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A FACILE PROCEDURE FOR EFFICIENT PLANTLET REGENERATION AND FRUITING WITHOUT VERNALIZATION IN *THELLUNGIELLA SALSUGINEA*

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Abstract

A facile and rapid system for efficient shoot regeneration and callus keeping was developed for *Thellungiella salsuginea* (Pall.) O. E. Schulz. Three kinds of explants including basal leaves, stems and anthers of vernalized plants were adopted here. Calli were formed from all these explants on MS medium supplemented with various plant hormone treatments. The highest callus inducing ratio for anther explants were observed on MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l ZT zeatin, while for both stem and leaf explants 1.0 mg/l 2,4-D, 1.0 mg/l IBA and 0.1 mg/l ZT were used. The highest shoot regeneration ratios of anther and leaf calli were observed on MS medium with 0.1 mg/l IBA and 2.0 mg/l ZT and for stem calli it was 0.1 mg/l IBA and 1.0 mg/l ZT. Anther calli could keep vigorous growth on MS medium with 1.0 mg/l 2,4-D and 0.1 mg/l ZT. Even after three times subculture, shoots regenerated from anther calli could also develop flowers without vernalization while shoots regenerated from stem and leaf calli couldn't. Without vernalization almost 100% of seedling regenerated from anther blossomed whereas only 7.3% and 3.0% of seedling from stem and leaf did respectively, which provided us not only a facile, rapid regeneration system but also an activator for research work of vernalization mechanism.

Introduction

In salt tolerance research a wild crucifer plant *Arabidopsis thaliana* (L.) Heynh., which has a short generation time, a small genome and is easily tansformed, was an important model system making it easy to study most cress response in plant. But *A. thaliana* is not a halophyte and it's difficult to reveal important unknown processes in this plant. In a recent discovery a new halophyte *Thellungiella salsuginea* (Pall.) O. E. Schulz was found to be a good model plant for salt tolerance research (Zhu, 2001). Salt cress is an extremophile native to harsh environments and can reproduce after exposure to extreme salinity (500 mM NaCl) or cold to -15°C. It is a typical halophyte that accumulates NaCl at controlled rates and also dramatic levels of Pro (150 mM) during exposure to high salinity (Günsu *et al*., 2004). This valuable plant has some similar characteristics as *A. thaliana*: a little plant, a short regeneration time, self-pollination and a simple genome. It also has a close relative to *A. thaliana*: similar phenotype and life circle, the 90% same cDNA sequence, very similar gene sequence arrangement, only two time the genome size of *A. thaliana*. Further more thousands of T-DNA insert mutants can greatly facilitate the study of salt tolerance genes (Zhu, 2001; Xiong & Zhu, 2002). Therefore *T. salsuginea* became a perfect model system in salt tolerance research without question.

But like *A. thaliana* has a low transform ratio (1/10000-1/1000) through flower infection method by *Agrobacterium tumefaciens*, *T. salsuginea* has a even low transform ratio at about 1/100000-1/10000 in our laboratory. On the other hand *T. salsuginea* need one month's vernalization for flower inducing which occupy one third of its life circle. Gene function studies through transformation have been restricted until a reproducible regeneration and transformation system was founded. Plant regeneration by tissue culture in *T. salsuginea* has not been reported so far. So here we report an efficient method to acquire a regeneration system of *T. salsuginea* without vernalization.

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Material and Methods

Seeds of *T. salsuginea* were sown on soil with cocos substrate, expanded perlite and pumice stone in proportion of 4:2:1 in autumn. Seedlings were kept outdoors and the humidity of the soil was kept at about 60% during the whole winter. The florescence came in middle March of the next year. Before the florescence stems with flower bud together but without basal leaves were planted onto full strength MS medium (Murashige & Skoog, 1962) supplemented with various plant regulator treatments (Wu *et al*., 2003). The basal leaves planted separately onto these same MS medium. All these explants were surface sterilized by a 10-15 min soaking in 0.2% (W/V) Mercuric chloride then rinsed several times in sterile double distilled water before planting. The MS medium was supplemented with 30 g/l sucrose, different concentration set of IBA, 2,4-D and ZT, and gelled with 0.7% agar (BBI). pH of the medium was modified to 5.7-6.5 before autoclaving (120^{o}C, 20 min). All these explants were cultured in a growth chamber at 20^{o}C (day time) / 15^{o}C (night time) with a light interval of 16 hr light / 8 hr darkness (Wang *et al.,* 2004). This condition was used throughout the experiment.

Several days later when flower buds swelled and became white in its colour, flowers were cut down and anthers taken out of the perianth. Then with filament upside yellow anthers were sown on MS medium supplemented with different concentrations of plant hormone. Basal leaves and stems with 3-4 cauline leaves were both inoculated on callus inducing medium for 3 weeks. At least 30 explants each kind were used per treatment and all the treatment were all repeated three times. The effects of different hormone treatment on callus inducing, shoot regeneration and callus keeping were compared in Sigmaplot 8.02 and Duncan's Multiple Range Test (α= 0.05). All these three kinds of callus were transplanted to regeneration medium with different concentrations of IBA and ZT. Regenerated shoots were then cut down and transplanted to rooting medium. Ventilation was performed once a week aseptically to facilitate growth and early fruiting. Seedlings were moved to soil 2-4 weeks after rooting.

Results and Discussion

Two weeks later green anther calli formed at the highest ratio of 94.5% on the medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l ZT followed by other two treatment 92.5% (MS + 1.0 mg/l 2,4-D + 1.0 mg/l ZT) and 90.0% (MS + 1.0 mg/l IBA, 1.0 mg/l 2,4-D and 1.0 mg/l ZT) respectively (Table 1). These anther calli diameter ranged from 2-3 mm with blasted filament could be seen on them (Fig. 1e). It seemed that high concentration of 2,4-D (≥1.0 mg/l) is important for callus inducing of these three kind of explants. IBA had no significant function for callus inducing at low concentration (≤1.0 mg/l) and had obstructive function for callus inducing at high concentration of 2.0 mg/l. Stems with 3-4 cauline leaves were inoculated on callus inducing medium for 3 weeks when callus was observed almost 100% (98.5%) around the stems on the medium surface with 1.0 mg/l IBA, 1.0 mg/l 2,4-D and 0.1 mg/l ZT (Table 1; Fig. 1a). At the same time leaf callus was formed at the highest percentage of 86.8 on medium with the same treatment of 1.0 mg/l IBA, 1.0 mg/l 2, 4-D and 0.1 mg/l ZT (Table 1; Fig. 1c).

Table 1. Effect of growth regulator concentration on percentage of callus induction[1,2].

IBA (mg/l)	2, 4-D (mg/l)	ZT (mg/l)	% of anther explants produced calli	% of stem explants produced calli	% of leaf explants produced calli
0.0	0.0	0.0	55.4^{g}	77.0^{de}	67.0^{cd}
0.0	0.0	0.1	61.0^{efg}	70.2^{e}	61.7^{f}
0.0	0.0	1.0	57.5^{fg}	76.5^{de}	65.7^{cde}
0.0	1.0	0.0	78.5^{bc}	88.8^{abc}	75.0^{bc}
0.0	1.0	0.1	86.9^{ab}	92.5^{ab}	81.5^{a}
0.0	1.0	1.0	92.5^{a}	94.5^{a}	82.0^{a}
0.0	2.0	0.0	75.0^{bc}	80.0^{bcd}	76.5^{b}
0.0	2.0	0.1	86.2^{ab}	86.0^{bc}	80.0^{bc}
0.0	2.0	1.0	94.5^{a}	87.5^{b}	75.4^{bc}
1.0	0.0	0.0	72.0^{cde}	82.5^{bc}	70.5^{bcd}
1.0	0.0	0.1	75.6^{cd}	85.0^{b}	66.7^{cd}
1.0	0.0	1.0	82.5^{bc}	84.5^{b}	72.5^{cd}
1.0	1.0	0.0	87.5^{ab}	95.0^{a}	84.3^{a}
1.0	1.0	0.1	83.6^{b}	98.5^{a}	86.8^{a}
1.0	1.0	1.0	90.0^{a}	97.5^{a}	83.4^{a}
1.0	2.0	0.0	81.7^{bc}	92.6^{a}	80.0^{bc}
1.0	2.0	0.1	86.3^{ab}	96.3^{a}	85.0^{a}
1.0	2.0	1.0	88.9^{ab}	91.4^{a}	82.5^{a}
2.0	0.0	0.0	66.0^{f}	82.4^{bc}	67.5^{cd}
2.0	0.0	0.1	72.0^{cd}	84.6^{bc}	72.3^{c}
2.0	0.0	1.0	64.5^{ef}	86.3^{b}	71.2^{cd}
2.0	1.0	0.0	84.5^{abc}	80.6^{bcd}	75.0^{bc}
2.0	1.0	0.1	86.1^{ab}	91.5^{a}	82.5^{ab}
2.0	1.0	1.0	88.0^{ab}	92.8^{a}	82.8^{ab}
2.0	2.0	0.0	85.0^{a}	89.3^{ab}	72.6^{bcd}
2.0	2.0	0.1	84.6^{ab}	87.5^{a}	75.0^{bc}
2.0	2.0	1.0	87.3^{a}	88.7^{ab}	77.5^{b}

Table 2. Effect of growth regulator concentration on anther callus growth and percentage of regeneration[1,2].

2, 4-D (mg/l)	ZT (mg/l)	Weight of callus after subculture (g)/ Weight of callus before subculture (g)		% of regeneration[3]	
		1st subculture	2nd subculture	1st subcultured callus	2nd subcultured callus
		4.53^{d}	4.80^{d}		
0.0	0.1	4.82^{d}	4.65^{d}	86.8^{a}	88.5^{b}
0.0	1.0	5.45^{cd}	5.45^{cd}	85.7^{ab}	86.5^{ab}
0.1	0.0	6.67^{b}	6.50^{bc}	80.5^{bc}	77.8^{c}
0.1	0.1	7.24^{ab}	7.20^{ab}	94.5^{a}	93.8^{a}
0.1	1.0	6.25^{c}	6.35^{c}	89.6^{a}	92.2^{a}
1.0	0.0	7.03^{b}	7.10^{b}	85.0^{b}	86.7^{ab}
1.0	0.1	8.53^{a}	8.56^{a}	94.3^{a}	95.1^{a}
1.0	1.0	8.18^{a}	8.05^{a}	89.5^{a}	92.0^{a}

Table 3. Effect of growth regulator concentration on percentage of regeneration of different callus [1,2].

IBA (mg/l)	ZT (mg/l)	Regeneration % of anther calli	Regeneration % of stem calli	Regeneration % of leaf calli
0.0	0.0	65.0^{d}	60.0^{d}	60.0^{e}
0.0	0.1	72.5^{bc}	72.5^{bc}	67.5^{cd}
0.0	1.0	85.3^{b}	72.5^{bc}	77.5^{b}
0.0	2.0	86.7^{ab}	85.0^{a}	77.5^{b}
0.1	0.0	71.5^{cd}	57.5^{d}	63.5^{de}
0.1	0.1	87.5^{abc}	65.0^{c}	65.0^{cde}
0.1	1.0	92.5^{a}	89.8^{a}	86.7^{ab}
0.1	2.0	95.5^{a}	85.0^{a}	92.4^{a}
1.0	0.0	60.3d^{e}	60.0^{d}	64.7^{cd}
1.0	0.1	57.5^{e}	70.0^{bc}	66.7^{cd}
1.0	1.0	85.3^{bc}	82.5^{ab}	84.4^{ab}
1.0	2.0	90.0^{ab}	82.5^{ab}	88.6^{a}

[1]Each experiment repeated three times, and at least 30 explants or calli were used in each treatment.
[2]Numbers followed by different letters are significantly different at 5% confidence level according to Duncan's Multiple Range Test.
[3]All subcultured anther calli were regenerated on MS medium with 0.1 mg/l IBA and 2.0 mg/l ZT.

Another 2 weeks later anther callus regenerated shoots with the highest percentage of 95.5 in medium with 0.1 mg/l IBA and 2.0 mg/l ZT (Table 3; Fig. 1g). New shoots were continuously induced afterwards and shoot number of each callus was no less than 18. Within 5 weeks most of the shoots regenerated from anther callus had differentiated flowers (Fig. 1h). Shoots were also regenerated from stem and leaf callus at percentages of 89.8 (0.1 mg/l IBA and 1.0 mg/l ZT) and 92.4 (0.1 mg/l IBA and 2.0 mg/l ZT) respectively in 3 weeks (Table 3; Fig. 1b, Fig. 1d). But flower initiation percentages of the later two kinds of shoot were low (7.3% and 3.0% respectively) even after another subculture and flowers clearly aborted on the slim tip of these shoots. This gave us an important hint about organ difference in tissue culture and relationship of vernalization and flower inducing.

Rooting ratio was just at about the same level in MS medium and 1/2 MS medium, 2 weeks after all these three kind of shoots cut from their calli, more than 90% shoots had 1-3 roots longer than 1 cm. But growth was forestalled by deficiency of inorganic nutrients later in 1/2 MS medium whereas siliquae grown on MS medium were not influenced before moved to soil (Fig. 1i). Anther callus keeping system was also studied for shorter regeneration duration purpose. Callus blocks weighting around 0.5 g were cultured on MS medium with different 2,4-D and ZT treatment for 3 weeks before the second weighing. Ratio of callus weight was used to evaluate the effects of growth regulator concentration on anther calli growth. Then subcultured calli with different 2,4-D and ZT treatment were transplanted onto MS medium with 0.1 mg/l IBA and 2.0 mg/l ZT to regenerate shoots. Treatment of 1.0 mg/l 2,4-D and 0.1 mg/l ZT was found facilitating the growth and regeneration of anther callus and in both the first and second subculture there's no significant difference (Table 2). Most of the seeds could ripe another 2-3 weeks later in soil.

It takes *T. salsuginea* more than 4 months to develop flowers for tansformation purpose including one month's vernalization in natural condition or by artificial low temperature treatment. Here we provide a facile procedure for inflorescence preparation, which can continuously regenerate flowering shoots in about 7 weeks time. This discovery also made it possible to found callus tansform system for *T. salsuginea*. Furthermore with the help of this regeneration procedure research work about effect of vernalization on flower initiation mechanism is in process in our laboratory.

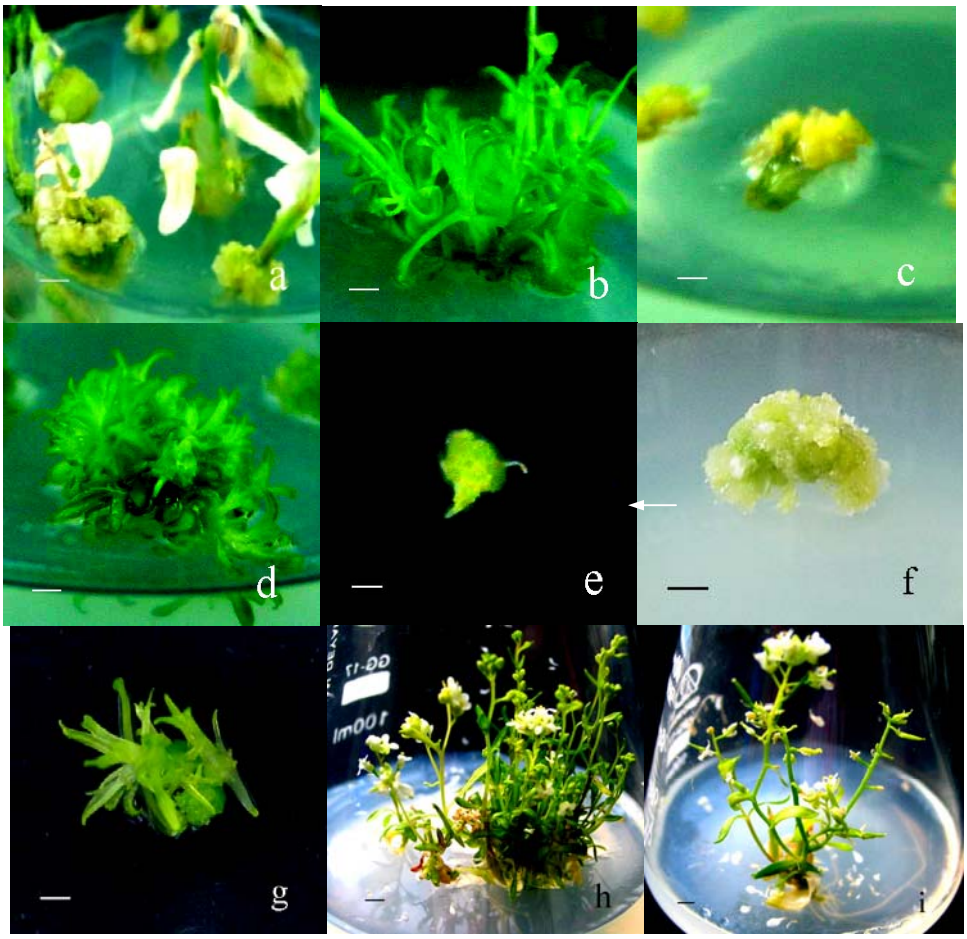

Fig. 1. Plantlet regeneration and flower inducing of *Thellungiella salsuginea*. (a) After 3 weeks' culture stem callus formed (bar 5 mm). (b) Shoots regenerated from stem callus after 3 weeks' culture (bar 2.5 mm). (c) Yellow leaf callus formed after 3 weeks' culture (bar 2.5 mm). (d) Shoots regenerated from leaf callus (bar 2.5 mm). (e) Green callus formed from anthers. The arrow shows blasted filament (bar 1 mm). (f) Three-time subcultured anther callus (bar 5 mm). (g) Shoots regenerated from anther callus (bar 5 mm). (h) Shoots regenerated from anther callus (bar 5 mm). (i) Shoots fruited 4 weeks later after be transplanted on MS medium (bar 5 mm).

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