Arabidopsis thaliana in vitro shoot regeneration is impaired by silencing of *TIR1*

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Abstract

Arabidopsis shoots regenerate from root explants through a two-step process consisting of pre-incubation on an auxin-rich callus induction medium (CIM), followed by transfer to a cytokinin-rich shoot induction medium (SIM). The auxin receptor gene *TIR1* was up-regulated when explants were transferred to SIM. The CIM pre-incubation is required for its up-regulation. The *tir1-1*, *TIR1* knockdown mutant, reduced the efficiency of shoot regeneration in tissue culture, while its over-expression mutant significantly improved efficiency. *TIR1* promoter::GUS fusion analysis demonstrated that *TIR1* expression was in the shoot and the newly emerging leaves. After 10 d on SIM, several cytokinin related genes (*CDKB1;1*, *CKS1*, *IPT4* and *ARR15*), which associate with shoot regeneration, were up-regulated in plants over-expressing *TIR1* and some of these were down-regulated in the *tir1-1* mutant. Thus, *TIR1* appears to be involved in regulating shoot regeneration.

Additional key words: auxin receptor gene, auxin-rich medium, cytokinin related genes, cytokinin-rich medium, root explants.

Introduction

Shoot regeneration from *in vitro* cultured *Arabidopsis thaliana* is routinely achieved from root and/or hypocotyl explants *via* indirect organogenesis (Valvekens *et al*. 1988, Che *et al*. 2002). Initially, callus is induced by culturing on an auxin-rich induction medium, and this is then transferred to a cytokinin-rich medium to encourage shoot formation (Che *et al*. 2006). During the pre-CIM incubation phase, when cells de-differentiate and proliferate, they acquire the competence to respond to shoot induction signals (Cary *et al*. 2001). However, new research has found that protuberances of cell mass initiated on CIM are in fact not true calli but organized structures displaying many root characteristics (Atta *et al*. 2009, Sugimoto *et al*. 2010). These protuberances are then able to develop into shoots once they have been hormonally induced to do so (Ozawa *et al*. 1998, Yamaguchi *et al*. 2003, Che *et al*. 2006). The CIM pre-incubation phase appears necessary for the efficient regeneration of shoots (Che *et al*. 2007). This protocol is also applicable for shoot regeneration of other plant species, such as *Brassica napus* (Tang *et al*. 2010), *Picrorhiza scrophulariiflora* (Bantawa *et al*. 2011) and others.

Cytokinin is important for shoot regeneration (Skoog and Miller 1957). In *A. thaliana*, the disruption of certain cytokinin synthesis and signaling pathway related genes, such as *ISOPENTENYLTRANSFERASEs* (*IPTs*), *CYTOKININ-INDEPENDENTs* (*CKIs*) and the set of *RESPONSE REGULATORs* (*ARRs*) leads to an alert in shoot formation efficiency (Kakimoto 1996, Hwang *et al*. 2001, Che *et al*. 2002, Sun *et al*. 2003). Over- expression of *CKI1* promotes green-callus and shoots formation, even in the absence of exogenously supplied cytokinin. Expression of *ARR15* is considered to be a marker for the acquisition of competence, and plays important roles in shoot regeneration (Che *et al*. 2007, Buechel *et al*. 2010). Additional, cyclin-dependent kinase (CDK) activity is a major determinant of cell differentiation, and is required to ensure the *in vitro* development of organs (Yamaguchi

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; GUS - β-glucuronidase; IAA - indole-3-acetic acid; 2-IP - 2-isopentenyladenine; MES - methylester sulfonate, X-Gluc - 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

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et al. 2003). Also, these genes are related to cell cycle regulation, for example, B-type *CDKs* (*CDKB1* and *CDKB2*) regulates cell cycle progression to the mitotic phase (Joubès *et al*. 2000). Over-expression of *IPTs* increases cytokinin levels to enhance local transcription of cell cycle related genes, particularly the cytokinin-responsive D-cyclins (Yanai *et al*. 2005). Thus, the effects on shoot regeneration of a number of cytokinin-related genes are well characterized. Previous research showed that auxin triggers organogenesis *in vivo* and in *vitro* (Dubrovsky *et al*. 2008, Pernisová *et al*. 2009). However, the participation of auxin-related genes in shoot regeneration still remains unclear.

The auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) mediates auxin degradation and releases the repression of a number of auxin-responsive genes in *A. thaliana* (Dharmasiri *et al*. 2005a, Quint *et al*. 2006). Its sequence is homologous to those of the three F-box proteins AFB1, AFB2 and AFB3 (Dharmasiri *et al*. 2005b). The *tir1* mutant is compromised with respect to both hypocotyl elongation and lateral root formation (Ruegger *et al*. 1998), and the presence of the wild type allele is necessary for normal somatic embryogenesis (Su *et al*. 2009). In this study, the goal was to investigate the effects of *TIR1* on shoot regeneration capacity, as well as potential molecular mechanisms.

Materials and methods

All *Arabidopisis thaliana* L. lines were based on the Col-0 ecotype. The seeds were germinated and allowed to grow for 7 d on Murashige and Skoog (1962; MS) medium in a growth chamber at temperature 22 °C, relative humidity of 60 %, 16-h photoperiod and irradiance of 120 μmol m^{-2} s^{-1}. Root and hypocotyl segments were transferred to callus induction medium (CIM), which was formulated from B5 (Gamborg *et al*. 1968) medium supplemented with 0.5 g dm^{-3} methylester sulfonate (MES), 2.2 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 μM kinetin and 0.8 g dm^{-3} agar. They were initially cultured on CIM for 4 d (PreCIM period), and then transferred to either shoot induction medium (SIM) containing 5.0 μM 2-isopentenyladenine (2-IP) and 0.9 μM indole-3-acetic acid (IAA), or onto fresh CIM. Seeds of *pTIR1::GUS*, *tir1-1* and *35S::TIR1* were obtained from Enrique Ibarra-Laclette (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico).

Total RNA was isolated using the *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA), and treated with RNase-free DNase I to remove contaminating genomic DNA. The first cDNA strand was synthesized from 3 μg total RNA in a 0.04 cm^3 reaction mixture using *Superscript II* reverse transcriptase (*Invitrogen*), and quantitative real time polymerase chain reaction (qRT-PCR) was performed using a *Bio-Rad* (USA) detection system with *SYBR Green I* master mix. Primer sequences were designed using *Primer 3* software (Table 1). All cDNA samples were analyzed in triplicate, and the cDNA was derived from two sets of independent experiments. Thermal cycling conditions consisted of 40 cycles of 94 ºC for 20 s, 60 ºC for 30 s, 72 ºC for 20 s, and a final extension at 72 ºC for 5 min. A tubulin gene was included in the assays as an internal control for normalizing the variations in cDNA amounts used. The relative changes in gene expression were quantified according to Xue *et al.*(2010).

To examine protuberances on root explants, the explants incubated on CIM for 6 and 14 d were viewed by stereo microscopy (*Nikon SMZ1500*, Tokyo, Japan).

To assess GUS activity in transgenic tissue carrying *pTIR1::GUS*, the tissue was first immersed for 20 min in 90 % acetone on ice, washed twice in 50 mM sodium phosphate, pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and then incubated at 37 °C for 12 - 16 h in the same solution to which 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc, obtained from *Duchefa*, Haarlem, The Netherlands) had been added. The material was then bleached by passing through an ethanol series (15 - 80 %), and inspected by light microscopy, as described previously (Seo *et al*. 2009).

Table 1. Real-time PCR primers used for analyzing expression of genes.

Gene name	Forward and reverse primer sequences (5' to 3')
TIR1	TACGAGAGAGTGATGTTGACGACG and CTCAGATGCTAAGCAAGATATATTGAG
ARR15	CAATGTATGATAGAAGGAGCAGAGG and GATGAATCAATGTCGTTTTGTAGGA
CDKB1;1	GCCTCTTGAGCCTTTTTTGATT and CACGACCAAGACCCAAATCAG
CKS1	TCTCTCCGAAAACGAATGGC and GCATTATGTGCGGCTCAGGT
IPT4	GGAGAAGTCACAGCGGCAGA and TCGGAGCAGATTGAGCCCT
FUS3	TAAGAATGGAAGATTTGGACGGT and ATGAGCATTCACAAAATCGCC
LEC2	CATCACTTCTTGCCTCCGTTT and GGATTACCAACCAGAGAACCGA
TUB	TTTGTGCTCATCTTGCCACGGAAC and CTCAAGAGGTTCTCAGCAGTACC

Results

TIR1 was down-regulated during days 2 - 7 of culture on callus induction medium (CIM), but by day 10, its level of expression had returned to the initial level. After 10 d of culture on shoot induction medium (SIM), the gene was gradually up-regulated to reach > 1.5 fold the level expressed after 10 d culture (Table 2). Furthermore, when root explants were directly transferred to SIM without CIM incubation, the up-regulation of *TIR1* was not found, but there was some down-regulation (Table 2).

The localization of its up-regulation during culture on SIM was tracked by expression of GUS in a transgenic plant carrying *pTIR1::GUS*. The signal was high in the root vasculature, especially at the root tip, at the beginning of the period of culture on CIM (Fig. 1*A*). During the preCIM culture and the early phase of CIM culture, the level of GUS expression dramatically decreased, and was only detectable at the root tip. As the period of CIM culture was prolonged to 10 d, GUS expression spread

Table 2. Real-time PCR assay of *TIR1* expression level during pre CIM, CIM and SIM incubation. PreCIM - the root explants were pre-incubated on CIM for 4 d and then separately transferred to SIM (for shoot regeneration) or fresh CIM (for callus induction). SIM^a - first pre-incubation on CIM for 4 d, and then incubation on SIM for different number of days; SIM^b - without pre-incubation on CIM, just incubation on SIM for different number of days. Relative amounts were calculated and normalized with respect to expression of *TIR1* at preCIM 0 d. Data are the means ± SD of three assays.

preCIM 0 d	preCIM 4 d	CIM 7 d	CIM 10 d	SIM^a 4 d	SIM^a 7 d	SIM^a17 d	SIM^b 4 d	SIM^b 7 d
1.00±0.24	0.73±0.11	0.62±0.04	0.86±0.06	1.13±0.13	1.48±0.10	1.57±0.06	0.86±0.09	0.55±0.10

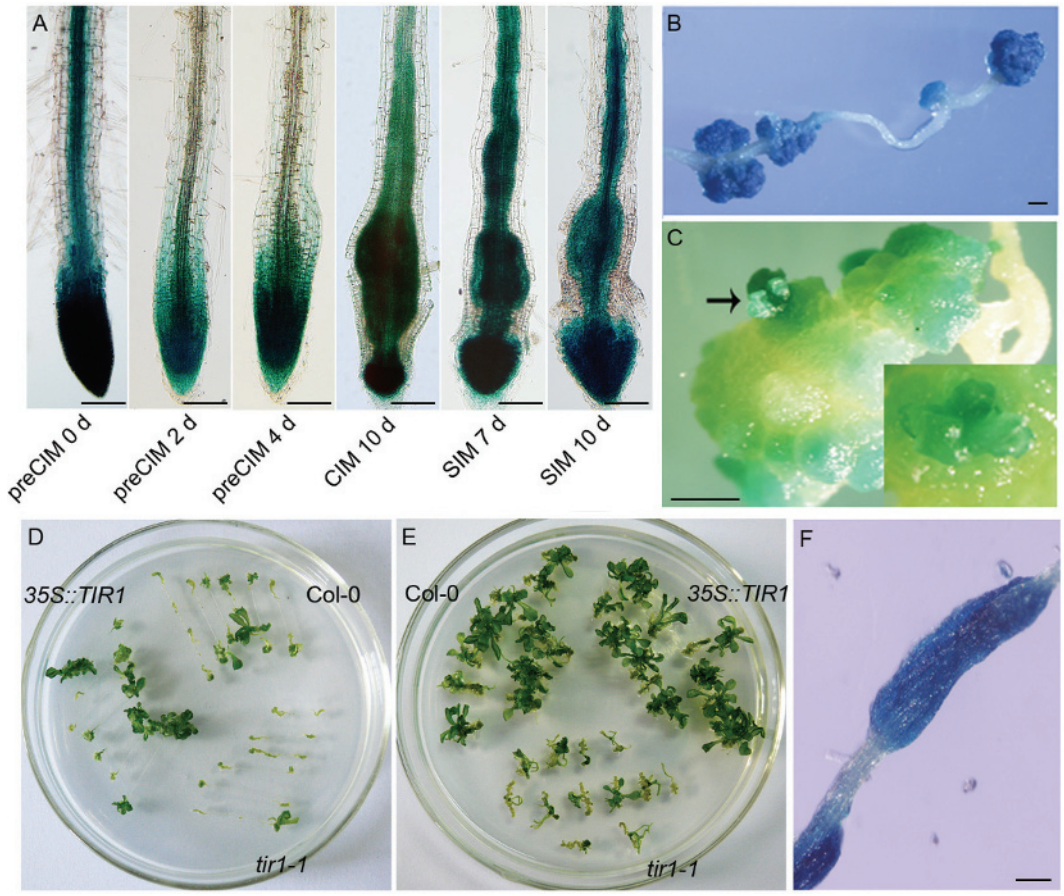

Fig. 1. Root explants from *pTIR1::GUS* seedlings were subjected to shoot regeneration conditions and stained for GUS expression at the times indicated. The root explants were first pre-incubation on CIM before transferring to SIM or fresh CIM. PreCIM - pre-incubation on CIM, *bar* = 100 μm (*A*). *pTIR1::GUS* root explants cultured on SIM for either 16 (*B*) or 18 (*C*) d with pre-incubation on CIM for 4 d. *Arrow* in *C* indicates *TIR1* expression, *bar* = 100 μm. Shoot regeneration from Col-0, *35S::TIR1* and *tir1-1* root explants (*D*) and hypocotyl explants (*E*). *TIR1* expression was localized to the protuberances developing from the root explants, *bar* = 100 μm (*F*).

Table 3. Tissue-specific expression of *TIR1* in plants. IFM - inflorescence meristem region; ST - stem; F - flower; RL - rosette leaf; CA - cauline leaf; SI - silique; R - root. Relative amounts were calculated and normalized with respect to expression of *TIR1* in SA. Data are the means ± SD of three assays.

IFM	ST	F	RL	CA	SI	R
1.00±0.13	1.12±0.03	0.47±0.10	1.98±0.45	1.84±0.32	0.42±0.01	1.02±0.20

from the root tip to elsewhere in the explant. Once the explant had been transferred to SIM, GUS expression markedly increased in the sites of cell proliferation (Fig. 1*A*). After 16 d of culture, the *TIR1* signal was particularly concentrated in protuberances on the verge of shoot regeneration (Fig. 1*B*). By 18 d of culture, the signal had become localized in places where new shoots or leaves were initiated (Fig. 1*C*).

A systematic assessment of *TIR1* expression within 28-d-old plants and over the plant life cycle (from 7 - 35 d) revealed that the highest levels of *TIR1* expression occurred in rosette and cauline leaves, while in the stems, roots and inflorescence meristems the level was intermediate, and in the flowers and siliques it was low (Table 3). In addition, it was expressed throughout the whole plant life, but was highest in younger seedlings and gradually decreased with plant age (Table 4).

Table 4. Growth stage-dependent expression of *TIR1*. Relative amounts were calculated and normalized with respect to expression of *TIR1* in 7-d-old plants. Data are the means ± SD of three assays.

7 d	14 d	21 d	28 d	35 d
1.00±0.15	0.78±0.24	0.76±±0.02	0.57±0.13	0.31±0.01

After 16 d of culture on SIM, 4.28 ± 1.81 shoots at the incision of each explant were regenerated in *35S::TIR1*, while only 0.33 ± 0.54 in the *tir1-1* mutant. The performance of the Col-0 wild type was intermediate (1.67 ± 0.82) (Fig. 1*D*). Hypocotyl segments of *35S::TIR1*, *tir1-1* and Col-0 (5 mm long) were pre-cultured on CIM for 4 d and then transferred to SIM for 16 d. Shoot induction was more rapid from these explants than from root explants, as also noted by Ozawa *et al*. (1998). A mean of 7.5 ± 0.8 shoots per explant was induced from *35S::TIR1*, 6.7 ± 0.65 from Col-0, but only 2.3 ± 0.33 from *tir1-1* (Fig. 1*E*). These data indicated that shoot regeneration was impaired by silencing of *TIR1*.

Previous research indicated that the process of acquiring competence was gradual. Without any pre-culture on CIM, Che *et al*. (2007) showed that shoot regeneration was poor, but after 1 - 2 d of culture on CIM, the competence to form shoots developed rapidly, and increased for the next two days. To examine whether longer time CIM incubation can rescue the defects in *tir1-1* mutant, root explants from the Col-0 wild type, *35S::TIR1* and *tir1-1* plants were cultured on CIM for longer times. After 5 - 8 d on CIM and 16 d on SIM, more shoots were regenerated from *tir1-1* root explants than after only 4-d incubation on CIM, but the total remained only ~20 % of the shoot regeneration frequency achieved by Col-0 (Table 5).

Table 5. Effects of different CIM incubation periods on shoot number per root explant. CIM incubation period was varied between 4 and 8 d before transferring explants onto SIM for 16 d. Means ± SD of three experiments with 30 replicates in each.

CIM	Col-0	*35S::TIR1*	*tir1-1*
4 d	1.67±0.82	4.28±1.81	0.33±0.54
5 d	3.32±0.78	4.34±1.04	0.58±0.43
6 d	4.21±0.71	5.09±0.74	0.83±0.53
7 d	5.23±1.13	6.23±1.35	1.32±0.53
8 d	6.05±0.83	7.42±0.84	1.75±1.70

Table 6. Relative transcription levels of cytokinin and somatic embryogenesis-related genes in Col-0, *35S::TIR1* and *tir1-1* root explants on 10th day on SIM. Relative amounts were calculated and normalized with respect to expression of each gene in root explants of Col-0. Values were the means ± SD of three assays.

Genes	Col-0	*35S::TIR1*	*tir1-1*
CDKB1;1	1.00±0.50	2.04±0.02	0.71±0.31
CKS1	1.00±0.41	2.58±0.61	0.46±0.14
ARR15	1.00±0.20	2.15±0.44	0.42±0.09
IPT4	1.00±0.05	1.67±0.06	0.67±0.04
LEC2	1.00±0.05	1.56±0.10	0.26±0.11
FUS3	1.00±0.24	1.70±0.28	0.71±0.03

The response of root explants to variation in the period of culture on CIM was assessed to determine whether *TIR1* expression affected protuberances formation *in vitro*. After 6 d of culture on CIM, both Col-0 and *35S::TIR1* formed rather large protuberances, while the *tir1-1* structures remained smaller and fewer (Fig. 2*A*). This maximum difference was after 14 d of culture on CIM (Fig. 2*B*). The activity of the *TIR1* promoter was visible in these protuberances, but not elsewhere in the explant after 10 d of culture on CIM (Fig. 1*F*).

The transcription in *35S::TIR1* and *tir1-1* of *CKS1*, *CDKB1;1*, *IPTs* and *ARRs* was compared after 10 d culture of root explants on SIM. That of *CDKB1;1*, *CKS1*, *IPT4* and *ARR15* was up-regulated in *35S::TIR1*, while *CKS1* and *ARR15* were significantly down-regulated in *tir1-1* (Table 6). This pattern was taken to indicate that either endogenous cytokinin synthesis or upstream cytokinin signaling was disturbed in both genotypes.

Expression of certain somatic embryogenesis related genes was also assayed, since *TIR1* expression is required for somatic embryogenesis, and these genes may also contribute to shoot regeneration. Both *LEC2* and *FUS3* were markedly up-regulated in the *35S::TIR1* explants and down-regulated in *tir1-1* ones (Table 6). Expression of *LEC2* was enhanced during the course of SIM culture, and its time course of expression was similar to that of *TIR1* (Table 7). Thus, effects of *TIR1* on shoot regeneration frequency may partly be caused by these genes.

Table 7. Real-time PCR assays of *LEC2* expression level during CIM and SIM incubation. Relative amounts were calculated and normalized with respect to expression of *LEC2* at CIM 0 day. Data are the means ± SD of three assays.

PreCIM 0 d	PreCIM 4 d	CIM 7 d	CIM 10 d	SIM 4 d	SIM 7 d	SIM 17 d
1.00±0.15	0.93±0.14	0.63±0.08	1.14±0.01	1.43±0.04	1.85±0.23	3.43±0.76

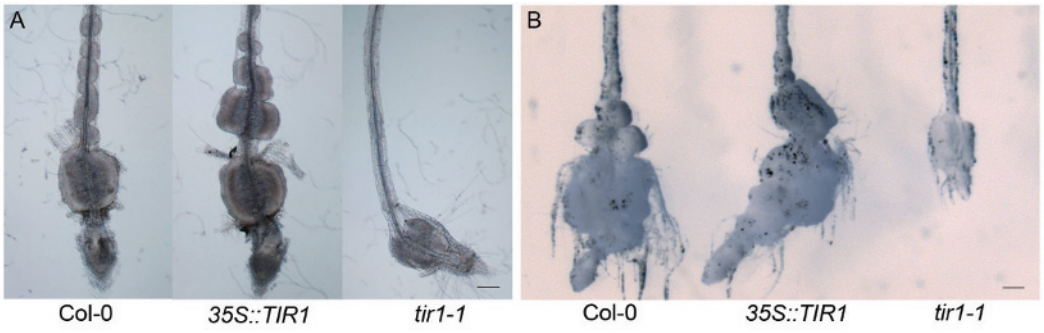

Fig. 2. Protuberances formed from Col-0, *35S::TIR1* and *tir1-1* root explants after 6 d (*A*) and 14 d (*B*) of culture on CIM, *bar* = 100 µm.

Discussion

Shoot regeneration is an essential component of *in vitro* propagation and genetic engineering, and thus its molecular basis has long been a topic for both basic and applied research. *TIR1* contributes to this process, since its silencing almost completely abolishes the capacity to regenerate shoots, while its over-expression improves regeneration efficiency (Fig. 1*D*, *E*). The loss of *TIR1* has only a modest effect on the auxin response and the development of *A. thaliana* (Dharmasiri *et al*. 2005b), which implies a level of *in vivo* functional redundancy amongst it and its homologues *AFB1*, *AFB2* and *AFB3*. Also, *in vitro*, the absence of either *AFB1* or *AFB2* expression has little effect on shoot regeneration capacity (data not shown). The behavior of the *tir1-1*, *afb1* and *afb2* mutants showed that abolished *TIR1* expression (as in *tir1-1*) and the ability to regenerate shoots was compromised.

TIR1 is auxin-inducible, however, it is negatively regulated on the auxin-rich medium and up-regulated on cytokinin rich medium (Fig. 1*A*; Table 2). This conflicting observation may be caused by special developmental contexts, because root explants had been cultured in CIM or SIM for a long time not several hours, and the cells occurred de-differentiated, proliferated and then re-differentiated. Thus the expression pattern of *TIR1* on CIM and SIM may not have been caused solely by auxin.

When cultured on CIM, explants cells initially de-differentiate and then proliferate (Cary *et al*. 2001), eventually forming a visible protuberances (Ozawa *et al*. 1998). Explants from *tir1-1* mutant plants showed a visible defect in the formation of protuberances on CIM (Fig. 2*A*,*B*). Expression of *TIR1*, as monitored by the GUS signal in the *pTIR1::GUS* transgenic plants, was localized in the protuberance cells as the period of CIM culture was extended (Fig. 1*F*). The implication drawn was that protuberance formation in root explants was mediated in some way by the TIR1 protein *in vitro*. In the *35S::TIR1* plants, not just *TIR1* but also the cytokinin related genes *CKS1*, *CDKB1*, *IPT4* and *ARR15* were up-regulated (Table 6), and some of these genes were down-regulated in *tir1-1*. All the genes were reported to be involved not only in shoot regeneration (Kakimoto *et al*. 1996, Hwang *et al*. 2001, Che *et al*. 2002, Sun *et al*. 2003), but also in cell cycle regulation (Joubès *et al*. 2000, Jasinski *et al*. 2005, Yanai *et al*. 2005). Thus, it is proposed that *TIR1* is related to the cell cycle and/or to the formation of root-like organized structures and shoot regeneration *in vitro*.

Certain somatic embryogenesis related genes, in particular *LEC2* and *FUS3*, were markedly induced by the *35S::TIR1* over-expression (Table 7). The expression profile of *LEC2* was rather similar to that of *TIR1* (Table 7). Stone *et al.* (2001) were able to show that *LEC* genes probably function downstream of auxin-induced somatic embryogenesis, while Su *et al*. (2006) reported that the *tir1-1* mutant formed an abnormal somatic embryo. Over-expression of *LEC2* induces somatic

embryo formation in seedlings and generates masses of shoots and roots (Stone *et al*. 2001). Thus, it is possible that these genes also contribute, at least partly, to overall shoot regeneration ability.

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