Expression of AtLEC2 and AtIPTs promotes embryogenic callus formation and shoot regeneration in tobacco

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Abstract

Background: LEAFY COTYLEDON 2 (LEC2) acts throughout embryo morphogenesis and maturation phase to maintain embryogenic identity. Our previous study stated that Arabidopsis thaliana LEC2 (AtLEC2) driven by glucocorticoid receptor-dexamethasone (GR-DEX) inducible system (AtLEC2-GR) triggers embryogenic callus formation in tobacco (Nicotiana tabacum).

Results: In this study, the adenosine phosphate isopentenyltransferase genes AtIPT3, AtIPT7 and the tRNA isopentenyltransferase gene AtIPT9 were overexpressed in the AtLEC2-GR transgenic background. In the AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR seedlings, high-quality embryogenic callus was obtained under the DEX condition, and the shoot regeneration efficiency was 2 to 3.5 folds higher than AtLEC2-GR alone on hormone free medium without DEX. Transcriptome analyses showed that up-regulated BBM, L1L, ABI3, and FUS3 might function during embryogenic callus formation. However, at the shoot regeneration stage, BBM, L1L, ABI3, and FUS3 were down-regulated and Type-B ARR were up-regulated, which might contribute to the increased shoot regeneration rate.

Conclusions: A novel system for inducing shoot regeneration in tobacco has been developed using the GR-DEX system. Induced expression of AtLEC2 triggers embryogenic callus formation and overexpression of AtIPT7 or AtIPT9 improves shoot regeneration without exogenous cytokinin.

Keywords: GR, Dexamethasone, AtLEC2, AtIPT7, AtIPT9, Embryogenic callus, Shoot regeneration, Transcriptome analysis

Background

Genetic improvement through the transgenic technology has been widely used for many crops, however, the transformation and regeneration of some crops were proved to be difficult and genotype dependent [1]. Establishment of a more efficient shoot regeneration system is of great significance for crop genetic improvement. Plant regeneration can be accomplished through somatic embryogenesis and organogenesis [2]. Traditional protocol for shoot regeneration is achieved by two steps: embryogenic callus initiation on the auxin-rich medium; shoot meristem formation on cytokinin-rich medium [3].

Transcriptional factors are known to play significant roles in plant cell differentiation and dedifferentiation. Overexpression of a number of transcriptional factor genes can improve somatic embryogenesis and enhance plant regeneration, such as LEAFY COTYLEDON 1 (LEC1) [4], LEAFY COTYLEDON 2 (LEC2) [5, 6], WUSCHEL (WUS) [7], BABY BOOM (BBM) [8] and AGAMOUS-LIKE 15 (AGL15) [9]. LEC1/LEC1-LIKE (LIL) with three B3 domain protein genes ABSCISIC ACID (ABA)-INSENSITIVE3 (ABI3), FUSCUA3(FUS3) and LEC2 is referred as LAFL network (LEC1/L1L, ABI3, FUS3 and LEC2) [10]. This network functions redundantly throughout the early embryo developmental process, embryo maturation and dormancy in a dose-dependent manner [11]. Ectopic expression of FUS3 and
AB13 enhances accumulation of embryo traits but without somatic embryogenesis [12, 13]. YUCCA4 (YUC4) encodes a protein that catalyzes the rate-limiting step in IAA biosynthesis [14]. LEC2 can interact with FUS3 and bind to the YUC4 promoter [15]. Ectopic expression of LEC2 rapidly activates YUC2 and YUC4 [15]. These observations suggest that the LEC2-induced embryogenic competence is tightly linked with the auxin. Furthermore, inactivation of gibberellic acids (GAs) biosynthesis enzymes or reduction of active GAs also enhances the embryogenic competence of tissue [16]. LEC2 directly activates the expression of AGL15 [9, 17]. AGL15 and FUS3 have been reported to decrease GAs contents through negatively regulation of gibberellin 20-oxidase1 (GA20ox1), GA3ox1 and GA3ox2 GAs biosynthesis enzyme encoding genes [13, 18]. Therefore, the embryogenic competence of LEC2 is also associated with GAs activity. In addition, the expression of LAFL genes is regulated at both chromatin level and transcriptional level. Two AINTEGUMENTA-LIKE (AIL) family transcriptional factors, BBM and PLETHORA2 (PLT2), may directly activate LAFL transcription [19]. VIVIPAROUS1/ABI3-LIKE 1 (VAL1) and chromatin remodeler PICKLE-RELATED 2 (PKR2) inhibit the LAFL gene expression [20–22]. CURLY LEAF (CLF), the member of Polycomb Repressive Complex 2 (PRC2) inhibits the LEC2 transcription [23, 24].

LEC2 can trigger vegetative to embryogenic transition, however, plant regeneration could not occur from embryogenic callus constitutively expressing LEC2 [5, 6]. The GR is a vertebrate steroid hormone receptor. It has been reported that the GR-DEX system is a good induction system in plants because DEX, a strong synthetic glucocorticoid, itself does not cause any pleiotropic effects in plants [25–27]. Under no DEX condition, transformation factor (TF)-GR as a cytoplasmic complex with heat shock protein (HSP90), and the binding of DEX to GR leads to the dissociation of HSP90, and causes nucleus localization of TF-GR [25]. GR, as a transcription factor, could also activate transcription of the glucocorticoid response elements (GREs) containing promoters, in the presence of a glucocorticoid [26–28]. Indeed, our previous study has reported that the tobacco plants with induced expression of AtLEC2 by the GR-DEX system (AtLEC2-GR) display an obvious somatic embryogenesis phenotype and shoots could be generated from the embryogenic callus under no DEX condition [6].

Cytokinins control cell division and cell differentiation, as well as shoot growth and apical dominance. It is well-known that the appropriate ratio of cytokinin and auxin promotes shoot formation [3]. Isopentenyltransferases (IPTs) catalyzes the rate-limiting step of the cytokinin biosynthesis [29]. IPT was initially found in Dictyostelium discoideum and Agrobacterium tumefaciens, it has been proved to convert adenosine-5′-monophosphate (AMP) and dimethylallyl pyrophosphate (DMAPP) into isopentenyladenine riboside 5′-monophosphate (iPMP) [30, 31]. Constitutive expression of IPT from the Ti-plasmid of A. tumefaciens significantly elevates the cytokinin levels in transgenic plants and results in excessive cytokinin abnormal phenotype [31, 32]. However, inducible expression of IPT from the Agrobacterium tumefaciens by the GR-DEX system can induce suitably elevated cytokinin levels and shoot formation [27]. In Arabidopsis, there are two types of isopentenyltransferases (IPTs): one type (AtIPT1 and AtIPT3 to AtIPT8) catalyzes adenosine phosphates (ATP/ADP or AMP) to react with DMAPP, another type (AtIPT2 and AtIPT9) catalyzes the isopentenylation of tRNA [33]. AtIPTs show diverse temporal and spatial expression patterns [34–36]. Overexpression of AtIPT4 enhanced shoot regeneration efficiency independent of external cytokinins [33]. Furthermore, the AtIPT8 gain-of-function mutant remarkably increases iPMP and Isopentenyl adenosine (iPA) levels, improves embryogenic callus formation and shoot regeneration [37]. The cytokinin response requires the participation of the hormone-dependent Cytokinin Receptor 1 (CRE1) and hormone-independent CYTOKININ-INDEPENDENT 1 (CKI1), the His-containing phosphotransfer factors (Arabidopsis thaliana histidine phosphotransfer proteins, AHPs), His kinases (HKs) and Arabidopsis response regulators (ARRs) [38]. These factors are involved in transferring of phosphoryl groups between their conserved His and Asp, to control gene expression and global physiological response [38–42]. Recent studies reveal that type-B ARRs (ARR1, ARR10 and ARR12) can activate the expression of WUSCHEL (WUS), to maintain the shoot apical meristem and axillary meristem. However, these proteins can negatively regulate YUCCAs (YUC1 and YUC4) to inhibit auxin accumulation [43–45]. However, shoot regeneration of the yuc1 yuc4 double mutant is dramatically decreased, suggesting that auxin is also indispensable in the determination of cell fate [43].

High regeneration and transformation efficiency are crucial for gene engineering-based crop genetic modification. Many studies have attempted to increase somatic embryogenesis and shoot regeneration rate through ectopic expressing key genes, such as LEC2, SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERKs), WUS and BBM [6, 7, 46–50]. Lowe et al. (2016) recently reported that co-expression of maize BBM and WUS2 genes significantly increases transformation frequency in commercial maize inbred lines, sorghum, sugarcane, and indica rice. Here, we described a simple and efficient regeneration system that combines the use of AtLEC2-GR and AtIPT7 or AtIPT9 overexpression. The system relied on the DEX-inducible expression of AtLEC2 to trigger
embryogenic callus formation and overexpression of AtIPT7 or AtIPT9 to promote shoot regeneration. This regeneration system enabled embryogenic callus formation under the DEX condition and shoot regeneration after the removal of DEX.

Results
Exogenous cytokinin promotes shoot regeneration from embryogenic callus

Induced expression of AtLEC2 under the DEX condition can generate embryogenic callus on the shoot apical meristem (SAM) in transgenic tobacco [6]. Shoots could be generated from the embryogenic callus on hormone-free MS medium (no DEX), but with a low regeneration efficiency [6]. In order to increase the shoot regeneration rate, exogenous 1-naphthylacetic acid (NAA) and 6-Benzylaminopurine (6-BA) were applied. We found that 6-BA could significantly promote shoot regeneration. The number of regenerated shoots was increased 1.2 to 2 folds when a low concentration of NAA was applied when compared with the hormone-free MS medium (Fig. 1 and Table 1). Remarkably, a 2.9 to 8.1 folds increase in the number of regenerated shoots was observed when the embryogenic callus treated with a low concentration of 6-BA compared with the hormone free MS medium (Fig. 1 and Table 1). We further found that the application of 0.05 mg/L NAA together with 1.0 mg/L 6-BA led to a maximum shoots regeneration rate (Fig. 1 and Table 1). These results demonstrated that exogenous cytokinin could significantly promote the shoot regeneration efficiency of embryogenic callus from the AtLEC2-GR transgenic line.

Generation of transgenic plants and phenotypic analysis

The prominent effect of exogenous cytokinin treatment encouraged us to promote the shoot regeneration efficiency from the AtLEC2-GR embryogenic callus through increasing endogenous cytokinin concentration. IPTs are key genes in the cytokinin biosynthesis pathway. AtIPT3, AtIPT7 and AtIPT9 were proved mainly expressed in phloem tissues or proliferating tissue of the growing seedling [36]. AtIPT3, AtIPT7 and AtIPT9 overexpressed vectors driven by the CaMV 35S promoter were constructed and then transformed into the AtLEC2-GR transgenic tobacco (AtIPT3-OE AtLEC2-GR, AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR, respectively) respectively by the Agrobacterium-mediated leaf disc transformation [51]. Totally, 13 AtIPT3-OE AtLEC2-GR, 15 AtIPT7-OE AtLEC2-GR and 18 AtIPT9-OE AtLEC2-GR independent transgenic lines were generated, respectively. As shown in Additional file 1: Figure S3 the AtLEC2, AtIPT3, AtIPT7 and AtIPT9 fragments were

![Fig. 1 Shoot regeneration from embryogenic callus derived from AtLEC2-GR lines with exogenous application of NAA and 6-BA. Embryogenic callus derived from AtLEC2-GR transgenic seedlings grown on MS medium with (0, 0.05, 0.1, and 0.2 mg/L) NAA and (0, 0.5, 1.0, and 2.0 mg/L) 6-BA. Embryogenic callus grown on MS medium with 0 mg/L NAA and 0 mg/L 6-BA were as control. Three independent experiments were performed, each experiment contains 5 replicates. Scale bars = 2 mm](image-url)
amplified by PCR using specific primers in the indicated transgenic lines. The result suggested that AtIPTs, AtIPT7 and AtIPT9 had been stably integrated into the genomes of AtLEC2-GR transgenic tobacco. In the subsequent experiment, three independent AtIPTs-OE AtLEC2-GR transgenic lines of each construct were selected for further studies.

Homozygous AtIPTs-OE AtLEC2-GR transgenic seeds were germinated on MS medium with or without the DEX induction. The comparisons of the AtIPTs-OE AtLEC2-GR lines with wild type and AtLEC2-GR lines were carried out. Under no DEX condition, no remarkable phenotype difference was observed among the lines of AtIPTs-OE AtLEC2-GR, AtLEC2-GR and wild type plants (Additional file 1: Figure S1a-S1e). The AtIPTs-OE AtLEC2-GR lines had slightly longer hypocotyls than the AtLEC2-GR and wild type seedlings at 10 DAG (days after germination), on the contrary, the primary root length of AtIPTs-OE AtLEC2-GR was 22.3, 34.6 and 16.9% shorter than the control seedlings, respectively (Additional file 1: Figure S1f). The AtIPTs-OE AtLEC2-GR plants grew well in the soil and more axillary buds could develop into shoots during the vegetative growth phase (Additional file 1: Figure S2a-S2d). In addition, we found that overexpression of AtIPTs dramatically increased the floral number at the reproductive stage (Additional file 1: Figure S2e-S2h).

When grown on MS medium with 20 μM DEX, we could not observe embryogenic callus formation on the SAM in all 13 AtIPT3-OE AtLEC2-GR lines (Fig. 2h, m, Additional file 1: Figure S5), while embryogenic callus appeared on the SAM of AtIPT7-OE AtLEC2-GR lines at 8–12 DAG (Fig. 2i, n, Additional file 1: Figure S5). Interestingly, compared to the AtLEC2-GR and AtIPT7-OE AtLEC2-GR plants, AtIPT9-OE AtLEC2-GR plants produced two pieces of embryogenic callus on the peripheral zone of SAM at 12 DAG and the normal growth of leaves was not affected (Fig. 2j, o, Additional file 1: Figure S5). The AtLEC2-GR plants had obviously shorter hypocotyls than wild type, but the AtIPTs-OE AtLEC2-GR plant hypocotyls were longer compared with the AtLEC2-GR background at 20 DAG (Fig. 2p left). AtIPT3-OE AtLEC2-GR and AtIPT7-OE AtLEC2-GR had longer primary roots than wild type, and AtIPT3-OE AtLEC2-GR had the longest primary root (Fig. 2p right). AtIPT9-OE AtLEC2-GR had the shortest primary root at 20 DAG (Fig. 2n, o, Additional file 1: Figure S5). Same as AtLEC2-GR, the AtIPT7-OE AtLEC2-GR seedlings had fleshy and unexpanded cotyledons, and the growth of seedling was ceased (Fig. 2l, n, Additional file 1: Figure S5). We also tested the phenotypes of all transgenic seedlings grown on MS medium with 50 μM DEX. Unexpectedly, no embryogenic callus was formed from 13 AtIPT3-OE AtLEC2-GR lines (Additional file 1: Figure S4b). Meanwhile, for the AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR transgenic lines, the embryogenic callus formation was the same as the 20 μM DEX condition (Additional file 1: Figure S4c-S4d).

We determined the trans-zeanin (tZ) -type cytokinin contents in the 20 DAG AtIPTs-OE AtLEC2-GR, AtLEC2-GR and wild type seedlings grown on MS medium with 20 μM DEX, but no significant change was observed (data not shown).

**Table 1 Shoot regeneration numbers on MS medium with exogenous hormones NAA and 6-BA.** Values are mean ± standard errors from 15 replicates. Three asterisks indicate statistically significant differences from the control (NAA contents 0 mg/l and 6-BA contents 0 mg/l) numbers (***P < 0.001, Student’s t-test)

| NAA contents (mg/l) | 6-BA contents (mg/l) | Shoot regeneration numbers |
|---------------------|----------------------|--------------------------|
| 0                   | 0                    | 7.7 ± 1.1                |
| 0                   | 0.5                  | 52.0 ± 2.5 ***           |
| 0                   | 1                    | 65.7 ± 4.0 ***           |
| 0                   | 2                    | 22.0 ± 2.5 ***           |
| 0.05                | 0                    | 17.5 ± 2.3 ***           |
| 0.05                | 0.5                  | 62.0 ± 4.3 ***           |
| 0.05                | 1                    | 69.5 ± 4.2 ***           |
| 0.05                | 2                    | 30.9 ± 1.9 ***           |
| 0.1                 | 0                    | 13.7 ± 2.2 ***           |
| 0.1                 | 0.5                  | 40.1 ± 2.6 ***           |
| 0.1                 | 1                    | 49.5 ± 2.7 ***           |
| 0.1                 | 2                    | 42.4 ± 1.8 ***           |
| 0.2                 | 0                    | 10.9 ± 2.6 ***           |
| 0.2                 | 0.5                  | 20.6 ± 1.8 ***           |
| 0.2                 | 1                    | 40.3 ± 2.1 ***           |
| 0.2                 | 2                    | 34.1 ± 3.0 ***           |

**Overexpression of AtIPTs promotes shoot regeneration efficiency**

Embryogenic callus derived from the 20 DAG seedlings of AtIPT7-OE AtLEC2-GR, AtIPT9-OE AtLEC2-GR and AtLEC2-GR under the 20 μM DEX induction were cultured on the hormone-free MS medium without DEX (Fig. 3a-c). Two days later, the proliferation of the embryogenic callus and many somatic embryo–like structures on the callus could be observed. Some of the somatic embryos could develop into shoots (Fig. 3). After 4 d, 3–5 shoots developed from AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR somatic embryos, but not from AtLEC2-GR callus (Fig. 3d-f, m). After 12 d, 3–14 shoots regenerated from the embryogenic callus derived from AtLEC2-GR, AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR transgenic plants (Fig. 3g-i, m). On average, 8 shoots could generate from one piece of AtLEC2-GR.
Fig. 2 (See legend on next page.)
GR embryogenic callus and 16 shoots from the AtIPT7-OE AtLEC2-GR embryogenic callus. Significantly, 28 shoots could generate from one embryogenic callus of AtIPT9-OE AtLEC2-GR (Fig. 3j-l, m). Shoots on the surface of embryogenic callus were able to develop into healthy plants (Additional file 1: Figure S6).

Transcriptome analysis of different transgenic plants
To understand the mechanism by which AtIPTs differentially influenced embryogenic callus formation, 20 DAG seedlings of wild type, AtLEC2-GR, and AtIPTs-OE AtLEC2-GR grown under the 20 μM DEX induction were used for gene expression profiling. To distinguish genes regulated by ectopic expression of AtLEC2 and AtIPTs, we carried out a comparative analysis of genes expression between these different lines (Fig. 4). When AtLEC2-GR was compared with the wild type (LEC2-GR/WT) plants, 6310 genes were up-regulated and 10, 789 genes were down-regulated (Fig. 4a). Gene Ontology (GO) analysis of differentially expressed genes revealed enrichment in isoprenoid metabolic process (GO:0006720), porphyrin-containing compound biosynthetic and metabolic process (GO:0006779 and GO:0006778) and so on, were enriched in the differentially expressed genes (Fig. 4c). Embryogenic storage protein genes including 11S2, 2S, 75 globulin and vicilins, and late embryogenesis abundant (LEA) protein genes were significantly upregulated. The expression of SUCROSE SYNTHASE 2 (SUS2) and FATTY ACID DESATURASE 2 (FAD2) genes were upregulated (Fig. 4b). In addition, genes implicated in the regulation of embryo development, somatic embryogenesis and seed maturation, including LAFL network genes, AGL15, and AIL family transcription factor genes (BBM and PLT2) were significantly upregulated in the AtLEC2-GR seedlings (Fig. 4b). The expression of many key regulators involved in auxin, abscisic acid, cytokinin and gibberellin acid metabolism and signaling were changed by ectopic AtLEC2 expression. For example, YUC4, encoding a flavin monooxygenase enzymes involved in auxin biosynthesis, was induced (Fig. 4b). AtLEC2 also activated PIN-FORMED (PIN) auxin efflux facilitators (PIN1, PIN2 and PIN3) and auxin response factors (ARFs) (ARF5, ARF6 and ARF8) (Fig. 4a, b). In contrast, the genes encoding GA20oxx1, GA20oxx2 and GA3ox1, which catalyzed the later steps of gibberellic acid biosynthesis, were downregulated (Fig. 4b). Three epigenetic repressor genes CURLY LEAF (CLF), VP1/ABI3-LIKE 1 (VALI) and CHD3-type chromatin-remodeling factor PICKLE RELATED 2 (PKR2), were up-regulated (Fig. 4b).

Overexpressing AtIPT3, AtIPT7 and AtIPT9 genes led to different phenotypes as described above. We also investigated the change of gene expression in three AtIPTs-OE AtLEC2-GR transgenic seedlings. In AtIPT7-OE AtLEC2-GR, 10697 genes were up-regulated and 6430 genes were down-regulated compared with wild type (IPT7-OE/WT) (Fig. 4a). GO enrichment was observed in the porphyrin-containing compound biosynthetic and metabolic process (GO:0006779 and GO:0006778) and response to stress (GO:0006950) and so on. (Fig. 4c). The AtIPT7-OE AtLEC2-GR seedlings exhibited a similar changing tendency compared with AtLEC2-GR. Consistent with the phenotype, only 14 up-regulated genes and 5 down-regulated genes were detected between AtIPT7-OE AtLEC2-GR and AtLEC2-GR lines (IPT7-OE/LEC2-GR) (Fig. 4a). In AtIPT9-OE AtLEC2-GR, a total of 1791 up-regulated genes and 3016 down-regulated genes were identified when AtIPT9-OE AtLEC2-GR compared with wild type plant (IPT9-OE/WT). GO terms of response to stimulus (GO:0050896), response to stress (GO:0006950) and so on, were enriched in the differentially expressed genes (Fig. 4c). Eight thousand thirty-two genes were up-regulated and 2804 genes were down-regulated when compared between AtIPT9-OE AtLEC2-GR and AtLEC2-GR (IPT9-OE/LEC2-GR) plants (Fig. 4a). Generation of precursor metabolites and energy (GO:0006091), isoprenoid biosynthetic and metabolic process (GO:0008299 and GO:0006720) and so on, were enriched in the differentially expressed genes (Fig. 4c). Gene expression in AtIPT9-OE AtLEC2-GR was compared with AtIPT7-OE AtLEC2-GR (IPT9-OE/IPT7-OE), 2846 genes were up-regulated and 8006 genes were down-regulated (Fig. 4a). Carbon fixation (GO:0015977), cofactor metabolic process (GO:0051186) and generation of precursor metabolites and energy (GO:0006091) and so on, were enriched in the differentially expressed genes (Fig. 4c). In AtIPT3-OE AtLEC2-GR seedlings, 1244 genes were up-regulated and 3565 genes were down-regulated when compared with
Fig. 3 Shoot regeneration from callus derived from different transgenic lines. a-c Embryogenic callus separated from SAM of 20 DAG seedlings under 20 μM DEX induction. Scale bars = 3 mm. d-f 4 d after embryogenic callus on hormone-free MS medium (no DEX). Scale bars = 3 mm. g-i 12 d after embryogenic callus on hormone-free MS medium (no DEX). Scale bars = 3 mm. j-l 20 d after embryogenic callus on hormone-free MS medium (no DEX). Scale bars = 3 mm. m Statistics of shoot regeneration number of embryogenic callus on hormone-free MS medium (no DEX). Cyan indicated embryogenic callus derived from three independent AtLEC2-GR transgenic lines (each line contains 20 replicates). Dark blue indicated embryogenic callus derived from three independent AtIPT7-OE AtLEC2-GR transgenic lines (line 9, 10 and 11, and each line contains 20 replicates). Yellow indicated embryogenic callus derived from three independent AtIPT9-OE AtLEC2-GR transgenic lines (line 8, 9 and 12, and each line contains 20 replicates). Values are mean ± standard errors from 60 replicates. Three asterisks indicate statistically significant differences (***P < 0.001, Student’s t-test). d, days after transferred to hormone-free MS medium.
Fig. 4 (See legend on next page.)

[Diagram showing gene expression analysis with scatter plots and heatmaps.]

**Legend:**
- **a:** Scatter plots showing log2 fold change for different gene expression patterns.
- **b:** Heatmap representing gene expression levels with fold change.

**Legend for Heatmap:**
- **pink:** Up-regulated genes
- **blue:** Down-regulated genes
- **gray:** No-regulated genes

**Gene Ontology (GO) Terms:**
- carbohydrate catabolic process
- carbohydrate metabolic process
- carbon fixation
- cellular homeostasis
- cellular lipid metabolic process
- cellular metabolic process
- cofactor biosynthetic process
- cofactor metabolic process
- generation of precursor metabolites and energy
- glucan metabolic process
- glycine metabolic process
- hexose catabolic process
- hexose metabolic process
- isoprenoid biosynthetic process
- isoprenoid metabolic process
- homeostatic process
- isoprenoid catabolic process
- lipid biosynthetic process
- lipid metabolic process
- monosaccharide catabolic process
- monosaccharide metabolic process
- photosynthesis
- photosynthesis, light reaction
- porphyrin-containing compound biosynthetic process
- porphyrin-containing compound metabolic process
- reactive oxygen species metabolic process
- regulation of biological quality
- response to stimulus
- response to temperature stimulus
- serine family amino acid metabolic process
- single-organism carbohydrate catabolic process
- single-organism carbohydrate metabolic process
- single-organism metabolic process
- small molecule catabolic process
- small molecule metabolic process
- terpenoid biosynthetic process
- terpenoid metabolic process
- tetraperpyrrole biosynthetic process
- tetraperpyrrole metabolic process
- tetramerpentenoid biosynthetic process
- tetramerpentenoid metabolic process
the wild type (IPT3-OE/WT). GO analysis of differentially expressed genes revealed enrichment in carbohydrate metabolic process (GO:0005975), generation of precursor metabolites and energy (GO:0006091) and response to stimulus (GO:0050896) (Fig. 4c). Ten thousand eight hundred sixty-five and 6717 genes were up- and down-regulated when compared with AtLEC2-GR (IPT3-OE/LEC2-GR) (Fig. 4a). Carbohydrate catabolic process (GO:0016052), carbon fixation (GO:0015977) and cellular homeostasis (GO:0019725) and so on, were enriched in the differentially expressed genes (Fig. 4c). However, in the AtIPT3-OE AtLEC2-GR seedlings, embryo storage proteins and LEA proteins encoding genes, key somatic embryogenesis and seed maturation regulator genes were up-regulated. Furthermore, PIN1, PIN2, GRETCHEN HAGEN3.6 (GH3.6), YUC4, ARF5, ARF8 and GA20ox2 were expressed oppositely when compared with AtLEC2-GR and two other AtIPTs-OE AtLEC2-GR plants (Fig. 4b). These results indicated that the SAM microenvironment for embryogenic callus formation was differentially affected by ectopic expression of these genes. Finally, 7175 genes were up-regulated and 10,948 genes were down-regulated in AtIPT7-OE AtLEC2-GR when compared to AtIPT3-OE AtLEC2-GR (IPT7-OE/IPT3-OE). Carbohydrate catabolic process (GO:0016052), carbon fixation (GO:0015977) and hexose metabolic process (GO:0019318) and so on, were enriched in the differentially expressed genes (Fig. 4c). Ten thousand eight hundred sixty-five genes were up-regulated and 6717 genes were down-regulated in AtIPT9-OE AtLEC2-GR when compared to AtIPT3-OE AtLEC2-GR (IPT9-OE/IPT3-OE) (Fig. 4a). Carbohydrate metabolic process (GO:0005975) was enriched in the differentially expressed genes (Fig. 4c). All results demonstrated that the down-regulation of embryo-specific protein genes, auxin synthesis and signaling genes, and the up-regulation of active GA biosynthesis enzyme genes negatively affected the formation of embryogenic callus in the AtIPT3-OE AtLEC2-GR seedlings (Fig. 4b).

To illustrate the mechanisms by which AtIPT7 and AtIPT9 promoted shoot regeneration efficiency, 20 days callus derived from AtIPT7-OE AtLEC2-GR, AtIPT9-OE AtLEC2-GR and AtLEC2-GR seedlings after regeneration on hormone-free MS medium (re: AtIPT7-OE AtLEC2-GR, re: AtIPT9-OE AtLEC2-GR and re: AtLEC2-GR, respectively) were used for gene expression profiling. Totally, 246 genes were up-regulated and 35 genes were down-regulated when compared re: AtIPT7-OE AtLEC2-GR with re: AtLEC2-GR (re: IPT7-OE/re: LEC2-GR, Fig. 5a). GO analysis of differentially expressed genes revealed enrichment in carbon fixation (GO:0015977) and cellular metabolic compound salvage (GO:0043094) and so on (Fig. 5c). In re: AtIPT7-OE AtLEC2-GR, cytokinin signaling genes (CRE1, HK4, ARR1, ARR10 and ARR12), LEA genes, AGL15, BBM, PLT2, REVOLUTA (REV), CLAVATA 1 (CLV1), WUSCHEL RELATED HOMEO-BOX 1 (WOX1), WOX3 and WOX4 were activated (Fig. 5b). ARR2 and two epigenetic regulators (CLF and PKR2) were down-regulated (Fig. 5b). In addition, up-regulated genes in re: AtIPT7-OE AtLEC2-GR had a similar expression pattern in re: AtIPT9-OE AtLEC2-GR, except for ARR12, WUS and WOX4 (Fig. 5b). When compared re: AtIPT9-OE AtLEC2-GR with re: AtLEC2-GR (re: IPT9-OE/re: LEC2-GR), 40 genes were up-regulated and only one gene was down-regulated (Fig. 5a). GO analysis of differentially expressed genes revealed enrichments in nucleic acid-templated transcription (GO:00097659), phosphorelay signal transduction system (GO:0000160), RNA biosynthetic process (GO:0032774) and so on (Fig. 5c). The expression of CKI1, ARR1 and PLT2 in re: AtIPT9-OE AtLEC2-GR line were up-regulated when compared to re: AtLEC2-GR. This result was consistent with the gene expression profiling in 20 DAG seedlings (Fig. 4b). The expression of 12 genes was up-regulated and 18 genes were down-regulated when compared re: AtIPT9-OE AtLEC2-GR with re: AtIPT7-OE AtLEC2-GR (re: IPT9-OE/re: IPT7-OE, Fig. 5a). Except for CKI1, ARR2, PLT2 and CLF, all genes listed in Fig. 5b were down-regulated.

**Discussion**

Biotechnology has become a useful means for crop genetic improvement through overexpressing or silencing the key genes. In most cases, the genes were driven by the 35S promoter or the ubiquitin promoter. However, a more flexible gene expression system is essential, especially for genes that would lead to abnormal phenotype when constitutively expressing. The GR-DEX system has been considered a desired induction system which is simple and efficient, and DEX could function within four hours through direct addition to the medium or spray on the explants [26]. Our previous research showed that
Fig. 5 (See legend on next page.)
induced expression of AtLEC2 could promote vegetative to embryogenic transition and formed embryogenic callus on the SAM of the transgenic tobacco [6]. This phenotype enabled us to obtain embryogenic callus through application of DEX. An indispensable requirement for shoot regeneration and growth is the removal of AtLEC2 expression on DEX free medium (Fig. 3 and Additional file 1: Figure S6) [41]. Similarly, constitutive expression of WUS or BBM trigger somatic embryogenesis [7, 8]. There are other ways to terminate the expression of the transgenes. For example, Lowe et al. (2016) used a drought-inducible promoter to drive CRE expression and terminate the gene expression by the removal of the Bbm and Wus2 sequences between the loxP sites [47].

In this study, multiple endogenous cytokinins could be affected by overexpressing the AtIPT genes and resulted in high efficiency of shoot regeneration. Plant transformation has been blocked by several bottlenecks, including genotype and specific culture medium dependence [1]. The expression of AtIPTs conferred cell ability to increase endogenous cytokinin levels and to regulate downstream target genes expression, which might play a more precise role than direct application of exogenous cytokinins and could make the regeneration process convenient, cost-saving and without selecting exogenous cytokinin species and concentrations. In previous researches, overexpressed IPT from A. tumefaciens usually triggers excessive cytokinin abnormal phenotype [31, 32]. Here, we found that overexpression of AtIPT7 and AtIPT9 enhanced shoots regeneration from embryogenic callus, especially the AtIPT9 (Fig. 3-I). Although we did not find an increased accumulation of the tZ-type cytokinin in three AtIPTs-OE AtLEC2-GR seedlings, other cytokinin derivatives might be increased [52]. The phenotype of AtIPTs-OE AtLEC2-GR plants with increased numbers of lateral branch also implies an enhanced cytokinin accumulation (Additional file 1: Figure S2a-S2 h). Sun et al. (2003) reported that overexpression of AtIPT8 only causes higher levels accumulation of iPMP and iP (isopentenyladenosine), rather than tZ or other cytokinins. Because no callus was obtained in AtIPT3-OE AtLEC2-GR, we are unable to confirm if AtIPT3 has the same effect on the shoot regeneration (Fig. 2m, Additional file 1: Figure S5). In our study, transgenic tobacco overexpressing AtIPTs didn’t exhibit excessive cytokinin phenotype. The plants grew well and have more axillary branches (Additional file 1: Figure S2a-S2d). In addition, overexpression of AtIPTs could increase the floral number at the reproductive stage (Additional file 1: Figure S2e-S2 h) and delay plant senescence (data not shown).

Transcription profiling analysis demonstrated that ectopic expression of AtLEC2 in tobacco activated many genes encoding embryo proteins and embryo storage proteins, consistent with its regulation roles during embryogenesis [17]. Up-regulated expression of FAD2 and SUS2 suggested that the ectopic LEC2 activity might increase the fatty acid and sucrose accumulation. Overexpression of AtIPTs in the AtLEC2 background differently affected embryogenic callus formation (Fig. 2a-o). Firstly, we found embryogenic callus was not formed in the AtIPT3-OE AtLEC2-GR seedlings (Fig. 2m). It has been reported that BBM, PLT2 and LAFL, AGL15 induce embryogenesis in a dose-dependent manner [17, 19]. Consistent with this phenotype, gene expression of embryo development regulators, BBM, PLT2, LAFL and AGL15, were all significantly down-regulated in the AtIPT3-OE AtLEC2-GR seedlings (Fig. 4b). According to the above results, we proposed that AtIPT3 or its product might inhibit the expression of BBM, PLT2, LAFL and AGL15 or their downstream target genes, but the detailed mechanism remains exclusive. Down-regulation of embryo storage proteins and embryogenesis abundant proteins genes also testified our point of view (Fig. 4b). In addition, the down-regulation of auxin biosynthesis genes (GH3.6 and YUC4), auxin polar transport genes (PIN1 and PIN2) and auxin signaling genes (ARF5, ARF8 and IAA33) in the AtIPT3-OE AtLEC2-GR seedlings implicated that the microenvironment on SAM for callus formation was affected (Fig. 4b). In contrast, in AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR seedlings, the genes encoding embryo development regulators, BBM, PLT2, LAFL and AGL15, embryo traits, auxin metabolism and signal transduction genes have a similar expression tendency compared with the AtLEC2-GR seedlings (Fig. 4b). It has been reported that biologically active GAs played negative roles in embryogenic callus formation [6, 53]. Here, we found that genes active GAs synthesis, like GA20ox1 and GA3ox1, were down-regulated in AtLEC2-GR, AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR when compared with the wild type plants. In contrast, GA20ox1, and GA20ox2 were up-regulated in AtIPT3-OE AtLEC2-GR when compared
with the wild type plants (Fig. 4b). Further analysis revealed that GA20ox1, GA20ox2 and GA3ox1 were up-regulated in AtIPT3-OE AtLEC2-GR when compared with AtLEC2-GR (Fig. 4b). Our results suggested that the up-regulation of GA biosynthesis enzyme genes might be another reason that caused the inhibition of embryogenic callus formation in the AtIPT3-OE AtLEC2-GR seedlings.

Previous studies have demonstrated that overexpression of LEC1, BBM and AGL15 promotes shoot regeneration [4, 8, 9]. Here, we found that the expressions of BBM, LAFL and AGL15 genes were significantly up-regulated in the AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR plants (Fig. 4b). This elevated expression level of BBM, LAFL and AGL15 in transgenic tobacco could be a key factor leading to the high rate of shoot regeneration; however, the excess expression could have an opposite effect. This explanation was consistent with our previous results that 10 μM DEX induction led to a higher regeneration rate than 30 and 50 μM DEX induction [6]. Callus formation was initiated on the DEX containing medium, while further development of the somatic embryo and shoot formation required removal of DEX induction. We found that BBM, LAFL and AGL15 had a lower expression level in AtIPT9-OE AtLEC2-GR than in AtIPT7-OE AtLEC2-GR (Fig. 4b). Even though no shoots were generated on embryogenic callus in this stage, the cell fate for shoot regeneration had been determined. Furthermore, they displayed a similar expression pattern in re: AtIPT9-OE AtLEC2-GR compared to re: AtIPT7-OE AtLEC2-GR (Fig. 5b). The up-regulation of two negative regulators, CLF and PRK2, consistent with the down-regulation of LIL and FUS3 in re: AtIPT7-OE AtLEC2-GR when compared with re: AtIPT7-OE AtLEC2-GR (Fig. 5b). Taken together, it is very likely that the expression patterns of BBM, LAFL and AGL15 are the major reasons for the embryogenic callus formation and promoted shoot regeneration, however, excess expression level of these genes might inhibit shoot formation.

Here, we found cytokinin receptors CRE1, HK4 and three type-B ARR1s (ARR1, ARR10 and ARR12) in AtIPT7-OE AtLEC2-GR and re: AtIPT7-OE AtLEC2-GR were activated when compared with AtLEC2-GR and re: AtLEC2-GR (Fig. 4b and Fig. 5b). In AtIPT7-OE AtLEC2-GR, CRE1, CKI1, HK4 and ARR10 were down-regulated whereas HK1, ARR1 and ARR12 were up-regulated when compared with AtLEC2-GR (Fig. 4b). In re: AtIPT7-OE AtLEC2-GR, we found that CRE1, HK4, ARR1, ARR10 and ARR12 were up-regulated (Fig. 5b). Meanwhile, CRE1, CKI1, HK4, ARR1 and ARR10 were up-regulated in re: AtIPT9-OE AtLEC2-GR (Fig. 5b). Above results might explain that the improved shoot regeneration efficiency when overexpression of AtIPT7 and AtIPT9 might be due to enhanced cytokinin response. HK4, ARR10 and ARR12 might contribute to the shoot regeneration improvement in re: AtIPT7-OE AtLEC2-GR. However, HK4 and ARR1 played the function in re: AtIPT9-OE AtLEC2-GR. The recent study revealed that B-type ARRs directly activated the expression of WUS and repressed the YUCs transcription [43-45]. WUS has been shown to participate in the stem cell specification and somatic embryogenesis. We found that WUS was up-regulated in AtIPT7-OE AtLEC2-GR, but down-regulated in AtIPT9-OE AtLEC2-GR when compared with AtLEC2-GR (Fig. 4b). WUS was up-regulated in re: AtIPT7-OE AtLEC2-GR and down-regulated in re: AtIPT9-OE AtLEC2-GR (Fig. 5b). These results indicated that there might have other factors responsive to cytokinin derivatives produced by AtIPT9 and promoted shoot regeneration. Down-regulation of CLV1 in re: AtIPT9-OE AtLEC2-GR than in re: AtIPT7-OE AtLEC2-GR further enriched WUS expression range and promoted the regeneration of additional shoots [54]. Recently, Zhang et al. (2017) demonstrated that WOX1, WOX2, WOX3 and WOX5 redundantly maintain the balance between the cytokinin and auxin pathways and function in the initiation of the stem cell. However, WUS is dispensable for this process in embryogenic shoot stem cell initiation. In addition, terminal branches and leaf-like structures could still form in Arabidopsis wus mutants [44]. All these reports further supported the existence of additional regulators redundantly in SAM initiation.

Conclusion
In this work, we established a simple and efficient regeneration system by co-expression of AtLEC2 and AtIPT7 or AtIPT9. The induced expression of AtLEC2 triggers embryogenic callus formation and overexpression of AtIPT7 or AtIPT9 improves shoot regeneration without exogenous cytokinins. This strategy might be useful for crop genetic improvement in the future.

Accession number
The accession numbers of the genes used in this article are as follows: AtLEC2 (AT1G28300), AtIPT3 (AT3G63110), AtIPT7 (AT3G23630), and AtIPT9 (AT5G20040).

Methods
Plant materials and growth conditions
Seeds of tobacco (Nicotiana tabacum cv. SR1) were stored in our laboratory. Seeds of Arabidopsis thaliana ecotype Columbia-0 (Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). Seeds were surface-sterilized in 75% ethanol for 30 s and then in 10% (v/v) H2O2 for 10 min, and washed four times in sterile distilled water. Arabidopsis seeds were sown on
solid 1/2 Murashige and Skoog (MS) medium containing 1% sugar. After 2 days at 4 °C in darkness, seeds were transferred to white light with 100 μmol m⁻² s⁻¹ for 16 h and darkness for 8 h daily at 22 °C. Tobacco seeds were sown on solid MS medium containing 4% sugar and grown at white light with 100 μmol m⁻² s⁻¹ for 16 h and darkness for 8 h daily at 25 °C.

Plasmid constructs and gene transformation
The coding sequences (CDS) of AtIPT3, AtIPT7 and AtIPT9 were amplified from A. thaliana using specific primers (Additional file 1: Table S1). The PCR amplification was performed with the following parameters: 95 °C for 5 min; 36 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min; and 72 °C for 10 min. The AtIPT3, AtIPT7 and AtIPT9 CDS fragments were confirmed by sequencing and clone into pCAMBIA2300. AtIPT3, AtIPT7 and AtIPT9 constructs were transferred into Agrobacterium tumefaciens (LBA4404) and transformed into Pro3SS:AtLEC2-GR transgenic tobacco (Nicotiana tabacum cv. SR1) using leaf disc method [55]. The AtIPT3-OE AtLEC2-GR, AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR transgenic tobacco were selected on MS medium containing 50 mg/L kanamycin. Genomic DNA was isolated from the T2 transgenic seedlings. The transgenic lines were further confirmed by PCR amplification using specific primers of AtLEC2, AtIPT3, AtIPT7 and AtIPT9 (Additional file 1: Table S1). PCR amplification was performed with the following parameters: 95 °C for 5 min; 36 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min; and 72 °C for 10 min.

DEX induction and shoot regeneration from transgenic seedlings
DEX (25 mM) stock solution was dissolved in absolute ethanol and added to MS medium at a final concentration of 20 μM. Homozygotic transgenic seeds were germinated on MS medium containing DEX for 20 days to obtain embryogenic callus and used for imaging or in vitro shoot regeneration. Callus from AtLEC2-GR, AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR was transferred to MS medium without DEX and exogenous hormone for shoot regeneration.

Microscopy and photograph
Images were captured with OLYMPUS SZX16 microscope and Canon EOS 500D camera. The lengths of hypocotyl and primary root were measured by the measuring tool in OLYMPUS SZX16 microscope. Adobe Illustrator and Photoshop were used for final image arrangement and annotations.

Transcriptome sequencing and data analysis
To gain insight into the mechanism by which AtIPTs influenced embryogenic callus formation, total RNA was isolated from wild type, AtLEC2-GR, AtIPT3-OE AtLEC2-GR, AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR seedlings grown for 20 d on MS medium containing 20 μM DEX for the RNA-sequencing (RNA-seq) experiment. To illustrate the mechanisms that AtIPT7 and AtIPT9 promote shoot regeneration efficiency, we isolated total RNA from AtLEC2-GR, AtIPT7-OE AtLEC2-GR and AtIPT9-OE AtLEC2-GR callus after grown on hormone-free MS medium for the RNA-seq experiment. RNA-seq was performed by BGISEQ-500 platform (Shenzhen, China). The data were provided by The Beijing Genomics Institute (BGI). The significantly regulated genes were screened according to Log2 of fold-change and -log10 of the p adj (adjusted p-value) ([Log2 (fold-change) > 1, p adj < 0.1]). Identification of significantly (p-value < 0.05) enriched GO categories was done using a web-based tool and database for GO analysis (http://www.geneontology.org/).

Additional files

**Additional file 1:** Figure S1. Phenotype of seedlings grown on hormone free MS medium without DEX. Figure S2. Phenotype of mature plants grown in soil. Figure S3. PCR amplified the AtLEC2, AtIPT3, AtIPT7 and AtIPT9 fragments. Figure S4. Seedlings grown on 50 μM DEX condition. Figure S5. Seedlings grown on 20 μM DEX containing medium. Figure S6. Shoot regeneration from the callus. Table S1. Primers used in the study. (DOCX 1921 kb)

Abbreviations
6-BA: N’-benzyladenine; ABI3: ABSCISIC ACID (ABA)-INSENSITIVE3; AGL15: AGAMOUS-LIKE 15; AHPs: Arabidopsis thaliana histidine phosphotransfer proteins; AMP: Adenosine-5’-monophosphate; ARFs: AUXIN RESPONSE FACTORS; ARRs: Arabidopsis response regulators; BBM: BABY BOOM; CKI1: CYTOKININ-INDEPENDENT 1; CLF: CURLY LEAF; CLV1: CLAVATA 1; CRE1: cytokinin receptor 1; DEX: Dexamethasone; DIMAPP: dimethylallyl pyrophosphate; FA2D: FATTY ACID DESATURASE 2; FUS3: FUSCA3; GA20ox1: Gibberelin 20-oxidase1; GA: gibberellic acids; GH3:6: GRETCHEN HAGEN3:6; GL: Glucocorticoid receptor; GRES: glucocorticoid response elements; HKs: Hist kinase; HSP90: Heat shock protein; IPA: Isopentenyl adenosine; iPMP: Isopentenyladenine riboside 5’-monophosphate; IPT: Isopentenyltransferase; L1L: LEC1/LEC1-LIKE; LAFL: LEC1/L1L, ABI3, FUS3 and LEC2; LEA: late embryogenesis abundant; LEC1: LEAFY COTYLEDON 1; LEC2: LEAFY COTYLEDON 2; NAA: 1-Naphthylacetic acid; PINs: PIN-FORMEDs; PKR2: PICKLE-RELATED 2; PLT2: PLETHORA2; PRC2: Polycomb Repressive Complex 2; REV: REVOLUTA; SAM: Shoot apical meristem; SBKS: SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE; SUS2: SUCROSE SYNTHASE 2; TRAN: Trans; VAL1: VIVIPAROUS1/ABI3-LIKE 1; WOXs: WUSCHEL RELATED HOMEBOXs; WUS: WUSCHEL; YUCs: YUCCAs; ZMP: Zeatin riboside-5’-monophosphate

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Availability of data and materials
The data sets supporting the results of this article are included within the article and its additional files.

Author details
All authors declare that they have no competing interests.

Competing interests
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