CHEMICAL ENGINEERING TRANSACTIONS
VOL. 46, 2015
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Peiyu Ren, Yancang Li, Huiping Song

ISBN 978-88-95608-37-2; **ISSN** 2283-9216
DOI: 10.3303/CET1546233

A Highly Efficient Castor Regeneration System Identified through WUSCHEL Expression

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The key problems that limit the application of molecular breeding tools in castor are the inconsistency and inefficiency of the existing regeneration response in this plant. In the present study, we have examined and attempted to correlate the WUS gene expression in the castor explants with their shoot bud forming efficacy following the induction of embryogenic cells under various culture conditions using RT-qPCR method. By pre-treating the seedling-derived epicotyls explants with appropriate cytokine, a budding rate of > 80% could be achieved when a treatment of 0.35 mg/l BA for 5 days was employed. The corresponding WUS expression in such explants was found to be concentration and pretreatment duration dependent. The relevance of regeneration system developed in this study is discussed in the light of genetic transformation studies being presently carried out in this plant system

1. Introduction

Castor (*Ricinus communis)* is a very important source for industrial oil. In today's world, thousands of chemical derivatives are being produced using castor oil as a raw material. Therefore, castor oil is one of the best choices for substituting common energy resources such as petroleum (Moshkin, 1986). The demand for castor oil has surged many attempts have been made to improve the production and quality of castor.

Transgenic techniques have been applied to castor breeding in order to improve its efficiency. Researchers have developed several important improvements over the years. Mckeon and Chen (2003) with their patented technique, carried out the first successful castor transformation. Sujatha and Sailaja (2005) successfully obtained the transgenic plant with *Agrobacterium*-mediated methods but only achieved a 0.08% transformation rate. Sailaja *et al*. (2008) acquired the transformed plant via the gene gun transformation method using the budded embryo as the explant and obtained a transformation efficiency of 1.4%. In recent years, there have been multiple reports on successful castor transgenic breeding; however, the transformation efficiency is problematic and is very low compared to other agriculture crops (such as rice, soybean, wheat, etc.) (Ahn *et al*., 2007; Kumari *et al*., 2008). The low transformation efficiency is due to the difficulty of castor regeneration (Sujatha *et al*., 2008). A highly efficient regeneration system is the basis for transgenic breeding.

Generating a somatic embryo, however, is a very complicated process. Through a series of investigations, many of the key genes involved in somatic embryogenic generation have been discovered. One of which is *WUSCHEL* (*WUS*). *WUS* was originally discovered in *Arabidopsis*, and it is specifically expressed in the central cells with embryogenic property (School *et al*., 2000).

Through various investigations, the primary function of *WUS* was determined to be maintaining the embryogenic property as well as the further development and differentiation capabilities of the cells (Ogawa *et al*., 2008). Therefore, the *WUS* gene is also used as the marker gene of the embryogenic cells. The relative amount of embryogenic cells can be inferred through detecting the *WUS* expression under different conditions and then determining the influence of that condition on the late-stage budding rate.

Our research compared the *WUS* gene expression of castor explants under different inducing conditions in order to screen for the optimal condition. Then, the castor regeneration system was further optimized through evaluating the late-stage budding effect to develop regeneration system evaluation methods based on embryogenic cell induction.

Please cite this article as: Li W., Li Z.J., Zhai Y.J., Wang C.L., 2015, A highly efficient castor regeneration system identified through wuschel expression, Chemical Engineering Transactions, 46, 1393-1398 DOI:10.3303/CET1546233

2. Materials and Methods

2.1 Plant material and explant preparation

The selected castor seeds were Jiaxiang NO.2 and were provided by the Shandong Academy of Agricultural Sciences, Zibo Branch. After being shelled, the seeds were immerged in a 0.1% $KMnO_4$ solution for 10 min for sterilization before being cleaned 2-3 times with sterile water. Then, the endosperms were carefully extirpated intact embryos, which were transferred into medium MS (Murashige and Skoog, 1962) supplemented with 0.2 mg l^{-1} 6-benzylaminopurine (BA). The embryos were cultured for 5 d in the dark at 26 °C. After the buds grew to around 7-10 mm with a visible embryogenic tip and hypocotyl, we removed the cotyledons and cut the epicotyls as the explant for the embryogenic cell induction.

2.2 Induction of embryogenic cells

We inoculated the isolated epicotyls into different pre-culturing mediums (MS supplemented with 0, 0.2, 0.35, 0.5 mg l^{-1} BA or 0.05, 0.1, 0.2, 0.4 mg l^{-1} TDZ) and then cultured under the day/night cycle (16/8 h) for 3, 5, 7 d at constant 26°C. The experiments were performed with 100 to 150 samples per condition, and each treatment consisted of three replicates. We observed the response of the explants under different conditions, obtained some of the explants, washed away any residual medium, and conducted *WUS* expression analysis.

2.3 Budding and shoot regeneration

We inoculated the pre-cultured explant into the shoot induction media (MS supplemented with BA 0.35 mg l^{-1}, IBA 0.25 mg l^{-1}) to induce budding. The explants were cultured in 26 °C with light and the medium was changed every 7 d. When the bud grew to around 2-3 cm, we carefully cut it off from the base and inoculated it into the root induction media (1/2MS supplemented with 0.2 mg l^{-1} IBA) to induce rooting. Rooted plantlets were transferred into humid soil (Nutrient soil: vermiculite 1:2), and cultured in a greenhouse at 28 °C.

2.4 WUS expression analysis

We tested the difference in *WUS* expression by using explants under different pre-culture conditions. The upper 5mm tissue of the explants (200 mg) was cut to extract the total RNA. (Plant RNA Kit, OMEGA). The RNA integrity and purity were observed through agarose electrophoresis and absorption detection. We then did a reverse-transcription with PrimeScript™ RT Master Mix (Takara) (used oligodT as a primer), synthesized cDNA, and added 75μL of DNase free water to dilute. The diluted template was used for the *WUS* semi-quantitative expression analysis.

Actin was used as the (GenBank: AY360221.1) internal control and designed the primer A-F：TGATGATGCTCCCAGGGC and A-R：GTGAGAAGCACAGGATGC. The detection primers were designed based on the castor *WUS* gene sequence (*WUS*-F： TCACCATCTGGCAACTAT, *WUS*-R：AATCCATCGCCTGCTCTA). PCR-amplification was conducted in a total volume of 20μL, containing the appropriate diluted cDNA, 0.2μM for each primer, and 10μL of 2×Q-PCRMix (with SYBR Green I; Takara). Negative controls are explants without the hormone stimulation cDNA template. Real-time quantitative PCR (qRT-PCR) was performed in an MxPCR (Agilent Technologies Inc) as follows: 1 min of pre-denaturation at 95°C and then 35 cycles of denaturation at 95°C for 20s, annealing at 56°C for 20s, and fluorescence collection at 72°C for 15s. The results of which were analyzed with MxPro (Agilent Technologies Inc).

2.5 Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Analysis of variance (ANOVA) was followed by Tukey's pair wise comparison tests, at a level of $P<0.05$, in order to determine the significant differences between means.

3. Results

3.1 Hormone response after pre-culture

After numerous experiments, we selected epicotyl, which has a relative low differentiation and high mitogen etic capability as the best explant. The pre-culture stage is used to stimulate cell division and inhibit differentiation, which can induce more embryogenic cells. The results showed that there exist substantial differences in the stress responses to various kinds of cytokine in the explants. The post-BA treatment explants showed obviously expanded tissue 3 days after culture with a relative verdant color (Fig. 1 a). With the duration of the treatments, some of the explants also showed a verdant bud-like protrusion at the morphological top (Fig. 1 b). The explants under the TDZ treatment showed expanded tissue as well as color changes due to some red pigments at the early stage of culturing (Fig. 1 d). Furthermore, with the duration of the TDZ treatment, some of the explants also exhibited a lot of budding-like protrusions. However, nearly 50% of the explants had a lot of white, loose calluses growing around them (Fig. 1 e). Once the callus is generated, the late stage budding rate of the explants will be greatly affected. In addition, the results also indicated that the hormone response in this stage is not concentration-correlated.

3.2 Difference in budding induction

We summarized the budding 3 weeks after transferring the pre-cultured explants into the budding induction medium (Table 1). The results indicate how the different pre-culture conditions influence the late stage bud induction. The explants with BA treatment showed an "increased then decreased" budding rate with the increased hormone concentration and cultivation duration. Furthermore, the new buds of the BA group are evenly distributed (Fig. 1 c). The maximal budding in the TDZ treatment group is higher than in the BA treatment group. The budding is dense and concentrated with vitrification-like phenomena on some of the buds (Fig. 1 f). While the budding rate was not obviously influenced by the cultivation duration, the budding rate decreased with increasing TDZ concentration. In summary, the budding induction in the BA pretreated group is better, in which the explants after 0.35 mg l^{-1} pretreatment for 5d have the best budding efficiency with an 80.7% budding rate.

Table 1: Effect of pre-treatment of explants on bud formation in castor. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at P = 0.05 according to DMRT. ('--' means not add)

Total no. of explants	Concentration of growth regulator (mg l^{-1})		Days of pretreatment	Bud frequency (%)	Max no. of shoots per explant
	BA	TDZ			
231			3	15.3p	3
242	0	0	5	15.1pq	5
216			7	16.6op	5
348			3	41.3h	5
371	0.2	--	5	71.3b	8
334			7	52.6g	5
453			3	63.3fg	8
382	0.35	--	5	80.7a	15
314			7	67.3df	12
307			3	64.0f	11
384	0.5	--	5	70.6c	8
411			7	68.0d	5
364	--	0.05	3	30.7	12
288			5	23.3l	18
311			7	33.3i	28
317	--	0.1	3	32.8ij	17
364			5	28.3j	11
245			7	25.6k	21
381	--	0.2	3	19.3n	23
355			5	17.3o	11
371			7	12.6qr	19
282	--	0.4	3	20.6m	21
341			5	4.3s	14
328			7	12.8q	19

3.3 The influence of BA on WUS gene expression

Explants cultured under different BA concentrations for 3, 5, and 7 d are used to obtain the cDNAs as the template for *WUS* expression analysis. The results indicated that the *WUS* expression is low without hormone induction and increases a little with the cultivation duration. After adding the BA stimulation, the *WUS* expression dramatically increased; the trend experienced an initial increase and then eventual decrease with the duration of the BA treatment (Fig.2, a). A similar trend of an initial increase and then decrease also occurs with increasing BA concentration. In addition, the *WUS* gene expression is highest in the explants 5 days after 2mg l^{-1} BA treatment.

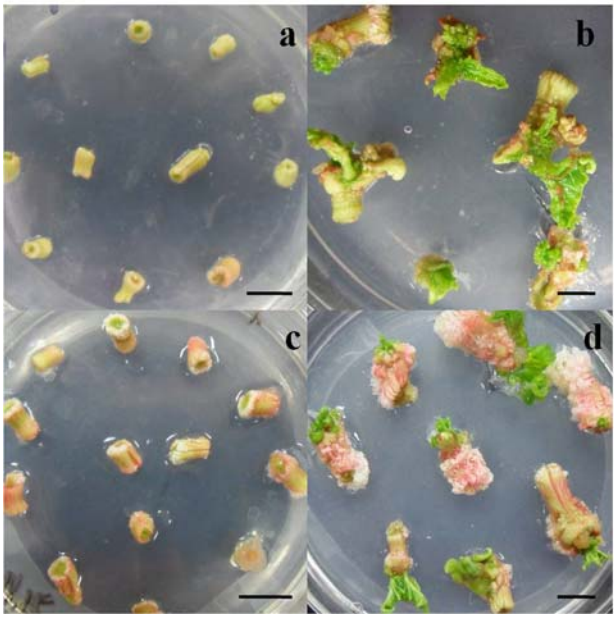

Figure 1: a - The explants preprocessed with 0.35 mg l^{-1} BA for 3d. b - The explants (preprocessed with 0.35mg l^{-1} BA) after 7 days of bud induction and culture. c - The explants preprocessed with 0.1mg l^{-1} TDZ for 3d. d - The explants (preprocessed with 0.1mg l^{-1} TDZ) after 7 days of bud induction and culture, which had a lot of white, loose calluses growing around. Bars=1 cm

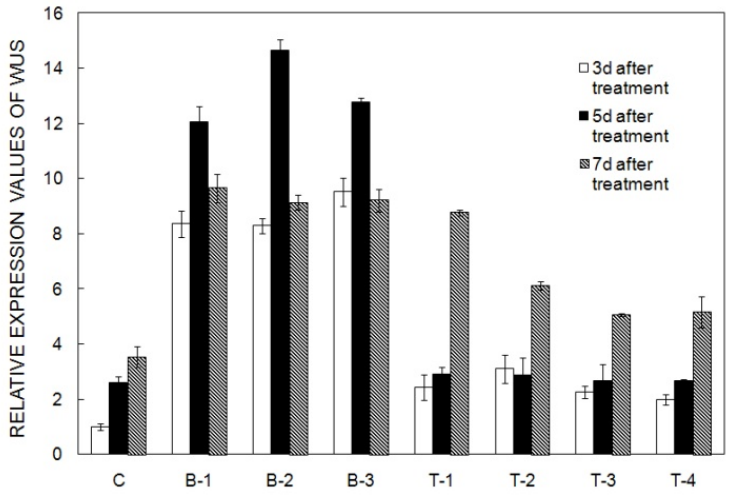

Figure 2: Expression analysis of WUS after BA and TDZ treatment. C was explants without hormone treatment. B-1, B-2, B-3 were explants treat with 0.2, 0.35, 0.5 mg/L BA. T-1, T-2, T-3, T-4 were explants treat with 0.05, 0.1, 0.2, 0.4mg/L TDZ. Data represent average of 3 experiments; error bars show standard deviations.

3.4 The influence of TDZ on WUS gene expression

Again, we used explants cultured under different TDZ concentrations for 3, 5, and 7 d to acquire the cDNA as the template for *WUS* expression analysis. The results of which showed that the *WUS* expression in the explants 3-5 days after adding TDZ is not different from that of the control group; however, the *WUS* gene expression significantly increased after 7 days of culture. Additionally, the explants that were cultured with 0.05 mg l^{-1} TDZ demonstrated the most obvious increase in *WUS* expression (Fig. 2, b). With this data, we suspect that TDZ's effect usually appears after 7 days of treatment, and culture duration has a greater influence than hormone concentration does.

3.5 Castor regeneration period

In this research, it was found that, during the castor regeneration, the elongation of new buds is very inefficient. A single explant, regardless of the number of buds it has, normally only 3-4 buds that would grow larger than 2cm. The new castor bud easily roots with a greater than 90% rooting rate under the conditions in our research. The intact explant can grow normally after the hardening treatment and being transferred into the soil. The regeneration period is about 50-60 d.

4. Discussion

4.1 The relationship between WUS gene expression and the budding rate

WUS is the key gene of embryogenic cells, and its expression directly represents the number of embryogenic cells in the plant tissue. Furthermore, an embryogenic cell is the foundation of plant regeneration. Therefore, a higher *WUS* expression should theoretically mean a higher budding rate. Our results also fit this premise. In comparing the budding induction after the BA pretreatment, we found that the changes in *WUS* completely correlated with the final budding rate. As the BA concentration increased, both the *WUS* expression and the budding rate showed a trend that initially increased and then decreased, which also demonstrates that the amount of embryogenic cell induction directly influences the budding rate. Also, the expression of *WUS* again showed a trend that initially increased and then later decreased with the duration of the BA treatment, which could be due to the reduction of the relative amount of the embryogenic cells or due to the *CLV3* feedback inhibition of *WUS*, thereby reducing the expression area [12]. The results also indicate that the budding induction should initiate before the *WUS* expression reduces in order to achieve a higher budding rate.

The *WUS* expression is not consistent with the final budding rate in the TDZ pre-cultured tissue. In the early TDZ induction stage (3-5 d), the explant shows expanded tissue with loose callus in the surroundings. Furthermore, the *WUS* gene expression is not changed at this stage, thus indicating that the plant cells are not embryogenic at this stage. This also means that in the early induction stage, TDZ can only promote division and not the generation of embryogenic property. The tissue culture experiments also show that with increased TDZ concentration as well as increased culture duration, the explants more easily exhibit a vitrification phenomenon, which could explain the mismatch of *WUS* expression and budding rate. Furthermore, with the increased TDZ culture duration, the explants might actually have a greater number of induced embryogenic cells; however, a large amount of cells appeared at the early stage and lead to the vitrification, which would then affect the differentiation of the embryogenic cells. This will lead to failed budding in addition to a reduced budding rate. The *WUS* expression increased significantly 7 days after TDZ treatment, which indicates that the culturing duration may be the key factor regulating TDZ-induced budding. Meanwhile, if vitrification can be reduced or even prevented, the budding rate could also possible be improved.

As mentioned above, during the *ex vivo* regeneration, the budding rate of the explant is related to the *WUS* activity. The higher the *WUS* activity is, the more embryogenic cells that will be induced, and the higher budding potential the explant will have.

4.2 The stability of budding rate

The budding is a very complex process. A normal cell needs to first be induced into an embryogenic state, then form the bud primordium, and finally develop into the visible bud [13]. The current evaluation of a regeneration system usually focuses on the budding efficiency. However, this kind of evaluation has its limitation and may actually be the cause of the low reproducibility and stability for building a plant regeneration system. In this research, we combined the mechanism of plant embryogenesis and began with the induction of embryogenic cells. By examining the *WUS* expression, we estimated the induction of embryonic cells, determined the optimal induction condition for embryogenic cells, improved the induction rate, and increased the stability of the budding rate. All these ensured the following up regeneration process. As such, our findings can stimulate the initiation of regeneration system construction, which ultimately provides the basis for building a better castor regeneration system.

4.3 Molecular evaluating methods

We compared the *WUS* expression and the budding rate as well as provided the molecular evaluation of a regeneration system, which is beneficial for building the plant regeneration system in the future. Besides *WUS*, numerous key genes that are involved in plant embryogenesis have been discovered using modern molecular biology: for example, the *LCE* family that influences embryo formation and the *PIN* family that influences the plant hormone trafficking (Lotan *et al*., 1998; Gaj *et al*., 2005). We can apply these findings to the construction of the plant regeneration system and modify the molecular evaluation of regeneration systems. Furthermore, this will assist in improving the efficiency of regeneration system construction as well as provide a more solid basis for a highly efficient regeneration system.

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