The TRANSPLANTA collection of Arabidopsis lines: a resource for functional analysis of transcription factors based on their conditional overexpression

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SUMMARY

Transcription factors (TFs) are key regulators of gene expression in all organisms. In eukaryotes, TFs are often represented by functionally redundant members of large gene families. Overexpression might prove a means to unveil the biological functions of redundant TFs; however, constitutive overexpression of TFs frequently causes severe developmental defects, preventing their functional characterization. Conditional overexpression strategies help to overcome this problem. Here, we report on the TRANSPLANTA collection of Arabidopsis lines, each expressing one of 949 TFs under the control of a β–estradiol-inducible promoter. Thus far, 1636 independent homozygous lines, representing an average of 2.6 lines for every TF, have been produced for the inducible expression of 634 TFs. Along with a GUS-GFP reporter, randomly selected TRANSPLANTA lines were tested and confirmed for conditional transgene expression upon β–estradiol treatment. As a proof of concept for the exploitation of this resource, β–estradiol-induced proliferation of root hairs, dark-induced senescence, anthocyanin accumulation and dwarfism were observed in lines conditionally expressing full-length cDNAs encoding RHD6, WRKY22, MYB123/TT2 and MYB26, respectively, in agreement with previously reported phenotypes conferred by these TFs. Further screening performed with other TRANSPLANTA lines allowed the identification of TFs involved in different plant biological processes, illustrating that the collection is a powerful resource for the functional characterization of TFs. For instance, ANAC058 and a TINY/AP2 TF were identified as modulators of ABA-mediated germination potential, and RAP2.10/DEAR4 was identified as a regulator of cell death in the hypocotyl–root transition zone. Seeds of TRANSPLANTA lines have been deposited at the Nottingham Arabidopsis Stock Centre for further distribution.

Keywords: *Arabidopsis thaliana*, transcription factors, conditional overexpression, functional genomics, TRANSPLANTA consortium, transgenic lines collection, functional screening.

INTRODUCTION

Transcription factors (TFs), with DNA-binding domains recognizing specific DNA sequences, play a central role in the regulation of gene expression. In plant genomes, approximately 7% of genes encode TFs (Udvardi *et al.*, 2007). In RNA polymerase-associated transcription regulatory complexes, TFs interact with other proteins. Whereas

some TFs are highly selective concerning the regulation of specific biological processes, some others affect a wide array of developmental programs and environmental responses in plants (Melzer and Theissen, 2011; Vaahteraa and Broschéa, 2011; Le Hir and Bellini, 2013; Licausi *et al.*, 2013; Nuruzzaman *et al.*, 2013). Upon completion of the Arabidopsis genome sequence (Arabidopsis Genome Initiative, 2000), the number of genes coding for TFs was first estimated to be about 1500 (Riechmann *et al.*, 2000), but soon after, the number rose above 2000. Several databases, such as AGRIS (Davuluri *et al.*, 2003), DATF (Guo *et al.*, 2005) and RARTF (Iida *et al.*, 2005) were established to collect the increasing information on Arabidopsis transcription factors. In addition, functional annotation of several other plant genomes sequenced to date (http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html; http://genomevolution.org/CoGe/) help to extend our knowledge on conservation of TFs in different plant species. The currently available classified information is accessible at several plant TF databases, such as PLACE (Higo *et al.*, 1998; http://www.dna.affrc.go.jp/PLACE/), PlnTFDB (Riano-Pachón *et al.*, 2007; http://plntfdb.bio.uni-potsdam.de/v3.0/) and PlantTFDB (Guo *et al.*, 2008; http://planttfdb.cbi.edu.cn/), facilitating the exploration of function and evolution of TFs in plants.

Several multilateral initiatives undertook genome-wide functional analyses of plant TFs. In Arabidopsis, the European consortium REGIA (Paz-Ares, 2002), aimed at determining the function of all Arabidopsis TFs, provided data on TF–protein interactions, contributing to other activities focusing on the definition of plant protein interactomes (Geisler-Lee *et al.*, 2007; Kerrien *et al.*, 2007, 2012; Cui *et al.*, 2008). Current information on protein interactions, providing useful help for deciphering the regulatory functions of TFs, is deposited at TAIR (http://www.arabidopsis.org/portals/proteome/proteinInteract.jsp), EBI-IntAct (http://www.ebi.ac.uk/intact/site/index.jsf) and AtPID (http://atpid.biosino.org/index.php) databases.

Despite a considerable quantity of information and numerous available tools, Arabidopsis researchers working on the functional characterization of TFs are often faced with the problem caused by functional redundancy between members of large TF families, which hinders their precise characterization. Overexpression of individual members of redundant TF families is used as an approach to specify their unique functions; however, this strategy is hampered by the fact that ectopic overexpression of TFs often results in deleterious effects (Kasuga *et al.*, 1999), or it potentially causes off-target effects thus causing misleading phenotypes. To overcome this problem, chemically inducible conditional overexpression of TFs offers a potentially useful solution. The possibility of time- and place-regulated overexpression by the controlled application of an inducing factor allows for the assessment of gene-triggered effects at precise times and locations. Here, we examine and assess the applicability of this strategy by generating a collection of homozygous Arabidopsis TRANSPLANTA (TPT) lines, in which the expression of TFs encoded by full-length cDNAs in a Gateway-compatible pER8GW vector (Papdi *et al.*, 2008) derived from the original pER8 vector (Zuo *et al.*, 2000) is inducible conditionally by β–estradiol. To document the versatile applicability and usefulness of the TPT lines, we show that conditional overproduction of RHD6, WRKY22, MYB123/TT2 and MYB26 TFs confers the proliferation of root hairs, dark-induced senescence, anthocyanin accumulation and dwarfism, respectively, in agreement with previous reports on overexpression phenotypes of these TFs (Baudry *et al.*, 2004; Menand *et al.*, 2007; Yang *et al.*, 2007; Zhou *et al.*, 2011). Moreover, by screening for gain-of-function phenotypes, we show that ANAC058 and a TINY/AP2 function as important modulators of ABA-mediated germination potential, whereas RAP2.10/DEAR4 is involved in the regulation of the cell death response in the hypocotyl–root transition zone. These examples illustrate how TPT lines can be used as a powerful resource to identify and analyze distinct functions and/or redundancy of known and yet uncharacterized Arabidopsis TFs in the regulation of different biological processes.

RESULTS

A collection of Arabidopsis transgenic lines conditionally overexpressing transcription factors

The TRANSPLANTA consortium aims to perform a systematic functional analysis of *Arabidopsis thaliana* transcription factors through several work packages, including the generation of different genomic tool resources and their further use in systematic screening for TF functions in different biological processes (http://bioinfogp.cnb.csic.es/transplanta_dev/). As a basic tool in the TRANSPLANTA project, a collection of Arabidopsis transgenic lines expressing single TFs under the control of an inducible promoter has been generated. The rationale behind using a system for conditional expression of the TF collection was to prevent the potential deleterious and off-target effects that the overexpression of TFs may cause, by affecting the development and physiology of the transformed plants. For this purpose, we started from a collection of pER8GW binary vector-based constructs expressing a collection of Arabidopsis TFs from the REGIA (Paz-Ares, 2002) and REGULATOR (Castrillo *et al.*, 2011) initiatives, under the control of a β–estradiol-inducible system. First, 1157 original cDNA clones in the pER8GW vector were quality controlled by full-length sequencing. About 30% of these clones carried either mutations causing changes in the sequence of encoded proteins or did not match completely with the expected sequence. All these clones were consequently discarded from the collection. After applying the

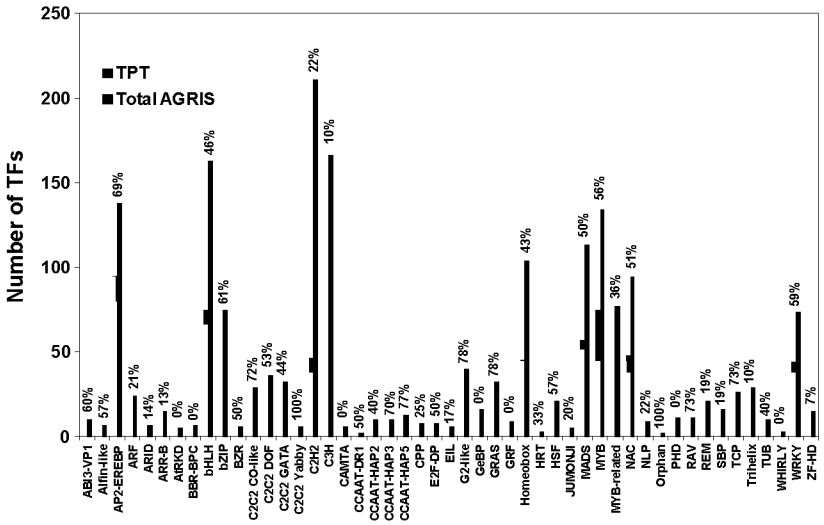

Figure 1. Coverage of transcription factor (TF) families defined by AGRIS in the TPT collection. The total number of TFs (black bars) and the number present in the TRANSPLANTA (TPT) collection (gray bars) in each family is shown. The percentage of TFs present in the TPT collection for every TF family is indicated above the bars. The total number of members in each family was obtained from the AGRIS website.

quality control filter, 806 mutation-free clones were selected for further work. This set was further enriched with the incorporation of 195 additional clones from public DNA collections. The latter cDNA clones were sequence-verified, and then recombined into the pER8GW binary vector. As performed with the rest of the clones, these new pER8GW-based clones were also full-length sequenced and all the mutation-free clones were added to the original basic collection to give a total of 949 TFs cloned in the pER8GW vector (Table S2). The complete collection of pER8GW:TFs was thus quality-controlled by full-length sequencing before being used for further genetic transformation of *A. thaliana*. The whole TF collection includes members of all TF families defined in AGRIS (http://arabidopsis.med.ohio-state.edu/AtTFDB), except for the small AtRKD, BRR-BPC, CAMTA, GeBP, GRF, PHD and WHIRLY families. The representation of TFs from each family ranges from 10 to 100%, although for most of the families over 30% of their members are present in the TRANSPLANTA collection (Figure 1). Moreover, some of the most important families including AP2/EREBP, ABI3/VP1, Alfin-like, BZR, C2C2 CO-like, DOF, YABBY, CCAAT, E2F-DP, G2-like, GRAS, HSF, MADS, MYB, NAC, RAV, TCP, WRKY and b–ZIP are represented with more than 50% of their members in the TPT collection (Figure 1).

About 8000 T_1 seeds for every TF clone were selected in hygromycin-supplemented MS media to recover between eight and 11 resistant (HygR) primary transformants for each clone. We found that this number of lines was sufficient to identify two or three events of single T–DNA integration for each TF construct. On average, nine T_1 HygR plants selected for every TF were individually propagated to generate T_2 seed populations. Further selection of T_2 seed populations on hygromycin-containing plates allowed us to identify those producing 75% of HygR individuals, indicative for carrying a single insertion of a TF construct.

Table 1 Summary of data from the production pipeline of TPT lines

	Total	Per TF construct
Number of TFs managed in TPT	949	–
T_1 seed pools generated upon Col–0 transformation	949	–
T_1 seeds selected in hygromycin plates	~80 000 000	8000
HygR T_1 seedlings selected	~9500	8–11 (average 9)
T_2 seed populations harvested	~9500	8–11 (average 9)
T_2 seed populations with single T–DNA insertion	5500	5–6
T_3 seed populations generated from T_2 seedlings	49 500	50
Predicted T_3 seed populations yielding 100% HygR	~2500	2.5
Predicted homozygous T_4 lines (bulk seed)	~2300	2.3

The individual propagation of nine individuals from each of the two or three T_2 populations with single TF construct insertion generated the T_3 seed populations that were further screened for homozygous lines. Those displaying 100% HygR T_3 individuals were selected as homozygous lines. About 10–12 HygR T_3 homozygous plants per TF were sown on soil to produce a bulk of T_4 seeds for every homozygous line. The details of the whole pipeline process are summarized in Table 1. Seeds of the collection of so-called TPT lines are being deposited in the NASC bank for further public usage by the Arabidopsis community. Presently, seeds of 1636 homozygous lines corresponding to 634 TFs, thus giving an average of 2.6 TPT lines per TF, are already available in the Nottingham Arabidopsis Stock Centre (NASC; Table S3). Around 33 and 41% of the TFs are represented with two or three independent homozygous lines, respectively. Approximately 8 and 4% of the

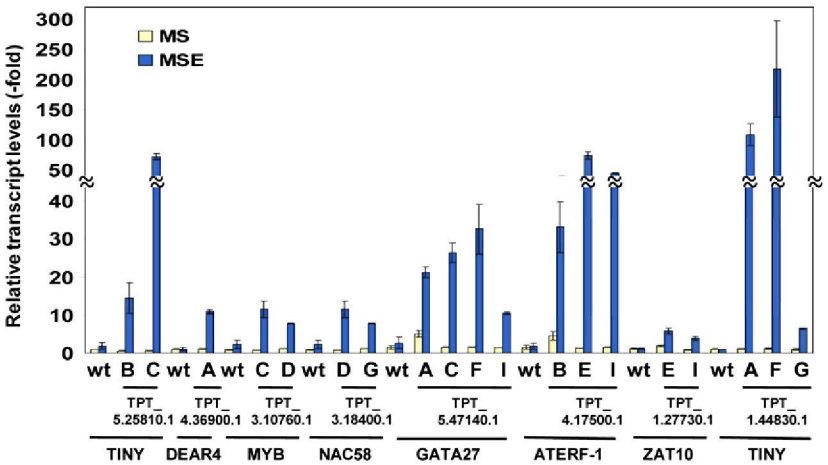

Figure 2. Conditional transgene expression in the TPT lines. Relative transcript levels quantified by RT-qPCR in untreated (yellow bars) and β–estradiol-treated (blue bars) seedlings of TPT lines corresponding to eight randomly selected transcription factors (TFs). Specific primers for every gene were used and their sequences are shown in Table S4. Values represent the mean of three independent replicates and error bars correspond to SDs.

TFs were represented by four or more than four independent transgenic lines, respectively. Finally, for 13% of the TFs only one homozygous transgenic line was generated. Details about the lines can be found on the TRANSPLANTA web page (http://bioinfogp.cnb.csic.es/transplanta_dev/). The lines can be ordered on the NASC website (accession number N210 1415, as a complete set or as individual lines; http://arabidopsis.info/).

Conditional expression of TFs in TPT lines

To assess whether TPT lines carrying the individual pER8GW:TF constructs generated in this work indeed show conditionally inducible expression, a double strategy was pursued. First, reporter pER8GW:GUS-GFP lines expressing a translational fusion of β–glucuronidase (GUS) and green fluorescent protein (GFP) under the control of the β–estradiol-inducible system were produced. Both GUS staining and GFP-associated fluorescence were observed in β–estradiol-treated seedlings (Figure S1a,b), but not without treatment (Figure S1a). These data are in agreement with the previously reported β–estradiol-inducible GFP fluorescence pattern of a pER8:GFP reporter line (Zuo *et al.*, 2000). Moreover, we also confirmed the β–estradiol-dependent accumulation of GFP protein by western blot (Figure S1b). Based on the results obtained with the pER8GW:GUS-GFP lines, the conditional expression systems appeared to work properly. Nevertheless, the robustness and efficiency of the conditional expression system was also tested by the selection of TPT lines corresponding to eight randomly selected TFs. The β–estradiol-dependent expression of different TFs was analyzed by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR), with specific primers for each TF tested (see Table S4 for the sequence of the specific primers). Figure 2 shows that most of the transgenic lines tested displayed a strong TF mRNA accumulation only upon β–estradiol treatment, thus confirming a robust and efficient conditional expression of TFs in the TPT lines. Moreover, for most of the TFs analysed, every available line showed β–estradiol-dependent expression of the transgene, although to a different extent (Figure 2), highlighting the need for at least two independent TPT lines per TF.

Conditional expression of TFs with previously reported functions correlated with the expected phenotypes in TPT lines

As a proof of concept for the β–estradiol-dependent expression of different TFs in Arabidopsis TPT lines, a number of lines conditionally overexpressing previously characterized TFs were used to assess the correlation between conditional TF expression and the reported phenotypes caused by their overexpression. TPT lines corresponding to four TFs were phenotypically analysed. We found that TPT lines conditionally expressing the *Root Hair Defective 6* (*RHD6*) transcription factor upon β–estradiol treatment displayed a large increase in the number and elongation of root hairs (Figure 3a), as expected given the currently reported function of RHD6 in controlling early stages of root hair cell differentiation (Menand *et al.*, 2007). The elongation of root hairs was not observed in roots of TPT *RDH6* lines in the absence of β–estradiol treatment, and in Col–0 roots grown in the presence or absence of β–estradiol (Figure 3a). We also confirmed that phenotypes dependent on the conditional expression of TFs are also observed in plant organs other than roots (i.e. in organs exposed directly to β–estradiol during induction). As shown in Figure 3b, an enhanced dark-induced senescence phenotype was observed in three independent TPT lines upon β–estradiol-induced expression of the *WRKY22* gene, which has been previously reported to promote dark-induced senescence in Arabidopsis (Zhou *et al.*, 2011). Another example of a shoot phenotype was observed upon β–estradiol-induced expression of *MYB123* cDNA encoding

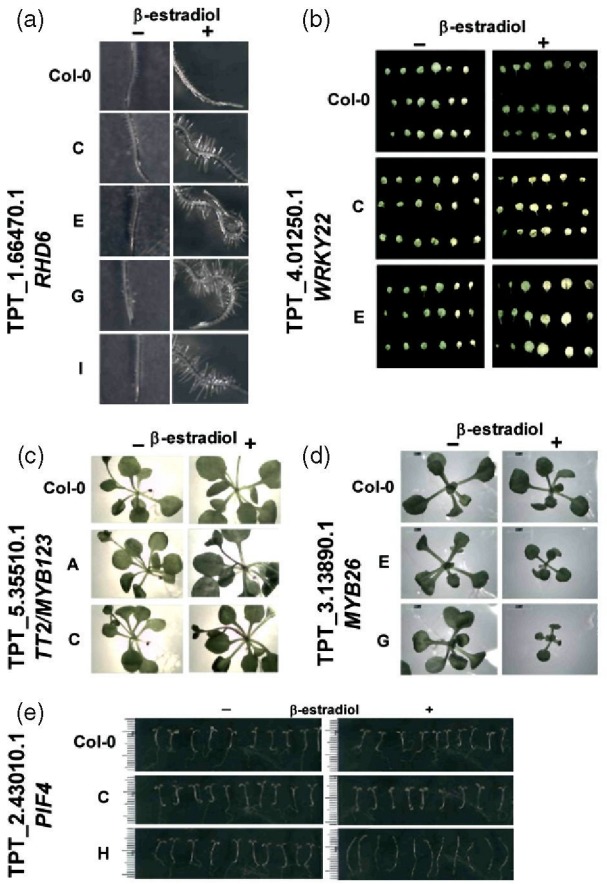

Figure 3. Phenotypes of β–estradiol-induced TPT seedlings overexpressing transcripts of transcription factors (TFs) with previously reported functions confirm the functionality of TPT lines.
(a) Proliferation and elongation of root hairs in four independent TPT lines conditionally expressing *Root Hair Defective 6* (*RHD6*, At1g66470).
(b) Enhanced β–estradiol-dependent phenotype of dark-induced senescence in two independent TPT lines conditionally expressing *WRKY22* (At4 g01250). Rosette leaf series of three individuals (per line) are shown in every panel.
(c) Accumulation of anthocyanins in leaves and stems of β–estradiol-treated seedlings of two independent TPT lines expressing *MYB123/TT2* (At5 g35550).
(d) Dwarf phenotype of β–estradiol-treated seedlings of two independent TPT lines conditionally expressing MYB26 (At3 g13890).
(e) Hypocotyls of red light-grown seedlings from two independent lines conditionally expressing *PIF4.*

the TRANSPARENT TESTA 2 protein involved in regulating proanthocyanidin biosynthesis (Baudry *et al.*, 2004). Increased anthocyanin content was observed in rosette leaves and, more markedly, in stems of two independent TPT lines conditionally expressing *MYB123/TT2* (Figure 3c). A 2.8- and 4.6–fold increased anthocyanin content was quantified in whole seedlings of two TPT independent lines conditionally expressing TT2 (Figure S2). As expected, this phenotype was again absent in Col–0 and or β–estradiol untreated TPT plants (Figures 3c and S2). Additional confirmation of the reliability of TPT lines was derived from the β–estradiol-induced expression of *MYB26* cDNA, previously reported to cause dwarfism upon overexpression (Yang *et al.*, 2007). As shown in Figure 3d, two independent TPT lines conditionally expressing *MYB26* displayed small seedling size only upon β–estradiol treatment. A reduction of 50% in the rosette diameter of two independent TPT lines was observed upon growth for 14 days in β–estradiol-supplemented media, when compared with seedlings grown in media without β–estradiol (Figure S2). Finally, we have also tested whether hypocotyl-related phenotypes can be confirmed, with TPT lines corresponding to TFs previously characterized as regulators of hypocotyl elongation. We examined whether TPT lines conditionally overexpressing *PIF4*, one of the members of the Phytochrome Interacting Factors (PIF) family of basic helix-loop-helix (bHLH) class transcription factors that promote hypocotyl elongation under red light conditions (de Lucas *et al.*, 2008), display the expected phenotypes. Figure 3e shows that only in β–estradiol-treated seedlings red light-grown hypocotyls of line TPT_2.43010.1H conditionally expressing *PIF4* were 1.7-fold longer than those from wild-type seedlings (Figure S2); however, hypocotyls of another line TPT_2.43010.1C were indistinguishable from wild-type hypocotyls under red light (Figures 3e and S2), suggesting that for some TFs the selected homozygous TPT lines may have significant differences in terms of transgene expression and the corresponding expected phenotypes. This strongly supports the need of using different independent homozygous lines for each TF, especially when TPT lines are going to be exploited for phenotypic screening.

Use of TPT lines to screen for phenotypes conferred by TFs that have not yet been characterized

An overall analysis of TPT seedlings in the absence of β–estradiol treatment showed that a very low proportion (<3%) displayed visible phenotypic alterations (i.e. probably as a consequence of insertional mutations induced by integrating pER8GW T–DNAs). Consequently, TPT lines can be useful to search for TFs that lead to specific phenotypes upon β–estradiol-induced expression. To demonstrate this, we used the TPT lines in phenotypic screens performed by directly germinating seeds on β–estradiol-supplemented media, or in screens in which the expression of TFs was induced only at different stages of plant development. As a typical example for a screen based on the permanent expression of TFs, the ABA sensitivity of TPT lines was tested during seed germination and seedling establishment. Seeds of wild type and TPT lines were sown on either MS medium or MS supplemented with 10 μM β–estradiol in the absence or presence of 0.3 or 1 μM ABA. Although most of the TPT lines showed ABA-mediated inhibition of seed germination and seedling establishment, similar to that observed for wild-type

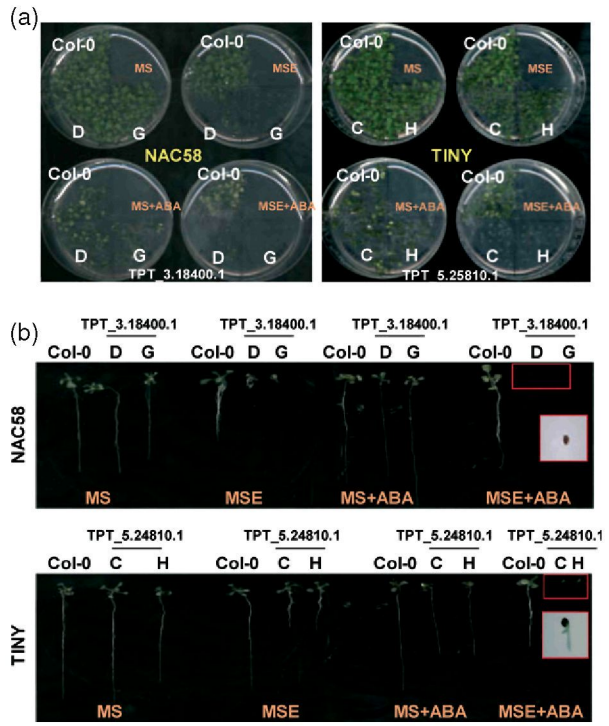

Figure 4. Use of TPT lines to screen for altered ABA sensitivity in seed germination assays.
(a) Wild-type Col–0 and TPT lines were sown in MS medium (MS), and media supplemented with 10 μM β–estradiol (MSE), 1 μM ABA (MS + ABA) or both (MSE + ABA). Seed germination and seedling establishment were analyzed at days 3 and 10 after sowing, respectively.
(b) Elongation of the primary roots was assessed by growing seedlings in vertically oriented square Petri dishes in the above mentioned media, as indicated. Photographs were taken by day 14 after sowing. D, G, C and H correspond to different TPT lines for the indicated TFs.

seeds, some lines displayed significantly different phenotypes. Our screen of 774 TPT lines, corresponding to 312 different TFs, for ABA sensitivity in germination assays led to the identification of 10 TFs showing altered responses to ABA. Figure 4a shows the germination assays for two examples of selected TPT lines conditionally expressing either ANAC058 or a TF belonging to the AP2 TINY family. Each of these TPT lines showed hypersensitivity to ABA with certain differences between TFs. The β–estradiol-induced expression of *ANAC058* led to arrested development after germination in the absence of ABA, whereas in the presence of ABA *ANAC058* caused arrested germination (Figure 4a,b). Conversely, β–estradiol-induced conditional expression of the *AP2/TINY* TF did not significantly affect the germination potential (Figure 4a), but instead induced shorter roots (Figure 4b). Moreover, combined treatment with β–estradiol and ABA did not block germination, but led to the severe arrest of root and shoot growth (Figure 4a,b). Therefore, expression of both TFs led to hypersensitivity to ABA, but affecting different processes.

We have also performed screenings using TPT seedlings, which were grown for 5 days on MS media, and then transferred to MS medium supplemented with 10 μM β–estradiol. Searching for seedlings with retarded or blocked root or shoot growth upon transfer to β–estradiol-supplemented media led to the identification of TPT lines with visible differential phenotypes, both in roots and shoots. As an example of TPT lines displaying severe root growth arrest upon β–estradiol treatment, Figure 5 shows the phenotype of a TPT line conditionally expressing *RAP2.10/DEAR4* cDNA encoding one of the TFs of the RAP2 family. Seedlings of the TPT_4.36900.1A line grown in MS medium for 5 days and then transferred to MS supplemented with β–estradiol displayed a complete arrest of root elongation (Figure 5a), which correlated with an 11–fold accumulation of *RAP2.10/DEAR4* mRNA (Figure 5b). A more detailed analysis of seedlings with arrested root growth highlighted a brown region in the hypocotyl–root transition zone, which is especially intense in the pericycle and vascular tissue of the upper part of the primary root (Figure 5c). The brown zones of TPT_4.36900.1A seedlings contained numerous dead cells, as demonstrated by Trypan blue staining (Figure 5d); however, it is noteworthy mentioning that cells stained with Trypan blue were, in turn, excluded from propidium iodide staining of DNA (Figure 5e), suggesting that cells in the brown area kept their plasma membrane intact. Dead cells with intact plasma membranes are common features of apoptotic cells in animals (Darzynkiewicz *et al.*, 1992). Interestingly, we found that brown areas of the hypocotyl–root transition zone were enriched in lipids, as demonstrated by the strong staining with Nile red (Figure 5f). It has been reported recently that sphingolipids can regulate apoptotic-like programmed cell death in plants (Alden *et al.*, 2011), which suggests the possibility that RAP2.10/DEAR4 could be involved in regulating cell death through lipid signaling. Interestingly, we have demonstrated that the cell death phenotype in the hypocotyl–root transition zone was specifically triggered by the expression of the *RAP2.10/DEAR4* gene in two independent TPT lines, but not in other TPT lines expressing related genes of the family, such as *RAP2.6*, *RAP2.6L* and *RAP2.12* (Figure 5g).

The phenotypic screenings described above demonstrated that TPT lines conditionally expressing TFs in a β–estradiol-inducible fashion are useful tools to search for TFs involved in a specific trait of interest. In fact, the available TPT lines are being systematically screened for different developmental and stress-related traits by members of the TRANSPLANTA consortium (for more details, see http://bioinfogp.cnb.csic.es/transplanta_dev/). The screens that have already been performed or are in progress include responses to phytohormones and plant growth regulators such as ABA, jasmonates, gibberellins, salicylates and nitric oxide. TPT lines are also being screened for

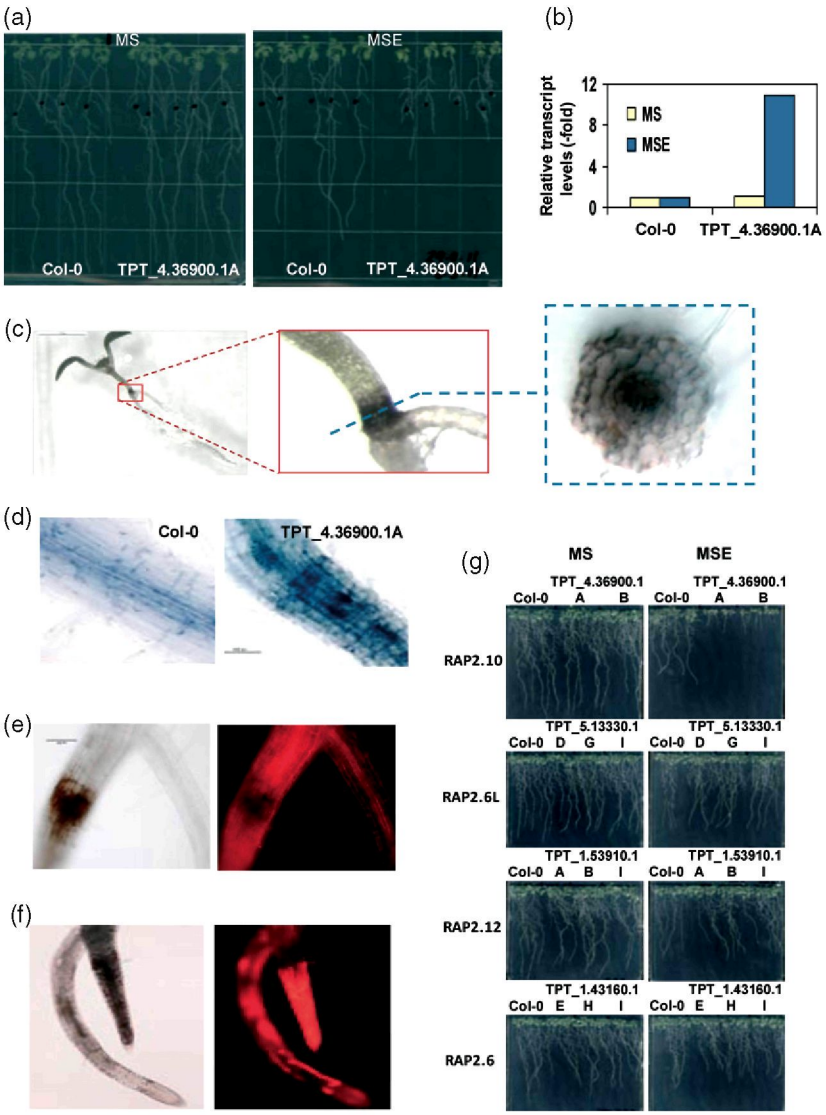

Figure 5. β–Estradiol induced root growth arrest in TPT lines conditionally expressing RAP2.10/DEAR4.

(a) Col–0 and TPT_4.36900.1A seeds were sown in vertical Petri dishes containing MS medium, and then grown for 5 days. The length of the primary roots at that time is indicated by black dots. Then, seedlings were transferred either to new MS medium or MS medium supplemented with 10 μM β–estradiol (MSE), and grown for an additional 14 days.

(b) Relative *RAP2.10/DEAR4* transcript accumulation was determined by RT-qPCR using total RNA isolated from Col–0 and TPT_4.36900.1A seedlings treated (MSE) or untreated (MS) with 10 μM β–estradiol, as indicated. Relative values to levels detected in untreated samples represent the means of three independent replicates ± standard deviations.

(c) A 5–day-old TPT_4.36900.1A seedling treated with β–estradiol showing brown necrotic-like areas in the hypocotyl to root transition zone. A close-up image of the necrotic area and a cross section of this zone are shown.

(d) Hypocotyl–root transition zones of Col–0 and TPT_4.36900.1A seedlings treated with β–estradiol were stained with Trypan blue, which stains only vascular bundles in Col–0 and vascular tissue and dead cells in TPT_4.36900.1A seedlings.

(e) Staining with propidium iodide and (f) Nile red of the hypocotyl–root transition zone of TPT_4.36900.1A seedlings shows the exclusion of the propidium stain and the enrichment of lipids in the dead cells.

(g) Elongation of the primary roots was assayed as described in (a) in two independent TPT lines expressing *RAP2.10/DEAR4* and three TPT lines for each of other three members of the RAP2 family, including *RAP2.6*, *RAP2.6L* and *RAP2.12*.

developmental traits such as seed germination, photomorphogenesis, leaf size and morphology, flowering time, fruit formation and senescence; however, the potential of TPT lines for phenotypic screening remains to be systematically explored, and we believe that the availability of these lines through NASC-mediated distribution for the researchers working with Arabidopsis will help to advance our knowledge on TF functions in many different biological processes of plants.

DISCUSSION

Many different approaches have been undertaken by the Arabidopsis research community to characterize the function of TFs. With the implementation of genomics, the functional characterization of TFs has benefited from the application of reverse genetic tools based on the exploration of T–DNA insertion mutant collections (FLAG, Brunaud *et al.*, 2002; MPIZ, Ríos *et al.*, 2002; SALK, Alonso *et al.*, 2003; GABI, Rosso *et al.*, 2003; WiscDsLox, Woody *et al.*, 2007), as well as other RNAi- (Agrikola, Hilson *et al.*, 2004) and artificial microRNA (amiR)-based gene silencing tools (Schwab *et al.*, 2006). Approaches based on the overexpression of TF coding genes are more limited in number, however, and have not been performed in a systematic way, but have instead focused on specific TF families (Weiste *et al.*, 2007). Systematic approaches using a selection for gain-of-function phenotypes largely relied on the use of T–DNA activation tagging technology (Weigel *et al.*, 2000; Tani *et al.*, 2004). Less successful results obtained from overexpression strategies are often related to deleterious effects or off-target effects triggered by the constitutively enhanced expression of TF coding genes. To fill the gap of genomic tools regarding the overexpression of TFs in a systematic approach, but avoiding the detrimental

effects of constitutive overexpresion, our strategy has been the production of transgenic lines conditionally expressing a collection of 949 Arabidopsis TFs. Conditional expression of transgenes has been approached with various strategies based on promoter sequences of genes responding to either chemicals, such as ethanol, dexamethasone and β–estradiol, or physical stimuli, such as heat shock (Zuo and Chua, 2000; Borghi, 2010). In this work, we decided to use the binary pER8GW vector (Papdi *et al.*, 2008) derived from the pER8 vector carrying an estrogen receptor-based chemical-inducible system. The *trans*-activating activity of the chimeric XVE factor, the expression of which is controlled by the strong constitutive promoter G10-90, is strictly regulated by the application of estrogens such as β–estradiol, in the case of pER8 (Zuo *et al.*, 2000). It has been reported already that this system of conditional expression is suitable for identifying stress regulatory genes (Papdi *et al.*, 2008; Rigó *et al.*, 2012).

Our approach based on the generation of individual transgenic lines is clearly more time and labour consuming than other strategies, such as those based on the generation of libraries of overexpressing plants by *Agrobacterium*-mediated plant transformation with pools of TF cDNAs (Weiste *et al.*, 2007). The production of TPT lines, however, represents a great advantage from both technical and practical points of view. The availability of several independent homozygous transgenic lines for every TF represents a widely available and stable tool, not only for genomic approaches but also for research focused on particular TFs. The availability of individual homozygous transgenic lines also opens up the possibility of crossing lines for further use of double heterozygous lines when the simultaneous conditional expression of two TFs needs to be achieved. We have checked that heterozygous lines containing only one copy of the transgenes also behave as conditional overexpressing plants.

A group of randomly chosen TPT lines was tested for transgene expression and found to express TFs stringently upon β–estradiol treatment, which permits the use of untreated TPT lines as reliable controls in every experiment. Although, in general, TPT lines treated with β–estradiol accumulate high levels of TF transcripts, the expression level is variable among different lines. The existence of lines with lower transgene expression may in fact represent an advantage for TFs causing deleterious effects when expressed to high levels. We have also found that a very low proportion of randomly selected TPT lines did not express the corresponding TF upon treatment with β–estradiol. On the other hand, we also realized that some of the TPT lines expressed TFs even in the absence of β–estradiol treatment, thus suggesting pER8GW-based clones may sometimes lead to leaky expression. Whether or not the expression of a TF can interfere with the function of the XVE repressor in Arabidopsis remains intriguing question for further studies. Despite these few exceptions, we believe that the vast majority of TPT lines behaved as conditional overexpressing plants, as expected.

In the frame of the TRANSPLANTA project, the TPT lines have been extensively tested in phenotypic screens that resulted in the successful identification of TFs involved in the regulation of a variety of biological processes. Here, we described some representative screens that were designed to identify TFs that modulate seed germination and seedling development by growing plants continuously in the presence of β–estradiol, providing constitutive overexpression or providing truly conditionally induced expression at different developmental stages to search for phenotypic alterations under different conditions and in different plant organs. The results obtained in different screens performed by the TRANSPLANTA project so far illustrate the potential of TPT lines to be applied successfully in many additional kinds of genetic screens. This is especially true for every screen designed for the use of young seedlings, where no limitation for the β–estradiol uptake is observed in our hands; however, the situation might be different when using adult plants grown in soil, where we detected some limitations in the application and subsequent uptake of β–estradiol, which will have to be addressed to optimize the use of this system for specific applications. In this regard, the application of β–estradiol should be optimized in each case, as well as the required dosages and the rate of treatments. This sort of work is being implemented within the TRANSPLANTA consortium for a large number of different screens. Those already performed that led to the identification of TFs regulating multiple developmental and stress-related processes in Arabidopsis will be soon reported.

Besides their application in phenotypic screens, TPT lines can also be useful tools for researchers interested in the study of particular TF families. The collection of TFs conditionally expressed in TPT lines has a wide coverage of some of the most important TF families in Arabidopsis. Families such as TCP, NF–Y, GRAS, MADS, MYB, NAC and WRKY, some of which contain the largest number of members (Riechmann *et al.*, 2000), are represented with a coverage of between 50 and 80% in the TPT collection. As an example, the TPT collection includes lines corresponding to each of the six members of the YABBY subfamily of Arabidopsis TFs (Bowman, 2000). We thus believe that TPT lines will also be valued as a complementary tool for researchers studying a particular TF or TF family using different genetic or genomic approaches. In such approaches, specific reporter constructs of TF-regulated target genes can easily be combined with particular TFs, e.g. by genetic crosses. As mentioned above, homozygous seeds of the TPT lines have been distributed to the international community through NASC, with the only requirement from TRANSPLANTA being to cite this article in the reference list of any publication derived from the use of TPT lines.

EXPERIMENTAL PROCEDURES

Plant material and genetic transformation

Arabidopsis thaliana wild-type accession Col-0 and TPT transgenic lines were grown on either MS medium supplemented with 1% sucrose or soil under long-day photoperiodic conditions of 16 h of light/8 h of dark at 21°C. Seeds were surface sterilized by exposure to chlorine fumes produced from bleach-treated hypochlorite solution, sown on media and stratified for 3 days at 4°C before transferring to photoperiodic light conditions.

The collection of TPT TFs in the pER8GW binary vector was used to transform the C58 *Agrobacterium tumefaciens* strain, and transformed agrobacteria were further selected on LB media supplemented with 50 mg L^{-1} rifampicin and 100 mg L^{-1} spectinomycin. Wild-type Col-0 plants were then transformed by the floral-dip method (Clough and Bent, 1998). Transformed seedlings were selected in MS media supplemented with 20 mg L^{-1} hygromycin. Transformation conditions were optimized to render a low (0.15–0.20%) transformation yield but a high proportion of single T-DNA insertion lines, which proved to be an important determinant for the execution of a high-throughput production of homozygous transgenic lines in this work.

Production and quality control tests of TF included in the TPT collection

Sequence-verified TF entry clones were recombined into the pER8GW vector, a Gateway version of β-estradiol-inducible expression vector pER8 (Zuo *et al.*, 2000), constructed by Bekir Ülker (Max-Planck Institute for Plant Breeding Research, Cologne), which carries an attR1 and attR2 recombination cassette between *Xho*I and *Spe*I cloning sites (Papdi *et al.*, 2008). Every pER8GW:TF clone was full-length sequenced before being used to transform Arabidopsis. Most TF entry clones were previously generated in the REGIA and REGULATOR consortiums (Paz-Ares, 2002; Castrillo *et al.*, 2011), and the collection was expanded with different TFs obtained from other public cDNA depositories (ABRC, (http://abrc.osu.edu/index.html), Peking-Yale Joint Center collection (http://www.pyc.pku.edu.cn, cited in Gong *et al.*, 2004) and Génoscope (http://www.genoscope.cns.fr/Arabidopsis). A GUS-GFP fusion was also cloned in the same vector to use it as a control of β-estradiol induction.

Treatments with β-estradiol and ABA, and analysis of TF transcript levels

β-Estradiol (E-8875; Sigma-Aldrich, http://www.sigmaaldrich.com) was added to MS media at a final concentration of 10 μM, unless otherwise indicated. ABA (A-1049; Sigma-Aldrich) was added to MS media at a concentration of either 0.3 or 1 μM, as indicated.

Transgene expression analysis in β-estradiol-treated and control untreated wild-type and TPT lines was performed by RT-qPCR with specific primers for each TF tested (Table S4). Transgene expression in the TPT reporter pER8GW-GUS:GFP lines was analyzed by staining for β-glucuronidase activity of the *uidA*/GUS reporter, by fluorescence GFP microscopy of seedlings or by western blot of total protein extracts with monoclonal anti-GFP antibody (632380; Clontech, http://www.clontech.com).

Seed germination and seedling growth assays of TPT lines

Stratified wild-type and TPT seeds were sown on MS medium free from (control) or containing β-estradiol. Germination rates were calculated at the indicated times as the percentage of seeds with radicle emergence through a broken seed coat. Root and hypocotyl lengths were measured using seedlings grown in vertically or horizontally oriented square Petri dishes, after scanning with ImageJ. Rosette diameters were also measured on scanned images of seedling populations with ImageJ (rsbweb.nih.gov/ij/index.html). The determination of anthocyanin levels was carried out as previously reported (Rabino and Mancinelli, 1986). When indicated, seedlings were stained with Trypan blue, propidium iodide and Nile red, as described previously (Greenspan *et al.*, 1985; Running *et al.*, 1995; Peterhänsel *et al.*, 1997).

ACKNOWLEDGEMENTS

This work has been performed in the frame of the TRANSPLANTA consortium, which is dedicated to the functional analysis of Arabidopsis TFs (http://bioinfogp.cnb.csic.es/transplanta_dev). We thank Toño García and Juana Monterrey for bioinformatic and administrative support, respectively. The REGIA cDNA clones were transferred into pER8GW by our collaborators at the Max-Planck Institute for Plant Breeding Research (Cologne, Germany) and the Institute of Plant Biology, Biological Research Center of the Hungarian Academy (BRC, Szeged, Hungary) within the DFG (Deutsche Forschung Gemeinschaft) project 436 UNG 113/172/0-1. This research was supported by the CONSOLIDER TRANSPLANTA CSD2007-00057 grant from the Ministerio de Ciencia e Innovación (MICINN), and by BIO2011-27526 and BIO2010-15589 grants from the Ministerio de Economía y Competitividad (to J.L. and J.A.J., respectively). The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Expression pattern of pER8GW:GUS-GFP reporter lines.

Figure S2. Quantification of the phenotypes of TPT lines conditionally expressing TT2/MYB123, MYB26 and PIF4.

Table S1. Name and affiliation of members of the TRANSPLANTA consortium involved in the construction of the cDNA library and the identification of homozygous TPT lines.

Table S2. List of Arabidopsis transcription factors encoding genes cloned in the pER8GW binary vector used to transform Col-0 plants in this work.

Table S3. TPT homozygous lines, conditionally expressing TFs under a β-estradiol-inducible system, which have been already deposited in the NASC seed bank.

Table S4. Primers used for RT-qPCR analysis of gene expression.

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