1.45	2.17	-1.28
AT2G27300	ANAC040/NTL8	NAC	Salt stress	2957.17	2.38	Steady expression	
AT2G30590	WRKY21	WRKY	SE <i>Dactylis glomerata</i> [121]	5.78	1.95	Steady expression	
AT2G31650		SET-domain	Histone methylation	4.99	1.55	3.41	-1.80
AT2G33480	ANAC041	NAC		2.73	1.78	Steady expression	
AT2G35700		AP2/EREBP	Biotic stress	2.19	-2.35	1.88	1.46
AT2G38470	WRKY33	WRKY	Biotic and abiotic stress	4.11	-3.20	-1.07	-1.92
AT2G39880	MYB25	MYB		5.39	-1.99	3.41	1.09
AT2G42280		bHLH		4.38	-1.01	Steady expression	
AT2G44430		MYB	Flower development	4.86	-1.32	Steady expression	
AT2G46770	EMB2301/NST1	NAC	ZE [49], cell wall	67.65	-1.06	44.32	2.64
AT2G47810	NFYB5	CCAAT-HAP3	Flower development	1.88	-2.20	Steady expression	
AT2G47890	COL13	C2C2(Zn) CO-like	Flower development	4.92	-29.04	Steady expression	
AT3G01220	ATHB20	HB	Auxin	9.13	3.01	Steady expression	
AT3G03200	ANAC045	NAC		2.04	-2.14	Steady expression	
AT3G04730	IAA16	Aux/IAA	Auxin	16.34	-105.42	Steady expression	
AT3G06490	MYB108/BOS1	MYB	JA, GA, stress	107.63	-1.34	Steady expression	
AT3G10470		C2H2	Flower development	625.99	-1.13	55.33	1.35
AT3G17600	IAA31	Aux/IAA	ZE [38]	7.62	3.43	Steady expression	
AT3G17730	ANAC057	NAC		12.64	1.08	Steady expression	
AT3G19070		GARP-G2-like	Cell wall	22.01	1.01	Steady expression	
AT3G21890	MZN24.1	C2C2(Zn) CO-like	Light	15.03	-2.22	Steady expression	
AT3G23240	ERF1B	AP2/EREBP	Ethylene	4.53	4.23	Steady expression	
AT3G24310	MYB71	MYB		195.36	-5.13	Steady expression	
AT3G27940	LBD26	AS2 (LOB) I		128.89	-7.41	199.47	-5.31
AT3G30260	AGL79	MADS	Root development	14.32	-2.60	6.63	-5.24

Table 3. Cont.

AGI	Gene name	TF family	Known or predicted function	Fold change $2^{\Delta\Delta Ct}$			
				Col-0		<i>tan</i>	
				5d-0d	5d-10d	5d-0d	5d-10d
AT3G50700	ATIDD2	C2H2		9.13	-9.00	Steady expression	
AT3G51080	GATA9	C2C2(Zn) GATA	ZE	3.63	-1.10	Steady expression	
AT3G53200		MYB		103.25	-1.41	6.63	-30.48
AT3G56660	BZIP49	bZIP	Stress	467.88	-1.65	Steady expression	
AT3G56770		bHLH	Biotic stress	13.55	3.18	Steady expression	
AT3G60490		AP2/EREBP	Stress	5.78	-1.16	-3.18	-8.46
AT3G61890	ATHB-12	HB	Water and salt stress	56.10	-6.68	Steady expression	
AT3G61910	ANAC066/NST2	HB	Cell wall	2.81	-1.57	Steady expression	
AT4G00940		C2C2(Zn) DOF		8.22	-13.74	Steady expression	
AT4G01260		GeBP		55.72	1.80	Steady expression	
AT4G01540	NTM1/ANAC068	NAC	Cell cycle; cytokinins	6.68	1.32	Steady expression	
AT4G05100	MYB74	MYB	JA; ethylene; stress	9.92	-1.09	Steady expression	
AT4G17460	HAT1	HB		24.93	-2.99	Steady expression	
AT4G20970		bHLH		64.89	-1.20	765.36	-1.22
AT4G22070	WRKY31	WRKY	SE <i>Solanum tuberosum</i> [26]	2225.63	5.74	Steady expression	
AT4G22680	MYB85	MYB	Vascular tissue, cell wall	124.50	6.32	Steady expression	
AT4G24540	AGL24	MADS	Flowering time	4.76	5.35	1.48	-1.12
AT4G27950	CRF4	AP2/EREBP	Ethylene, stress	10.93	-1.57	Steady expression	
AT4G28110	MYB41	MYB	ABA, water and salt stress	6.02	-1.93	Steady expression	
AT4G28500	ANAC073/SND2	NAC		116.97	-1.02	Steady expression	
AT4G30080	ARF16	ARF	ZE	6.23	1.16	4.17	-1.93
AT4G32280	IAA29	Aux/IAA	Auxin; root development	94.35	1.52	Steady expression	
AT4G32730	MYB3R1	MYB	Cell cycle; cytokinins	5.54	1.05	Steady expression	
AT4G38620	MYB4	MYB	ZE [49]	11.55	1.23	2.43	1.11
AT4G38910	ATBPC5	BPC/BRR		7.52	5.66	34.78	1.85
AT4G39250	ATRL1	MYB-related	Seed/embryo development	115.36	-1.47	167.73	35.26
AT4G39410	WRKY13	WRKY		4.17	11.31	1.73	-1.84
AT5G01200		MYB-related		41.07	-3.18	Steady expression	
AT5G02350		CHP-rich	Root development	5.03	1.04	-1.15	4.00
AT5G04390		C2H2		60.55	4.11	10.85	-1.29
AT5G06500	AGL96	MADS	ZE [49]	10.63	1.65	Steady expression	
AT5G06510	NF-YA10	CCAAT-HAP2	Seed/embryo development	24.59	-2.99	17.51	4.14
AT5G06650	GIS2	C2H2	GA	6.23	-2.22	Steady expression	
AT5G10030	OBF4	bZIP	ABA, SA, biotic stress	24.42	2.50	Steady expression	
AT5G11190		AP2/EREBP	Ethylene, biotic stress	136.24	-1.15	Steady expression	
AT5G14000	ANAC084	NAC	ZE [49]	8.51	-2.55	Steady expression	
AT5G15130	WRKY72	WRKY	ZE [49]	1652.00	-1.21	229.13	1.19
AT5G18000		B3	Flower development	1184.45	1.93	Steady expression	
AT5G22890		C2H2	Root development	94.35	-2.25	Steady expression	
AT5G23260	AGL32/TT16	MADS	Seed/embryo development	20.53	3.14	Steady expression	
AT5G24110	WRKY30	WRKY		11746.96	-5.46	Steady expression	
AT5G26870	AGL26	MADS	Root development	2.08	3.56	Steady expression	
AT5G26950	AGL93	MADS		11.16	-2.73	Steady expression	
AT5G27070	AGL53	MADS		18.64	-1.09	Steady expression	
AT5G27130	AGL39	MADS	Seed/embryo development	10.13	-1.57	1067.48	1.93
AT5G27580	AGL89	MADS		14.22	2.50	Steady expression	
AT5G27910	NF-YC8	CCAAT-HAP5		8.46	-1.46	1.26	-2.93

Table 3. Cont.

AGI	Gene name	TF family	Known or predicted function	Fold change $2^{\Delta\Delta Ct}$			
				Col-0		tan	
				5d-0d	5d-10d	5d-0d	5d-10d
AT5G38800	ATbZIP	bZIP	Epidermal developmental, cell wall	243.88	-1.31	Steady expression	
AT5G39760		ZF-HD		5.82	1.06	4.29	-2.16
AT5G40220	AGL43	MADS		80.45	2.17	Steady expression	
AT5G43175		bHLH		1120.56	-1.11	9741.98	1.58
AT5G50570		SBP		5.66	1.03	Steady expression	
AT5G50670		SBP		4.86	-1.12	Steady expression	
AT5G51780		bHLH		5.66	-2.64	Steady expression	
AT5G52260	MYB19	MYB		44.63	1.67	Steady expression	
AT5G56200	DEL1/E2L3	C2H2	Endoreduplication	103.97	1.83	1.21	-47.84
AT5G58010	LRL3	bHLH	Root development	13.64	-2.43	Steady expression	
AT5G60440	AGL62	MADS	Seed/embryo development	5.46	1.47	Steady expression	
AT5G62165	AGL42	MADS		55.72	-15.56	103.25	-1.33
AT5G66870	ASL1/LBD36	AS2 (LOB) I	Flower development	12.55	1.11	3.18	2.45
AT5G66980		B3	Flower development	250.73	1.55	Steady expression	
AT5G66990		NIN-like		2.04	-709.18	2.85	-41.36
AT2G17150		NIN-like		4.53	-1.45	Steady expression	
AT2G23740	SUVR5/SET6	C2H2	Flower development; histone methylation	5.86	2.38	2.17	-1.28
AT1G33760	ERF022	AP2/EREBP	Ethylene, stress	-155.42	-2.60	Steady expression	
AT1G43640	TLP 5	TUB	Protein degradation	-6.32	1.36	-14.32	-33.36
AT1G49190	ARR19	GARP-ARR-B	ZE [38]	-25.28	1.27	Steady expression	
AT1G77200	ERF037	AP2/EREBP	Callus differentiation <i>O. sativa</i> [43]	-3.48	2.11	Steady expression	
			ZE globular stage [49]				
AT2G25900	ATTZF1	C3H	ZE [49]	-15.35	-2.57	Steady expression	
AT2G42150		MYB	Seed/embryo development	-4.00	1.60	Steady expression	
AT3G02310	AGL4/SEP2	MADS	Flower development	-7.89	-3.12	-3.10	-1.24
AT3G02940	MYB107	MYB	ZE [38]	-65.80	-14.83	Steady expression	
AT3G03760	LBD20/ASL21	AS2 (LOB) I		-6.68	-4.11	Steady expression	
AT3G27810	MYB21	MYB	JA, GA	-162.02	1.38	Steady expression	
AT3G50060	MYB77	MYB	ZE [45], auxin response, lateral root growth	-3.20	1.83	Steady expression	
AT3G57600	DREB2F/ERF051	AP2/EREBP	Water stress	-1.84	2.64	-2.77	7.84
AT4G01250		WRKY	Biotic stress	-3.25	3.39	Steady expression	
AT4G14540		CCAAT-HAP3		-12.64	0.00	4.82	3.51
AT4G32800		AP2/EREBP	Stress	-4.17	1.21	Steady expression	
AT4G36900	DEAR4/RAP2.10	AP2/EREBP	Root development; biotic stress	-12.38	-1.45	-1.89	-1.48
AT4G38000	DOF4.7	C2C2(Zn) DOF	Flower development	-7.67	1.58	Steady expression	
AT5G04400	ANAC077	NAC		-89.88	1.02	Steady expression	
AT5G15800	AGL2/SEP1	MADS	Flower development	-34.30	-2.75	Steady expression	
AT5G39660	DOF5.2	C2C2(Zn) DOF	Flowering time, root development	-4.72	-3.51	Steady expression	
AT5G51990	DREB1D/CBF4	AP2/EREBP	Water stress	-121.10	-3.51	-110.66	1.05
AT5G65100		EIL	Flower development	-3.12	8.51	-71.51	-2.27
AT5G65590		C2C2(Zn) DOF		-25.99	-1.13	Steady expression	
AT5G27810		MADS		-15.56	-1.42	Steady expression	
AT5G43840	HSFA6A	HSF	Heat stress	-6.19	-1.69	Steady expression	
AT4G28790		bHLH		-1.99	5.17	-1.95	1.21
AT1G69180		YABBY	Flower development	-2.97	-48.84	344.89	-2.03

Table 3. Cont.

AGI	Gene name	TF family	Known or predicted function	Fold change $2^{\Delta\Delta Ct}$			
				Col-0		tan	
				5d-0d	5d-10d	5d-0d	5d-10d
AT2G14210	AGL44/ANR1	MADS	ZE [49]	-2.64	-52.71	Steady expression	
AT3G46770	REM22	B3	Flower development	-106.15	-213.78	Steady expression	
AT4G00870		bHLH	Flowering time	-1.10	-7.16	3.20	-1.93
AT4G25480	DREB1A/CBF3	AP2/EREBP	ABA, water stress	-3.61	-13.55	Steady expression	
AT1G19040		NAC		424.61	639.15	Steady expression	

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Functional Test of SE-modulated Transcription Factors

To further elucidate the involvement of TFs in SE we analysed the capacity for SE induction in mutants carrying T-DNA insertions in 17 TF genes of SE-modulated expression (**Table S4**). Twelve of them (~70%) were found to display a significantly impaired embryogenic response manifested by a reduced number of explants undergoing embryogenic transition (**Figure 10A**). The SE-defective phenotypes suggest that the mutated TFs contribute to SE induction; however, the precise molecular functions of most of the genes are unknown. Among the mutants showing reduced embryogenic potential were those affected in genes related to auxin signaling (*AUX/IAA*). All *iaa* mutants analysed (i.e., *iaa16*, *iaa29*, *iaa30* and *iaa31*) displayed significantly impaired SE

efficiency, manifested by a lower frequency of explants undergoing SE induction compared to the Col-0 wild type (**Figure 10A**). Furthermore, one of them (*iaa30*) also produced significantly fewer somatic embryos per responding explant (**Figure 10B**).

In addition to the analysis of the insertion mutants, the capacity for SE was evaluated in eight transgenic lines overexpressing TFs of SE-modulated expression under the control of a β -estradiol-inducible promoter (**Figure 10C,D**). We observed a significantly reduced embryogenic response in cultures overexpressing *DOF5.2*; both, SE efficiency and SE productivity were impaired, i.e. fewer explants underwent SE induction and a lower number of somatic embryos were produced by the responding explants, indicating that *DOF5.2* acts as a negative regulator of SE. This conclusion is

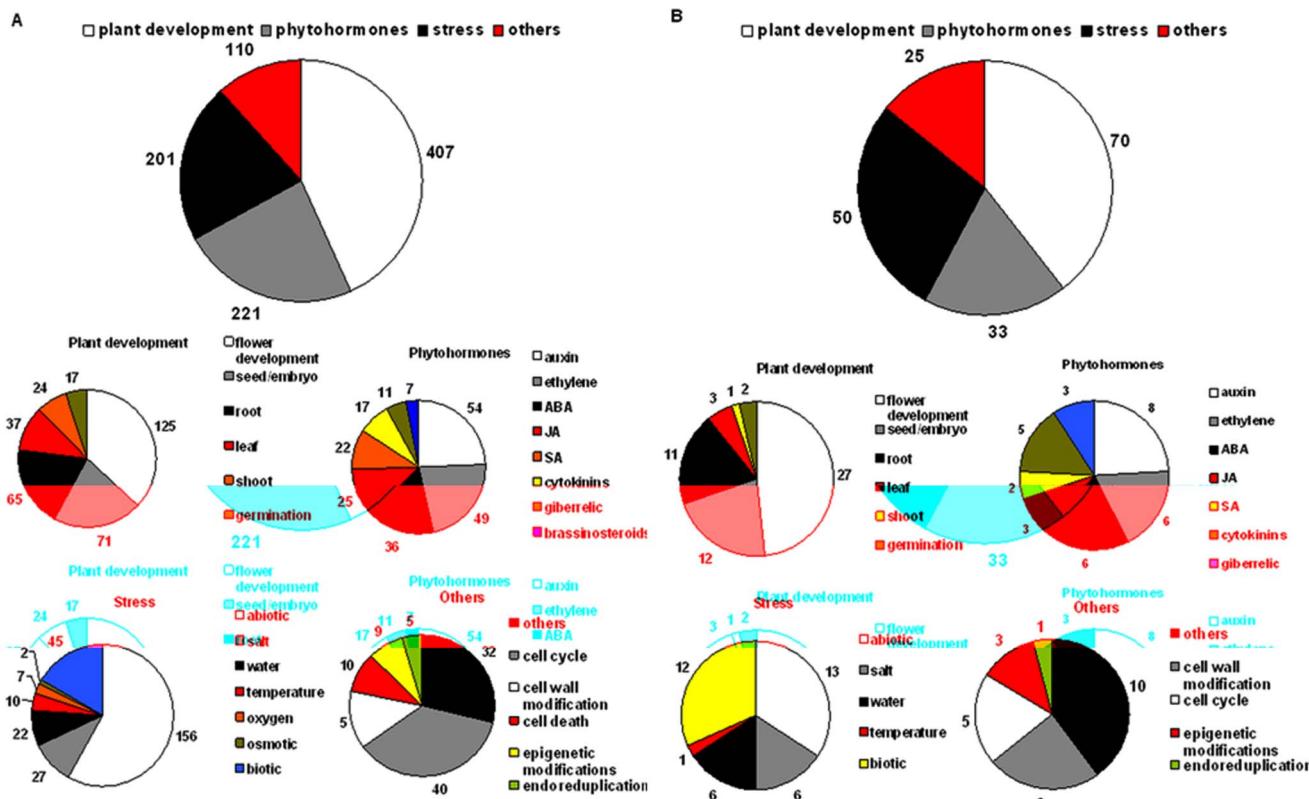


Figure 8. Functional categories of differentially expressed genes. A) TFs differentially expressed during SE. B) SE-associated TFs. TFs were annotated to four major categories (plant development, phytohormones, stress and others) and various subcategories. Given are the numbers of TFs in the different functional categories.

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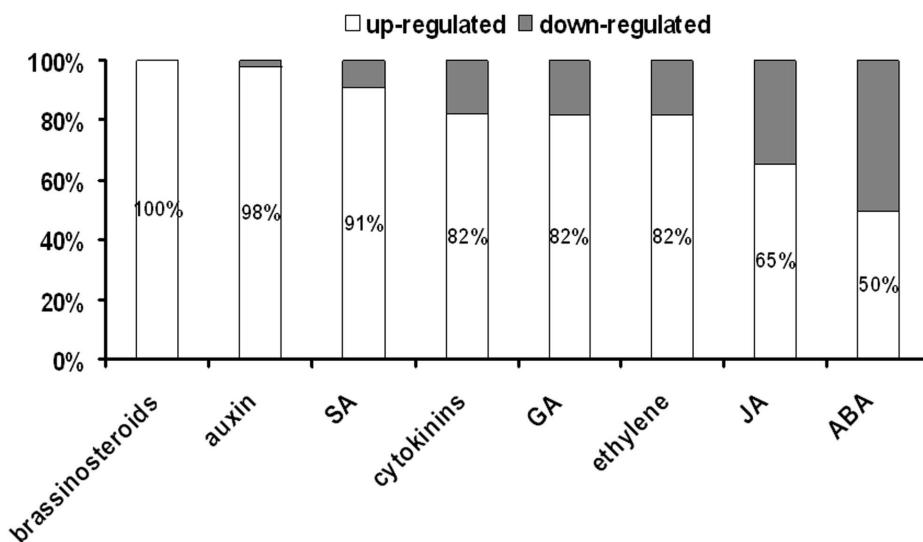


Figure 9. Hormone-related TFs. The graph shows the percentages of hormone-related TFs up- or downregulated in embryogenic Col-0 culture. A great majority of the hormone-related TFs are up-regulated including those related to brassinosteroids, auxin, SA, cytokinins, GA, ethylene, JA and ABA. doi:10.1371/journal.pone.0069261.g009

consistent with the observation, that *DOF5.2* expression declines during early somatic embryo formation, compared to explants (0 d). In contrast, overexpression of *bHLH109* resulted in significantly increased SE productivity, in accordance with the fact that *bHLH109* transcript abundance strongly increases during SE (Figure S2).

AUX/IAA Genes

The *AUX/IAA* genes negatively affecting SE induction potential when mutated (i.e., *IAA16*, *IAA29*, *IAA30* and *IAA31*) were subjected to a closer analysis and their transcript levels were evaluated at different time points in cultures derived from the IZE explants. To reveal relations between gene expression and auxin treatment, explants treated with auxin and undergoing SE induction were compared to those of developing seedlings on

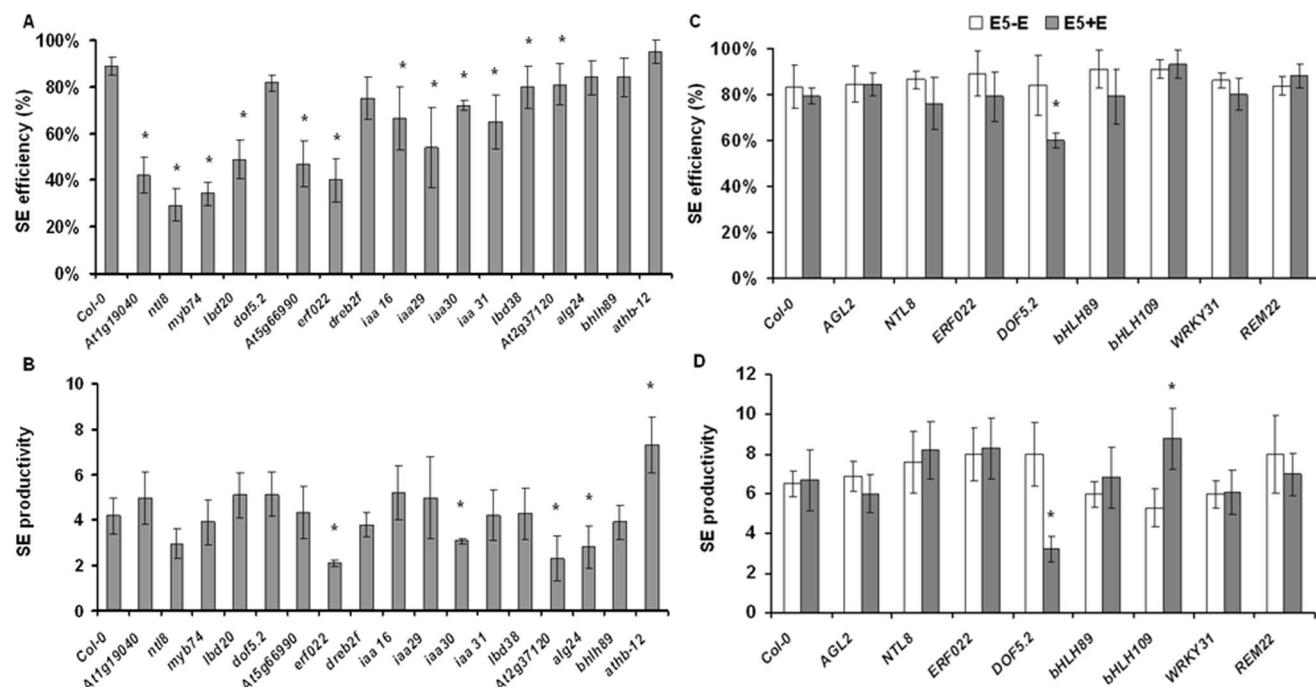


Figure 10. Functional test of SE-modulated transcription factors. Embryogenic capacity of TF T-DNA insertion mutants (A, B) and transgenic lines expressing the indicated TFs under the control of a β -estradiol-inducible promoter (C, D) was analysed and SE efficiency (A, C) and SE productivity (B, D) were evaluated. Values significantly different from the parental Col-0 genotype are marked by asterisks ($n = 3$; means \pm SD are given; Mann-Whitney's U test; $p < 0.05$). doi:10.1371/journal.pone.0069261.g010

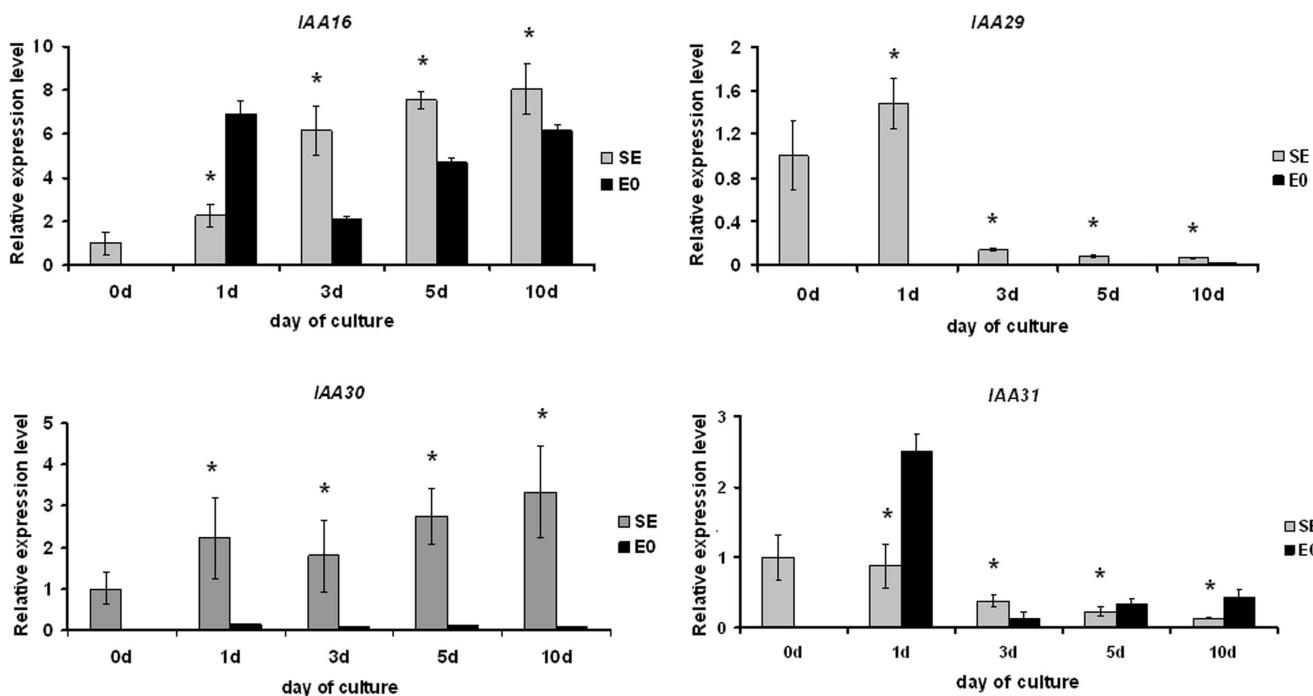


Figure 11. Expression profiles of AUX/IAA genes. Shown are expression levels of AUX/IAA genes (*IAA16*, *IAA29*, *IAA30* and *IAA31*) in explants induced towards alternative morphogenic pathways, i.e. somatic embryogenesis (SE) and seedling development (E0). Values significantly different from E0 are labeled by asterisks (n = 3; means ± SD are given; Mann-Whitney's U test; p<0.05).

doi:10.1371/journal.pone.0069261.g011

auxin-free medium. The qRT-PCR analysis indicated that expression patterns during SE varied between the genes; two of the genes (*IAA16* and *IAA30*) were up-regulated while two others (*IAA29* and *IAA31*) were down-regulated during SE (Figure 11). Among the *AUX/IAA* genes analysed, *IAA16* displayed the highest increase in transcript level in embryogenic culture. We found that transcript levels of the studied *IAA* genes were significantly influenced by auxin and expression of most of them (*IAA16*, *IAA29* and *IAA30*) was distinctly stimulated on auxin medium.

Discussion

An Extensive Up-regulation of the TF Transcriptome Accompanies SE Induction

This study provides the first, to our knowledge, comprehensive analysis focused on TFs and their expression during the time course of SE. Our analysis indicates that in embryogenically induced somatic tissue of *Arabidopsis* a large part of the TF transcriptome (over 1,600 TFs) is active. Similarly, over 1,300 TFs were expressed throughout seed development in *Arabidopsis* and TF genes were found to constitute a much higher fraction (17%) in seed-specific than global (6%) transcriptomes [38]. Thus, tissues undergoing embryogenesis, both in *in planta* and *in vitro*, appear to be highly enriched for TF transcripts supporting the model that regulatory genes have a strong impact on plant developmental processes and in particular, embryogenesis. In support of this, the transcriptome of embryogenesis-related tissues in *Medicago truncatula* includes a high number of TF mRNAs, and 91% vs. 77% of the TF genes were found to be expressed in pods containing developing seeds vs. leaves [32]. Similarly, transcriptome data for reproductive cells in *Brassica napus* showed a distinctly increased number of TF genes expressed in microspores of high embryogenic potency than in non-embryogenic pollen [39].

To identify SE-related TF genes we focused on transcripts differentially expressed during the time course of the embryogenic culture and found that 729 TFs display differential expression in embryogenic culture. Likewise, in shoot organogenesis induced in poplar, 588 TFs (23% of the total) were found differentially expressed [40]. These data reflect the massive genetic reprogramming of somatic cells associated with the induction of new morphogenic paths under *in vitro* conditions and indicate that the control of gene expression at the transcriptional level greatly contributes to the morphogenic switches induced *in vitro*.

Strikingly, when global mRNAs were analysed in embryogenic cultures of other plants much fewer transcripts than found in the present study were reported to be differentially expressed. In rice cultures induced towards different regeneration processes including SE, only 1–3% of the genome was reported to be differentially expressed [41]. Likewise, in soybean and potato 2.6% and 4% of all transcripts were found to be modulated, respectively [25,26]. The results obtained by global transcriptome analyses suggested a relatively low frequency of differentially expressed TF transcripts [26,42,43].

The relatively high number of modulated genes observed in the present study may in part be due to the higher sensitivity of qRT-PCR over hybridization-based approaches, as reported earlier [28,44]. In accordance with this we identified over twice as many TF mRNAs (1730) in IZE explant tissue than previously discovered (847) by microarrays in the mature green stage of zygotic embryos [38]. Our study furthermore revealed that up-regulation of TF gene expression dominated over down-regulation; up-regulated TFs were almost four times more frequent than down-regulated ones. In ZE, only a moderate predominance (slightly over 50%) of up- over down-regulated mRNAs was observed in early stages of seed development spanning from globular to bent cotyledon embryos [45]. Likewise, recent analysis

on several marker genes in pine, including TF mRNAs, documented generally higher gene expression level during SE than during ZE [46].

Similar to our results on the TF transcriptome, global transcriptome analysis in an embryogenic culture of *M. truncatula* indicated a distinct prevalence of up- over down-regulated transcripts [47]. Similarly, differentially expressed genes in cotton embryogenic cultures were also found to be upregulated in most cases [42]. In differentiating embryogenic rice callus, activation of gene expression was more common than repression, but a distinct prevalence of up- versus down-regulated genes was not observed [43]. Few reports indicated that TFs were mostly down-regulated, in contrast to global mRNA profiles [25,26]. However, the overall relatively small number of TF transcripts detected in these experiments (possibly due to technical limitations associated with microarrays used in those studies) may explain these earlier results.

TFs Strongly Modulated during SE-induction

The next striking feature of the TF transcriptome during SE induction revealed here was the drastic change (by at least 10-fold) of the expression of almost half (49%) of the modulated transcripts. In contrast, highly up-regulated transcripts were much less frequent in the global ZE transcriptome and constituted only 1–5% of the differentially expressed mRNAs [38]. It can perhaps be assumed that a rapid, massive and strong stimulation of TF expression occurring *in vitro* in SE-induced tissue results from a genome response to auxin treatment. Likewise, in potato, the most dramatic modulation of the transcriptome was observed during the SE induction phase enforced on auxin-containing medium [26], while a drastic fall in gene expression levels was observed in oil palm embryogenic culture after auxin removal from the medium [48].

Early versus Advanced Stages of SE

Our analysis demonstrated that different TF expression patterns discriminated early from advanced stages of embryogenic culture. In contrast to the embryo induction stage, stabilization of the transcriptome was observed at the more advanced culture stage associated with embryo formation, and most genes (58%) that changed expression by more than 2-fold during the embryo induction stage (i.e., between 0 d and 5 d) retained their expression level thereafter, thus changed expression by less than 2-fold between 5 d and 10 d. Divergent expression profiles were also reported for early and late stages of embryogenesis during seed development [39,49]. However, data on gene expression profiles specific to different stages of embryogenic cultures are generally scarce. In potato, similar to our results, the differentially expressed transcription-related genes are distinctly less abundant during advanced embryo formation than in the embryo-induction phase [26]. Also studies in maize and *Medicago truncatula* revealed a lower frequency of highly expressed genes in more advanced embryogenic cultures [23,50].

Apart from distinctly different expression profiles of early and advanced embryogenic cultures, it must be stressed that the great majority (>1,600) of the TFs were expressed across both stages of SE, and the number of TFs exclusively expressed at either the early or advanced SE stage was found to be very small (below 10). Also in ZE, many genes, including TFs, were expressed across multiple embryogenic stages [38,45] and only a small number of genes was specifically active in each given ZE stage [51,52]. Likewise, in *Brassica napus*, 30% of the genes expressed in microspore cultures upon embryogenic transition were also associated with developing androgenic embryos [53]. These observations thus indicate an extensive overlap in the transcription

regulatory machinery of SE-competent (explant) and SE-responding tissue and that many regulatory genes and their associated biological processes are shared across different stages of embryogenic culture.

SE-associated TFs

A common approach in screens for SE-associated genes is to contrast transcriptome profiles of embryogenic and non-embryogenic tissues and select the genes differing in expression profiles [22,25,50,54]. This strategy eliminates the genes expressed in response to auxin but not directly involved in the embryogenic switch. A similar approach used here identified 141 genes of distinctly different expression profiles in cultures derived from the highly embryogenic Col-0 accession versus the non-embryogenic *tanmei* mutant. A subset of the 141 genes includes regulators previously found to affect embryogenic development, including sixteen genes reported to be expressed during ZE [38,45,49].

Considering the suggested similarities between the genetic programmes governing zygotic and somatic embryogenesis [2], the number of genes required for somatic embryo development was assumed to be convergent to that in ZE. In ZE, the number of genes essential for embryo development in *Arabidopsis* was estimated to be 500–1000, including 220 *EMB* genes identified as required for normal zygotic embryo development [55,56,57]. However, in a recent analysis of the ZE global transcriptome less than 2% of the genes were found to be seed-specific and among them 48 TF genes were reported to be active exclusively, or at elevated levels, in seeds [38]. Strikingly, the majority of the seed-specific TFs [38] were not identified here among the TFs of SE-modulated expression in embryogenic *Arabidopsis* cultures. We found that only three of them (*ARR19*, *MYB107*, *IAA31*) displayed SE-specific expression, whilst 12 other seed-specific TFs were modulated in Col-0 embryogenic culture. This apparently lower than expected similarity between SE- and seed-specific gene expression was also stressed in a study on cucumber embryogenic cultures [58]. In addition, comparative expression profiling of some genes during ZE and SE in pine indicated some differences in the level and pattern of expression, including TF genes [46]. The differences in the gene expression patterns in ZE and SE likely reflect specificities of molecular mechanisms underlying embryogenic development in zygotic vs. somatic cells. Furthermore, the heterogeneity of the cell population analysed in embryogenic cultures may, in contrast to the more homogenous cell populations in ZE, substantially affect the gene expression profiles in tissues undergoing SE.

Stress-responsive TFs

The induction of SE was considered as a tissue response to stress imposed by *in vitro* culture [59,60,61]. In support of this, the activity of many stress-related genes was found to be associated with embryogenic cultures in different plants [25,47,48,50,62,63]. Similarly, in our study numerous stress-responsive TFs were expressed in *Arabidopsis* embryogenic cultures, representing half of the transcripts with SE-specific expression. The great majority (80%) of the stress-related TFs were up-regulated especially at the early stage of SE. Activation of such a large number of stress-related genes during *in vitro* embryo induction is unlikely to indicate a specific mechanism relevant to SE, but rather reflects a general response of the plants genome to the environment imposed *in vitro*. A significant proportion (39%) of the stress-related TFs modulated in embryogenic culture belong to the AP2/EREBP, WRKY and NAC families that are commonly activated in response to biotic and abiotic stresses [64,65,66,67].

A massive involvement of TF genes in stress responses can be expected as transcriptional control provides a crucial mechanism of plant responses to various stresses [68]. Several exogenous factors can trigger the expression of stress-related genes under *in vitro* conditions, and 2,4-D used in SE-induction medium is supposed to act as a powerful ‘stressor’ [59,60,69]. The strong response of stress-related genes in somatic cells under 2,4-D treatment observed here is in accordance with reports on other plant cultures [25,26,70,71,72]. Other tissue culture-related conditions can also be expected to influence gene expression *in vitro*. Recently, *WIND1* (*WOUND INDUCED DEDIFFERENTIATION1*) encoding a TF involved in establishment and maintenance of the dedifferentiated status of somatic cells in the absence of exogenous hormones was reported to be activated by tissue wounding [73]. Increased expression of *WIND1* in embryogenic cultures was detected here and in other plant cultures [50,74].

Hormone-related TFs

Our analysis revealed a large number of hormone-related TFs that changed their expression during SE, indicating an extensive involvement of hormone-related signaling pathways in this process.

Auxin-responsive genes. Auxin is a key trigger of SE in most plants, including Arabidopsis [75]. In accordance with this we observed a large number of auxin-responsive genes to be modulated in Arabidopsis embryogenic culture and similar observations were documented during SE in other plants [26,41,43,76,77]. Members of the ARF and AUX/IAA transcription regulator/signalling families act in concert to modulate expression of auxin-responsive genes [78,79]. We found that expression of over half (27/42) of all *AUX/IAA* and *ARF* genes changed during SE in Arabidopsis. In ZE of Arabidopsis, the majority of *AUX/IAA* and *ARF* genes were found active [80,81]. Transcripts of these genes constituted up to 4% of the seed-specific transcriptome [38] and, as indicated in the present study, *AUX/IAA* and *ARF* transcripts constituted a similar fraction of the SE-associated transcriptome.

Within the group of ARF regulators, *ARF5* (*AT1G19850*) encoding the MONOPTEROS (MP) auxin response factor, was up-regulated in embryogenic cultures of Arabidopsis (this study) and similarly in soybean [25]. *MP* constitutes a key gene in the control of zygotic embryo patterning via affecting polar auxin transport through activation of the auxin efflux carrier gene *PIN1* [82]. Significant activity of *MP* in embryogenic cultures may indicate that, similar to ZE, polar auxin transport and patterning are associated with somatic embryo induction and development. In support of this, mutations in both, *MP* and *TIR1* (*TRANSPORT INHIBITOR RESPONSE1*) were found to partly impair SE induction in Arabidopsis IZE explants (Malgorzata D. Gaj and A. Trojanowska, unpublished data). An important role of polar auxin transport for proper embryogenesis is supported by the fact that embryo development is impaired *in vivo* [83] and *in vitro* [84,85,86,87] when auxin transport is disturbed.

We also observed an upregulation of several other *ARF* genes in embryogenic cultures, including *ARF6*, *ARF8*, *ARF16* and *ARF17*. We found *ARF6* to be co-expressed with *ARF8*, similarly to what has been reported for ZE [45,49]. *ARF8* has been suggested to control the level of free IAA (indole-3-acetic acid) in a negative feedback fashion by regulating expression of *GH3* genes [88]. Expression of *ARF16* and *ARF17* was also modulated during ZE [45,49]. *ARF17* has been implicated as a regulator of *GH3*-like early auxin response genes [89]. *ARF16* together with *ARF10* and *IAA17/AXR3* regulate distal stem cell differentiation in Arabidopsis roots acting upstream of *PLETHORA* (*PLT*) [90]. Of note, these

genes (*ARF10*, *ARF16*, *IAA17*, *PLT1* and *PLT2*) were up-regulated in embryogenic Arabidopsis cultures.

Similar to *ARFs*, reports on *AUX/IAA* expression in embryogenic cultures of plants are rare; of note, however, homologs of the Arabidopsis *IAA9* and *IAA8* genes were found expressed during SE in *Cyclamen persicum* and *Gossypium hirsutum* [91,92]. In the present analysis almost 70% of the *AUX/IAA* family members displayed modulated expression in embryogenic cultures suggesting their involvement in SE. In support of this we found *iaa* mutants (*iaa16*, *iaa29*, *iaa30* and *iaa31*) to be significantly impaired in the embryogenic response.

AP2/EREBP TFs and ethylene responses. The SE-modulated TF transcriptome was highly enriched for members of the AP2/EREBP family. Numerous *AP2/EREBP* genes were previously shown to control SE and shoot organogenesis *in vitro*, and several members of the family were reported to promote embryo development in somatic tissues when overexpressed, including e.g. *BABY BOOM* (*BBM*) [13], *AGAMOUS-LIKE15* (*AGL15*) [15,93] and *EMBRYOMAKER* (*EMK*) [19]. Expression of *AP2/EREBP* TFs was frequently found to be modulated in embryogenic cultures of different plants [25,50,76,77,94,95] including Arabidopsis (this report). Many members of the ERF subfamily are involved in ethylene responses [65]. Hence, enhanced expression of *AP2/EREBP* genes during the *in vitro* culture may reflect a general stress response of the tissues as e.g. induced by wounding or hormonal treatment [68], while some *ERF* genes may be specifically involved in the induction of SE. The role of ethylene for somatic embryo development was demonstrated in *Medicago truncatula*, where *SOMATIC EMBRYO-RELATED FACTOR1* (*MiSERF1*), an ERF subfamily TF affecting ethylene biosynthesis, is crucial for embryo induction [50]. Likewise, in *Pinus silvestris* an increased content of endogenous ethylene appears to be required for somatic embryo development [95]. Recently, ethylene biosynthesis and perception were also reported to be involved in SE induction in Arabidopsis [96]. In support of this, the extensive modulation of many (49) ethylene-related TFs of the ERF, MYB, bHLH, NAC and WRKY families was observed here for embryogenic Col-0 cultures, and mutations affecting *ERF022* (encoding an ERF TF; **Figure 10**) and *ACC SYNTHASE4* (*ACS4*; involved in ethylene biosynthesis) appeared to significantly decrease explant capacity for SE (data not shown). Our preliminary analysis indicates regulatory relationships between *ERF022* and genes acting in ethylene signaling and biosynthesis (Katarzyna Nowak and Małgorzata D. Gaj, unpublished). Another ethylene related gene, *RAP2.6L* (*RELATED TO AP2 6L*; *AT5G13330*) of the AP2/EREBP family, was found here to be up-regulated in embryogenic cultures. *RAP2.6L* expression is also induced during shoot organogenesis [94], in proliferating cells of newly formed tissues after wounding, and by stress hormones and abiotic stresses [97,98].

Cytokinin-related TFs. Although cytokinin is not included in SE-induction medium, the involvement of cytokinin-related TFs in embryogenic development may be expected due to widespread crosstalk between auxin and cytokinin signalling [99,100,101,102]. We here observed 16 cytokinin response-associated TFs to be affected in the auxin-induced embryogenic Col-0 cultures, including key cytokinin regulatory genes, i.e. *CYTOKININ RESPONSE FACTORS* (*CRFs*) and *Arabidopsis RESPONSE REGULATORS* (*ARRs*). Of eight *CRFs*, four (*CRF2*, 3, 4 and 5) were up-regulated in Col-0 embryogenic cultures. CRFs mediate a large fraction of the transcriptional response to cytokinin to regulate development of embryos, cotyledons, and leaves and they function together with type-B *ARRs* [103]. Two type-B *ARR* genes, i.e. *ARR19* and *ARR10*, had altered expression in Col-0 cultures.

ARR10 transcripts were up-regulated in early and advanced stages of SE and similarly, up-regulation of the *ARR10* homolog *MtRR1* (*Mtr.43735.1*) was reported in embryogenic cultures of *M. truncatula* [47]. *ARR10*, together with *ARR1* and *ARR12*, is proposed to play a general role in cytokinin signal transduction [104].

Gibberellin-related TFs. In Arabidopsis, the endogenous level of gibberellins in somatic tissue seems to be negatively correlated with embryogenic potential. The *lec* mutants, displaying increased GA content [105], were found to have a drastically reduced ability for SE [106]. Similarly, the *pickle* mutant which has elevated levels of bioactive GAs displays reduced embryogenic potential in cultures of IZEs, and exogenously supplied GA₃ was demonstrated to decrease tissue capacity for SE induction [106].

In support of the inhibitory effect of GA on embryogenic capacity in Arabidopsis, several genes important for the negative regulation of GA responses were found to display an SE-specific up-regulation, including the DELLA-encoding genes *RGL1* (*RGA-LIKE1*, RGA for repressor of ga1-3) and *RGL2*. DELLA proteins interact with multiple environmental and hormonal response pathways and restrain plant growth [107]. The stimulation of DELLA-encoding genes in Col-0 embryogenic cultures may also be associated with stress responses as DELLA accumulation was reported to elevate the expression of genes encoding ROS detoxification enzymes, thus reducing ROS levels [108]. Another suppressor of GA responses, *SHORT INTERNODES (SHI)*, was found to be up-regulated in Col-0 embryogenic cultures; in intact plants, *SHI* affects the development of shoot and root primordia [109].

Role of TFs in SE

To increase the probability of finding TFs functionally relevant for SE, we included the *tanmei* mutant in our transcriptome analysis. As *tanmei* lacks the capacity for SE, TFs differentially expressed between Col-0 and the mutant may represent candidate regulators of SE, although genes not specifically associated with SE may also be expressed at different levels in the two genetic backgrounds. Considering the results of our global expression analysis we selected 21 genes (18 of which showed altered expression in Col-0 *vs. tanmei*, and three genes displayed differential expression in embryogenic culture) to test their potential relevance for somatic embryo formation, using T-DNA insertion mutants and transgenic lines expression the TFs under the control of a β-estradiol-inducible promoter [110]. The majority (70%) of the mutants analyzed were significantly impaired in their SE capacity suggesting an involvement of the tested TFs in this process.

We found that various T-DNA insertion lines impaired in SE were actually mutated in genes related to stress responses, including *ERF022*, *NTL8*, *DREB2F*, *ATHB-12*, *LBD20* and *MYB74*. Mutating *ERF022* increases the plants sensitivity to osmotic and salinity stress, whilst overexpressing it triggers the opposite phenotype (Katarzyna Nowak and Małgorzata D. Gaj, data not shown). *NTL8* of the NAC TF family was reported to regulate gibberellic acid-mediated salt signalling during Arabidopsis seed germination [111]. Expression of *DREB2F* is affected by abiotic and biotic stresses (eFP browser: <http://www.bar.utoronto.ca/efp/cgi>). *ATHB12* together with *ATHB7* was reported to encode a potential regulator of growth in response to water deficit [112]. *LBD20* (*LOB DOMAIN-CONTAINING PROTEIN20*) has recently been suggested to be involved in transcriptional regulation of plant defence responses against pest or pathogen attack [113]. *MYB74* is a close homolog of *MYB102* which was demonstrated to be induced by osmotic stress and wounding [114]. Summarizing, the SE-impaired phenotypes observed in mutants of stress-related genes strongly support the notion that SE

induction shares, at the molecular level, processes that are also relevant to general stress responses.

In contrast to the insertion mutants, phenotypes of transgenic lines overexpressing TFs under the control of a chemically inducible promoter were generally less informative. However, for two TFs, i.e. *DOF5.2* and *bHLH109*, we observed a clear function in SE. The phenotype observed upon induced overexpression of *DOF5.2* (reduced SE capacity) together with the fact that expression of the gene decreases during early stages of somatic embryogenesis suggests that *DOF5.2* functions as a negative regulator of SE induction. Currently, the exact molecular function of *DOF5.2* is unknown, however, the gene was shown to be specifically expressed in the quiescent centre of roots and a role for stem cell niche maintenance in the root meristem possibly by affecting auxin flux was postulated [115].

The other gene found to affect SE is *bHLH109*, which in contrast to *DOF5.2* appears to act as a positive regulator of somatic embryo formation. Accordingly, expression of *bHLH109* was found to be highly upregulated in embryogenic cultures, and auxin strongly enhanced its expression. Identifying the downstream target genes controlled by *bHLH109* will help to better understand through which regulatory networks the bZIP TF promotes embryogenic development in the future.

Conclusions

Our study provides the first comprehensive analysis of the global TF transcriptome of plant somatic tissue undergoing embryogenic induction during *in vitro* culture. TF genes of drastically different expression in embryogenic vs. non-embryogenic cultures were selected as candidates for further studies aiming at the characterization of genes with decisive roles in SE.

The results presented here indicate the presence of a regulatory burst at the gene expression level that is associated with early stages of somatic embryo development. The global TF transcriptome associated with SE induction reflects the combinational effects of stress and hormone signalling related to the *in vitro* environment imposed during culture. Accordingly, among the TFs showing SE-specific expression those involved in stress and hormone responses, plant and especially flower development were found most frequent. The use of Arabidopsis for this study opens new avenues for advanced analysis of the selected SE-associated candidate genes based on genomic data, mutant collections, transgenic lines and other genomic tools available for this model species. The study provides guidelines for further research on functional genomics of SE.

Materials and Methods

Plant Material and Growth Conditions

Two *Arabidopsis thaliana* (L.) Heynh. genotypes of different embryogenic capacity were analyzed, i.e. the highly embryogenic Col-0 ecotype and the SE-impaired *tanmei* (*tan1-2*) mutant [34]. Additionally, mutants carrying T-DNA insertions [64] in selected TF genes were analysed with respect to their capacity for somatic embryo formation. The parental Col-0 ecotype and the insertion mutants were obtained from NASC (The Nottingham Arabidopsis Stock Center; <http://arabidopsis.info/>). T-DNA insertion lines (**Table S4**) originated from the SALK and SAIL collections; homozygous plants carrying insertions in TF genes were selected from a segregating T₃ population according to standard procedures. Seeds of the *tan1-2* mutant were kindly provided by J. J. Harada (University of California, Davis, USA). Plants were grown in Jiffy-7 peat pots of 42 mm diameter (Jiffy) in a ‘walk-in’

type phytotron, under controlled conditions: 22°C, 16h/8h (light/dark), 100 µE/m²s light intensity.

Estradiol-inducible TF Overexpression Lines

To generate transgenic plants expressing TFs under the control of an estradiol-inducible promoter, the coding regions of the selected genes (*NTL8*, *ERF022*, *bHLH99*, *bHLH109*, *REM22*, *AGL2*, *WRKY31*, *DOF5.2*) were amplified by PCR from Arabidopsis leaf or zygotic embryo cDNA using primers IOE-fwd and IOE-rev (Table S5), inserted into pBluescript SK (Stratagene) and then cloned via *Xba*I (or *Apa*I) and *Sph*I sites into the pER8 vector [110]. *Agrobacterium tumefaciens* strain GV3101 was used for *A. thaliana* (Col-0) transformation. Seedlings of selected homozygous transgenic lines were used for expression analysis. RNA was isolated (TriPure Reagent; Roche) from β-estradiol-treated (5 µM, 2 d) and mock-treated (0.01% ethanol) seedlings, and cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas). The resulting cDNA was used for qRT-PCR (Table S6). LightCycler Fast-Start DNA Master SYBR Green I (Roche) and appropriate primers were used for qRT-PCR reactions.

Induction of Somatic Embryogenesis

A standard protocol was used to induce somatic embryogenesis in Arabidopsis under *in vitro* conditions [116]. In brief, explants, i.e., immature zygotic embryos (IZEs) at the late cotyledonary stage of development, were excised from siliques 10–12 days after pollination. Siliques were surface-sterilized with sodium hypochlorite (20% commercial bleach) and washed thoroughly with sterile water. Then IZEs were isolated and placed on E5 solid medium containing B5 salts and vitamins [117] and supplemented with 5 µM 2,4-D, 20 g l⁻¹ sucrose and 3.5 g l⁻¹ Phytagel (Sigma). To induce overexpression of TFs in pER8-TF-transformed transgenic cultures, E5 medium was supplemented with 5 µM of β-estradiol.

Cultures were maintained in the controlled conditions of a growth chamber: 22°C, 16h/8h (light/dark), light intensity 50 µE/m²s. At selected time points of the culture (0, 5 and 10 d), explants of Col-0 and *tan1-2* were sampled for transcriptome analysis.

The capacity for SE in T-DNA insertion mutants and transgenic lines overexpressing TFs was evaluated after 21 days of *in vitro* culture. Embryogenic potential of mutants and transgenic lines was evaluated by calculation of SE efficiency (i.e., the percentage of explants forming somatic embryos) and SE productivity (i.e., the average number of somatic embryos produced per SE-responding explant). SE efficiency and productivity of the analysed genotypes was compared to Col-0-derived cultures. All experiments were conducted in three independent replicates, and at least 30 explants (10 explants/Petri dish) were analysed per replicate.

Statistical Analysis

Kruskal-Wallis ANOVA rank and Mann-Whitney's U statistical tests were applied to calculate significant differences (at p = 0.05) between combinations.

Transcriptome Profiling by Multi-parallel qRT-PCR

Quantitative RT-PCR was used to compare the expression levels of 1,880 Arabidopsis TF genes in the SE cultures of Col-0 and *tan1-2*. Total RNA was isolated at 0, 5 and 10 d of wild-type-(WT) and mutant-derived cultures, using RNAqueous kit (Ambion). The isolates were digested with Turbo DNA-free kit (Ambion) to remove DNA contaminants. SuperScript III reverse transcriptase (Invitrogen) was used for cDNA synthesis. qRT-PCR was done as described [31,118,119]. PCR reactions were run on

an ABI PRISM 7900 HT sequence detection system (Applied Biosystems Applera, Darmstadt, Germany).

Data analysis was performed using SDS 2.2.1 software (Applied Biosystems). All amplification curves were analysed with a normalized reporter (R_n: the ratio of the fluorescence emission intensity of SYBR Green to the fluorescence signal of the passive reference dye) threshold of 0.3 to obtain the C_T (threshold cycle) values. Four replicates of the reference control gene, *UBQ* (*AT1G55060*), were measured in each PCR run, and their median C_T was used for relative expression analyses. Expression data were submitted to the NCBI Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo/) under accession number GSE45697.

To find significant changes between the genotypes (Col-0 and *tan1-2*) and the time points, ANOVA followed by false discovery rate (FDR) correction was applied using a custom R script (<http://www.r-project.org>). Only TFs which displayed an FDR corrected p-value < 0.05 were considered for further analysis. Furthermore, different comparisons between genotypes and time points were performed using Students t-test (p < 0.05). The analysis was performed in two ways: (1) to identify differentially expressed TFs that are specific for the different time points in Col-0, and (2) to identify TFs differentially expressed between Col-0 and *tan1-2* at each time point. The fold change was calculated using (2)^{-ΔΔCT}, where ΔΔCT represents ΔC_T reference condition – ΔC_T compared condition. The obtained results were transformed to log₂ scale. Candidates were extracted using thresholds of 2- and 10-fold change.

Principal component analysis (PCA) was performed using the prcomp function of the “stats” package in R [120].

Supporting Information

Figure S1 TF families among differentially expressed and SE-associated genes. For each TF family the percentage of genes differentially expressed or being SE-associated is indicated. (TIF)

Figure S2 Expression levels of *bHLH109* and *DOF5.2* TFs in explants induced towards alternative morphogenic pathways, i.e. somatic embryogenesis (SE) and seedling development (E0). Values significantly different from E0 are marked by asterisks (n = 3; means ± SD are given; Mann-Whitney's U test; p < 0.05). (TIF)

Table S1 TFs exclusively or highly expressed in embryogenic Col-0 explants compared to non-embryogenic *tanmei* mutant explants.

(DOC)

Table S2 Expression values of 729 TFs modulated in Col-0 embryogenic culture.

(XLS)

Table S3 TFs showing an at least 10-fold expression change during early culture stages.

(DOC)

Table S4 T-DNA insertion lines used for the functional analysis of selected TFs.

(DOC)

Table S5 Primers used for the amplification of open reading frames.

(DOC)

Table S6 Expression level of transgenes in seedlings treated with β-estradiol (5 µM) for 2 days.

(DOC)

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Author Contributions

Conceived and designed the experiments: MDG SB BMR. Performed the experiments: MG KN SB. Analyzed the data: MG KN SB. Contributed reagents/materials/analysis tools: MDG BMR. Wrote the paper: MDG KN SB BMR.

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