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# *Agrobacterium* Mediated Transformation of Tomato and Production of Transgenic Plants Containing Gene Increased Sucrose Transport *SoSUT1*

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## *Abstract*

*The development of transgenic tomato to increase of sucrose translocation can be done through transformation by genetic engineering techniques. The process of sucrose transport from leaves to the stem/fruit is facilitated by transport proteins (sucrose transporter/SUT). In the year 2008 has been isolated SoSUT1 gene to increase of the rate of sucrose transport, so the accumulation of sucrose in storage organs (stems/fruit/sinks) increases. Cotyledon and axillary bud from L. esculentum cv. Zamrud were co-cultivated with Agrobacterium strain GV3010 that harbored a pAct binary vectors carrying genes for both Hygromycin phosphotransferase (HPTII) and S. officinarum SPS (SoSUT1). This study aims to determine transformation and obtain transgenic tomato plants transformed with the SoSUT1 gene using Agrobacterium tumefaciens. Transformation efficiency was obtained at 10 mg/l hygromycin antibiotic as a selection agent with the percentage of explants forming shoots 6.2%. PCR results showed that the plasmid pKYS_SoSUT1 already integrated in the explants and has obtained five clones of transgenic tomato plants.*

*Keywords: Tomato (Lycopersicon esculentum), SoSUT1 gene, Transformation, Agrobacterium tumefaciens.*

## 1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops and a genetic model for improving other dicotyledonous plants [1,2]. The development of transgenic tomatoes can be done by genetic engineering through recombinant DNA methods of gene transformation techniques using *Agrobacterium tumefaciens*. Tomato transformation using *Agrobacterium tumefaciens*, has developed very rapidly since 1986 [2]. Factors such as plant variety [1,3,4], explants material [2], plant growth regulators [5], bacterial concentration [6], binary vector [7], Agrobacterium strains [8] and concentration antibiotics [9,10], have a influence on transformation efficiency.

Based on assimilation of carbon (C), tomato plants were classified as C3 plants. In the Calvin cycle of $CO_2$ reacted with ribulose bisphosphate (RuBP) to form compound 3-phospho glyceric acid (3-PGA) catalyzed by ribulose bisphosphate carboxylase oxygenase (rubisco), the Calvin cycle occurs in mesophyll cells. The end product of carbon assimilation in the process of photosynthesis is sucrose and starch [11].

Sucrose is the largest part of the final product of photosynthesis in the leaf vascular tissue [12]. Sucrose was translocated in plant leaves (source) to the organ (sinks) such as stems, fruits, roots and flowers [13]. Isolation of DNA SUT has successfully conducted from spinach [14], tobacco [15], Arabidopsis, potato and tomato [16], cumis [1] and sugar cane [15].

The development of biotechnology by using cDNA SUT transformed the plant is expected to increase the translocation of sucrose to sink organs. The researchers suspect that by doing the over-expression of genes coding for sucrose transporters can increase the rate of sucrose transport, thus increasing its ability to accumulate sucrose in the sink [15]. Hackle [17], showed that LeSUT1 and antisense LeSUT2 on tomato plant able to produce normal tomato fruit. This study aims to determine transformation efficiency and obtain transgenic tomato plants transformed with *SoSUT1* gene using *Agrobacterium tumefaciens*.

## 2. Experimental Details

### *Plant Material*

Seed of tomato var. Zamrud were obtained from Research Institute for Vegetables (Balitsa) Lembang. The Seeds were soaked in warm water for 5 minutes, and surface-sterilized with 5% hypochlorites solution for 30 seconds and rinsed 3 times with sterile distilled water. Then, seeds were implanted planted on germination medium (G): (MS + 30 g/L sucrose + 3 g/L phytagel pH 5.8 before autoclaving) at 25°C under 16-h and 8-h

cycle with a light intensity of 1,600 lux illumination. Both axillary bud and cotyledons segment from 14 d-old in vitro plants were used as explants sources.

*Effect of hygromycin on regeneration frequency from axillary shoot, cotyledon and hypocotyls explants*

To determine optimal hygromycin concentration for callus induction, the axillary shoot, cotyledon and hypocotyls explants excised from non-transformed 14-d-old seedling were cut into 0.5 cm, 1x0.5 cm and 0.5 cm segments, respectively. Explants segments were cultured on callus induction medium (I): (MS + 30 g / l sucrose + 0.2 $mgl^{-1}$ IAA + 2 mg/L BAP + 3 g / l phytagel, pH 5.8 before autoclaving) supplemented various concentrations of hygromycin (0, 10, 20 and 30 mg/L). Each experiment was repeated three times. Observations were carried out by observing the percentage of dead explants for 28 days and 7 days intervals.

*Agrobacterium strain and plasmid vector*

*Agrobacterium tumefaciens* strain LBA4404, harbouring plasmid pAct, which contained hptII asa plant selectable marker, and *SoSUT1* gene construct, was utilized for *Agrobacterium*-mediated transformation. In this construct, the expression of hptII gene was under the control of the nopaline synthase (NOS) promoter and NOS terminator and *SoSUT1* gene was driven by Rice-actin (Figure 1).

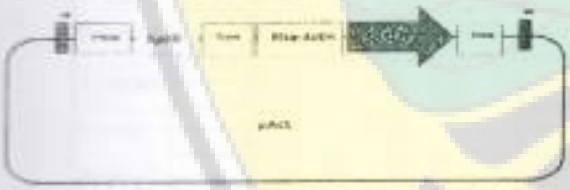

Figure 1. Map of pAct: *SoSUT1* construct. cDNA-SoSUT1 under the control of Rice-actin promoter and resistance gene (*hptII*) for hygromycin.

*Transformation mediated by Agrobacterium tumefaciens*

Single colonies of *Agrobacterium tumefaciens* LBA4404, harbouring plasmid pAct on Luria Betani (LB) medium plate (10 $gl^{-1}$ pepton, 10 $gl^{-1}$ yeast extract, 5 $gl^{-1}$ NaCl, 14 $gl^{-1}$ agar, pH 7.0) were inoculated in 2 ml liquid Yeast Extract Peptone (YEP) medium supplemented with 50$mgl^{-1}$ kanamycin, 30 $mgl^{-1}$ Streptomicyin and 50 $mgl^{-1}$ refamicyin and incubated overnight at 28°C with continuous shaking at 150 rpm. *Agrobacterium* resubcultured into 50 ml liquid YEP medium containing 50 $mgl^{-1}$ kanamycin, 30 mg/L Streptomicyin and 50 $mgl^{-1}$ refamicyin and incubated 4-h at 28°C to a final density of 0.3-0.4 $OD_{600}$. *Agrobacterium* cells were harvested by centrifugation at 5,000 rpm for 10 min and resuspended in liquid MS medium.

The explants were infected by immersing in *Agrobacterium* suspension for 30 min with gentle shaking three to five times during the infection process. Subsequently, the infected explants were dried on a steril filter paper and transferred onto (C) medium (MS salts + 30 $gl^{-1}$ sucrose + 0.2 $mgl^{-1}$ IAA + 2 $mgl^{-1}$ BAP + 3 $gl^{-1}$ phytagel + 50 $mgl^{-1}$ acetosyringone, pH 5.8 before autoclaving) for 2-d for cocultivation in dark at 25°C. Following cocultivation, the infected explants were washed with 50 ml of liquid MS containing cefotaxime 500 $mgl^{-1}$, drained on steril filter paper and transferred onto E medium (MS + 30 $gl^{-1}$ sucrose + 0.2 $mgl^{-1}$ IAA + 2 $mgl^{-1}$ BAP + 3 $gl^{-1}$ phytagel + 500 $mgl^{-1}$ cefotaxim , pH 5.8 before autoclaving) at 25°C under 16-h light, 8-h dark cycle for 5 days.

Axillary bud and cotyledon explants were cultured subsequently on S medium (MS + 30 g / l sucrose + 0.2 $mgl^{-1}$ IAA + 2 $mgl^{-1}$ BAP + 3 g / l phytagel + 500 $mgl^{-1}$ cefotaxim + 50 mgl kanamycin, pH 5.8) for shoot induction, R medium MS + 30 $gl^{-1}$ sucrose + 0.25 $mgl^{-1}$ BAP +0.25 mgl GA3 + 3 $gl^{-1}$ phytagel + 500 $mgl^{-1}$ cefotaxim + 5 $mgl^{-1}$ kanamycin, pH 5.8) for shoot enlargement and R medium (MS + 30 $gl^{-1}$ sucrose + 0.15 mgl NAA + 3 $gl^{-1}$ phytagel + 500 $mgl^{-1}$ ceftaxim + 50 $mgl^{-1}$ kanamycin, pH 5.8) for rooting induction.

*DNA isolation and PCR analysis*

Genomic DNA was isolated from nontransformed (control) and transformed leaves. PCR analysis were carried out to detect the presence of the hptII genes, using primer hpt-F, R, 480 bp. Lingkage analysis of these gene in transgenic plants was conducted using PCR amplification.

## 3. Results and Discussion

*Effect of hygromycin on the survival from tomato explants*

The sensitivity of axillary bud, cotyledon and hypocotyls to hygromycin was tested according to the survival and regeneration frequency of explans on H medium with various concentrations of hygromycin. Hygromycin is an antibiotic that is used as a selection agent. Explants grown on media containing hygromycin, because the target gene construct contained the gene for resistance to hygromycin (hpt). For Axillary bud explants, cotyledon and hypocotyls after 28-h of cultur were not death on H medium without hygromycin.

Regeneration frequency drastically declined as the hygromycin concentration increase from 10, 20 and 30 $mgl^{-1}$. The results showed that hygromycin concentration, 10, 20, 30 mg/L for 14-h was death at 100% on all explants. After 14-h, all explants turned brown and became completely necrotic on media with 10, 20 and 30 $mgl^{-1}$, respectively. No callus and shoot was produced from all explan st 10 mg/L. Thus the concentration of hygromycin 10 $mgl^{-1}$ is effective for selection in all explants (Table 1).

Hygromycin phosphotransferase (hpt) for resistance to hygromycin is aminoglycoside antibiotic resistance marker gene in plan transformation. Hygromycin inhibits protein synthesis by causing mistranslation and interferes with protein translocation [18]. Hygromycin are poison to kill non-transformed cells, but the transformed cells are able to grow normally because the regeneration of plants are not disturbed [19]. Meng *et al.* [20] reported that concentrations of 7.5 and 20 inhibit the initiation of callus on the cotyledon and hypocotyl explants of cotton. Explants are not transformed at concentrations higro 10 and died on 15 $mgl^{-1}$ hygromycin, the seed concentration 20 inhibits elongation of roots and shoots.

**Table 1**. Percentage mortality axillary bud explants, cotyledon and hypocotyl of tomato grown on MS medium containing hygromycin

| Explant Type | Concentration Hygromycin (mgl$^{-1}$) | Percentage mortality explants on the day of | | | |
|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 |
| Axillary bud | 0 | 0 | 0 | 0 | 0 |
| | 10 | 40 | 60 | 100 | - |
| | 20 | 60 | 100 | - | - |
| | 30 | 60 | 100 | - | - |
| Cotyledon | 0 | 0 | 0 | 0 | 0 |
| | 10 | 50 | 100 | - | - |
| | 20 | 50 | 100 | - | - |
| | 30 | 70 | 100 | - | - |
| Hypocotil | 0 | 0 | 0 | 0 | 0 |
| | 10 | 50 | 100 | - | - |
| | 20 | 70 | 100 | - | - |
| | 30 | 80 | 100 | - | - |

**Transformation Efficiency**

That the cotyledon explants produced shoots and putative transformant plant by 6.1% and 6.2%, respectively (Table 2). Explants that survive on S media are assumed that the target gene has been transformed. Observations on explants not infected with Agrobacterium, then grown in medium containing no hygromycin (control -) and containing hygromycin (control +) showed that explants grown on negative control to 85%, whereas explants in positive control media showed that all explants was death until the third selection.

Transformed explants with *Agrobacterium* showed that the explants are able to grow on selection media until 5 cycles 13.2% (Table 3). Frary [6], showed that the transformation efficiency of 10.6% in tomato cultivar Moneymaker. Cortina [5] also reported that the transformation efficiency reached 12.5% on tomatoes.

**Table 2**. Percentage Plantlet Putative Transformants

| Explant Type | Number of Explant | Percentage Plantlet putative transformants (%) |
|---|---|---|
| Axyllary bud | 100 | 6.1 |
| cotyledon | 100 | 6.2 |

**Table 3**. Percentage survival of shoots explants on selection media

| Treatment | Co-cultivation [%] | Elimination [%] | S1 [%] | S2 [%] | S3 [%] | S4 [%] | S5 [%] |
|---|---|---|---|---|---|---|---|
| GV+Hor | 100.0 | 100.0 | 100 | 80.0 | 47.7 | 25.6 | 13.2 |
| Control (+) | 100.0 | 85.0 | 40.0 | 20 | 0 | 0 | 0 |
| Control (-) | 100.0 | 400 | 100 | 95.0 | 90 | 85.0 | 85.0 |

**PCR amplification and linkage of hpt gene with SoSUT1 in T-DAN region**

Five putative transgenic tomato plants were analyzed by PCR amplification to detect the presence of hpt gene (Figure 2). The result showed that a 480-bp fragment of hpt gene was amplified in the entire sample except in the nontransformed.


**Figure 2**. Result of PCR with primer F/R HPTII (M): Marker 1 kb DNA Ladder (intron), (K+) control positive (pAct), (1,2,3,4,5): transgenic tomato plants and (W): control negative (*wild type*)

## 4. Conclusion

Transformation efficiency was obtained at 10 $mgl^{-1}$ hygromisin antibiotic as an selection agent with the percentage of explants forming shoots 6.2%. PCR results showed that the plasmid pKYS: SoSUT1 already integrated in the explant and has obtained five clones of transgenic tomato plants.

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## 6. References

[1]. Ling H.Q., Kruscius D., Ganal, M.W. (1998). Effect of Ticarcillin/Potassium Clavulanate on Callus Growth and Shoot Regeneration in *Agrobacterium*-mediated Transformation of Tomato (lycopersicon esculentum Mill.). *Plant Cell Reports* 17 : 843 - 847.

[2]. McCormick S., J. Niedermeyer, J. Fry, A. Barnason, R. Horsch, and R. Fraley (1986), Leaf disc transformation of cultivated tomato *(L. esculentum)* using *A. grobacterium tumefaciens*. *Plant Cell Reports*, 5:81-84.

[3]. Santoso T.J., A. Sisharmini dan M. Herman (2009), Respon Regenerasi Beberapa Genotipe Dan Studi Transformasi Genetik Tomat (*Lycopersicon esculentum* Mill.) Melalui Vektor *Agrobacterium tumefaciens*, Balai Besar Penelitian dan Pengembangan Bioteknologi dan Sumberdaya Genetik Pertanian.

[4]. Ellul P, Garcia-Sogo B, Pineda B, Rios G, Roig LA & Moreno V. (2003), The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* L. Mill.) is genotype and procedure dependent, *Theor. Appl. Gen.*, 106 (2): 231–238.

[5]. Cortina C. & F. A. Culiañez-Maci' (2004), Tomato transformation and transgenic plant production, *Plant Cell, Tissue and Organ Culture* 76: 269–275.

[6]. Frary, A. and E. D. Earle (1996). An examination of factors affecting the efficiency of *Agrobacterium-mediated* transformation of tomato, *Plant Cell Reports*, 16:235-240.

[7]. An, G. (1985), High efficiency transformation of cultured tobacco cells, *Plant Physiol*, 79:568-570.

[8]. Roekel J. S. C. van, B. Damm, L. S. Melchers, and A. Hoekema (1993), Factors influencing transformation frequency of tomato *(Lycopersicon esculentum)*, *Plant Cell Reports* 12: 644-647.

[9]. Hu W. and G. C. Phillips (2001), A combination of overgrowth-control antibiotics improves Agrobacterium tumefaciens-mediated transformation efficiency for cultivated tomato (L. esculentum), *Invitro Cell. Dev. Biol. Plant*, 37:12-18.

[10]. Qiu D., G. Diretto, R. Tavarza, G. Giuliano. (2007), Improved protocol for Agrobacterium mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene CsZCD, *Scientia Horticulturae* , 112 : 172–175.

[11]. Huber, S.C. and J.L. Huber (1996), Role and regulation of sucrose-phosphate synthase in higher plant, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 431 – 444.

[12]. Barker,L., Kuhn, C., Weise, A., Schulz, A., Gebhardt, C., Hirner, B., Hellmann H., Schulze, W., Ward, J.M. and Frommer, W.B. (2000), SUT2, a putative sucrose sensor in sieve elements, *Plant Cell*, 12 : 1153-1164.

[13]. Sugiharto, B., H. Sakakibara, Sumadi, T. Sugiyama (1997), Differential expression of two genes for sucrose phosphate synthase in sugar cane: Molecular cloning of the cDNAs and comparative analysis of gene expression, *Plant Cell Physiol*, 38: 961-965.

[14]. Riesmeier, J.W., Hirner, B., and Frommer, W.B. (1993), Potato sucrose transporter expression in minor veins indicates a role in phloem loading, *Plant Cell* 5 : 1591 - 1598.

[15]. Wang, N.F.Q. (2003), Analysis of sugar transport-related gene products expressed in developing seeds of *Vicia faba* and *Hordeum vulgare*. Dissertation of Universitat Halle-Wittenberg, Germany.

[16]. Weise, A., Barker, L., Kuhn, C., Lalonde, S., Buschmann, H., Frommer, W.B. and Ward, J.M. (2000), A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants, *Plant Cell* 12 : 1345 – 1355.

[17]. Hackel, A., N. Schauer, F. Carrari, A. R. Fernie, B. Grimm and C. Kuhn (2006), Sucrose transporter LeSUT1 and LeSUT2 inhibition affects tomato fruit development in different ways, *Plant Journal*, 45 :180–192.

[18]. Gonzales, A., Jimenez, A., Vazquez, D., Davies, J.E., Schindler, D. (1978), Studies on the mode of action of hygromycin B, an inhibitor of translocation in eucariotes, *Biochim. Biophys. Acta*, 521:459-469.

[19]. Nasir, M. (2002), Bioteknologi Molekuler Teknik Rekayasa Genetik Tanaman, PT Citra Aditya Bakti, Bandung.

[20]. Meng, Z., A. Liang and W. Yang (2007), Effects of hygromycin on cotton cultures and its application in Agrobacterium-mediated cotton transformation, *Invitro, Cell Dev Biol.*, 43:111-118.

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This is to certify that

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