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Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation

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Abstract The effects of timentin on shoot regeneration of tobacco (*Nicotiana tabaccum*) and Siberian elm (*Ulmus pumila* L.) and its use for the suppression of *Agrobacterium tumefaciens* in *Agrobacterium*-mediated genetic transformation were determined. Timentin is a mixture of ticarcillin and clavulanic acid, and at concentrations of 200–500 mg/l with ratios of ticarcillin:clavulanic acid of 50:1 and 100:1, it had little effect on shoot regeneration of tobacco or Siberian elm. Timentin was as effective in suppressing *A. tumefaciens* as carbenicillin and cefatoxime at concentrations commonly used in transformation. The disarmed *A. tumefaciens* strain LBA4404 in infected tobacco leaf tissues was visually undetectable after three subcultures on medium with 500 mg/l of timentin and 250 mg/l carbenicillin. Timentin was stable in solid agar medium and remained effective for at least 70 days, but was unstable when stored as a mixed stock solution or as separate ticarcillin and clavulanic acid stock solutions at –20°C or –80°C freezer for 4 weeks. Timentin may be an alternative antibiotic for the effective suppression of *A. tumefaciens* in genetic transformation.

Key words *Agrobacterium tumefaciens* · Antibiotic · Genetic engineering · Timentin

Abbreviations *BA* 6-benzylaminopurine

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Introduction

Agrobacterium tumefaciens or *A. rhizogenes*-mediated transformation has been used widely for introducing agriculturally important genes into crops and trees (Gasser and Fraley 1989) and used as a tool to study gene expression and regulation (Edwards and Coruzzi 1990). For transformation, plant tissues are infected by co-cultivation with a disarmed *A. tumefaciens* or *A. rhizogenes* carrying a gene of interest in an antibiotic-free medium for 1–3 days. After co-cultivation, the bacterium needs to be suppressed so as not to interfere with the growth and development of the transformed plant cells. This is normally done by transferring plant tissues to a selective medium containing *Agrobacterium*-suppressing antibiotics (Horsch et al. 1985). Currently, carbenicillin and cefatoxime are the most commonly used antibiotics for this purpose. Tissues need to be subcultured a number of times on the medium with these antibiotics to maintain suppression of *Agrobacterium*. These antibiotics are expensive, have to be used in high concentrations either alone or in combination (carbenicillin at 500 mg/l, cefatoxime at 200–300 mg/l) (Hammershlag et al. 1995), and often are ineffective (Shackelford and Chlan 1996; Z.-M. Cheng unpublished). Therefore, an effective, inexpensive alternative antibiotic to carbenicillin and cefotaxime would be useful.

Recently, Shackelford and Chlan (1996) tested ten antibiotics (carbenicillin, cefatoxime, erythromycin, spectinomycin, polymyxin B, chloramphenicol, methicillin, augmentin 500, augmentin 250, and moxalactam) for their activity against *A. tumefaciens*. Cefatoxime was found to be the most effective against *A. tumefaciens* LBA4404, and moxalactam the most effective against strain EHA101. However, in their assay, tissues that were infected by *A. tumefaciens* and grown on medium with antibiotics were not transferred to an antibiotic-free medium. Therefore, whether or not *Agrobacterium* was completely eliminated was unknown.

Timentin (SmithKline Beecham Pharmaceutical, Philadelphia, Pa.) has been widely used in human and animal

clinics to inhibit a variety of gram-positive and gram-negative bacteria (Neu 1982). It is a mixture of ticarcillin and clavulanic acid and is commonly used at a ratio of 50 (ticarcillin) :1 (clavulanic acid) (w/w) (Sweeney et al. 1988). Ticarcillin is a semisynthetic penicillin, and like penicillin is sensitive to and becomes inactivated by certain β-lactamases produced by a wide variety of bacteria (Labia et al. 1986). This problem can be overcome by the addition of clavulanic acid, a potent β-lactamase inhibitor (Fuchs et al. 1984; Verbist and Verhaegen 1986). Many clinical tests on human and domestic animals have shown that a combination of ticarcillin and clavulanic acid is effective and safe for use in a wide variety of infections (Sparks et al. 1988). In plants, some researchers have used ticarcillin or timentin to inhibit systemic bacteria in tissue culture (Tor et al. 1992; Buckley and Reed 1994), and to suppress *Agrobacterium* in genetic transformation (Zimmerman 1995). The objective of the research reported here was to determine the efficacy of timentin as an alternative antibiotic to carbenicillin and cefotaxime for the inhibition of *A. tumefaciens* in *Agrobacterium*-mediated genetic transformation.

Materials and methods

Tobacco (*Nicotiana tabaccum* L., 'Havana') and Siberian elm (*Ulmus pumila* L.) leaf tissues were used. The tobacco plants were maintained in Magenta GA7 vessels (Chicago, Ill.) containing 40 ml of MS medium (Murashige and Skoog 1962) free of plant growth regulators. Siberian elm seedling plants were grown in the greenhouse at 22°–24°C (Kapaun and Cheng 1997). Ticarcillin and clavulanic acid (kindly provided by SmithKline Beecham Pharmaceutical, Philadelphia, Pa.) stocks were made with potassium phosphate buffer at pH 6.0 and used within 1 day, unless otherwise indicated.

Agrobacterium tumefaciens strains LBA4404 and EHA105, both harboring pBI121, and a wild-type strain, C58, were grown in 2YT broth (tryptone 16 g/l, yeast extract 10 g/l, and NaCl 5 g/l) for 24 h. The suspensions were diluted to A_{600}=0.06. For the initial test of timentin on *A. tumefaciens* inhibition, strains LBA4404, EHA105 and C58 were streaked onto 15×100-mm petri plates containing 20 ml of MS medium supplemented with 4.4 μM benzylaminopurine (MS1 hereafter) and 250 mg/l timentin. Timentin was added at ratios of 30:1, 50:1 and 100:1 of ticarcillin:clavulanic acid. Each treatment consisted of 3 petri plates, which were placed under fluorescent light (54 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 16-h photoperiod. *A. tumefaciens* growth was evaluated after 3 weeks.

To determine the effect of timentin on tobacco shoot regeneration, tobacco leaf tissues were cut into about 0.5×0.5-cm segments and were grown on MS1 with timentin. In the first experiment, timentin was tested at 0 and 250 mg/l. In the second experiment, timentin was added at 0, 50, 100, 150, 200, and 250 mg/l. For each concentration of timentin, three ratios (30:1, 50:1, and 100:1) of ticarcillin:clavulanic acid were used. Each treatment had three replicates, each consisting of six leaf segments. Shoot regeneration was evaluated at the end of 3 weeks.

For Siberian elm, regeneration followed the procedure of Kapaun and Cheng (1997), with the basal medium being MS supplemented with 10 μM BA and 3% (w/v) sucrose. The medium was solidified with 0.3% (w/v) Phytogel (Sigma Co. St Louis, Mo.). Timentin was used at 0, 200, 300, 400 mg/l at ratios of 50:1 and 100:1 (ticarcillin:clavulanid acid). A completely random design was used. Each treatment was replicated three times each with eight leaf segments. The experiment was conducted twice. Data were recorded 4 weeks after inoculation to medium and analyzed using the general linear model procedure with the protected LSD mean separation technique at the 0.05 confidence level (SAS 1990).

Table 1 The effect of timentin on shoot regeneration of Siberian elm leaf tissues[a]

Timentin		Percentage of explants regenerated ±SE	Percentage of shoots regenerated per explant ±SE
(mg/l)	Ratio (ticarcillin: clavulanic acid)		
0	50:1	60±15 ab	1.7±0.5 a
200	50:1	65± 9 ab	1.7±0.4 a
	100:1	42±10 bc	1.6±0.5 a
300	50:1	44± 7 bc	1.2±0.3 a
	100:1	48± 9 bc	1.5±0.4 a
400	50:1	38±16 bc	1.4±0.4 a
	100:1	31±15 c	1.3±0.3 a

[a] Results were from two separate experiments, each with three replications per treatment. Data were taken 4 weeks after inoculation to medium. Treatment means with the same letters are not significantly different at P=0.05. The basal medium was MS+10 μM BA+3.0% (w/v) sucrose+0.3% (w/v) Phytogel

Timentin had little effect on tobacco and Siberian elm shoot regeneration, therefore, the efficacy of timentin for inhibition of *A. tumefaciens* in genetic transformation was examined. Tobacco leaf segments were infected by dipping in a suspension of *A. tumefaciens* strain LBA4404. Tissue segments were blotted on sterile paper towels and cultured on MS1 medium without antibiotics as described by Horsch et al. (1985). After 2 days of co-cultivation, leaf segments were transferred to medium with either 0 or 250 mg/l timentin at ratios of 30:1, 50:1 or 100:1 in the first experiment, or to medium with 0, 50, 100, 150, 200, and 250 mg/l timentin at the same three ratios in the second experiment, to determine the minimum effective concentration of timentin. Each treatment had 3 plates, each with six leaf segments. After 3 weeks, regenerating leaf segments on medium with 200 or 250 mg/l timentin, which showed no growth of *A. tumefaciens,* were excised and transferred to antibiotic-free medium for 10 days to determine whether the bacterium was suppressed or killed. The third experiment tested timentin concentrations of 500, 1000, 2500, and 5000 mg/l at the ratio of 100:1. All regenerating tissues were transferred to timentin-free medium after 3 weeks to evaluate bacterial growth. Each treatment had 2 plates, each with six leaf segments, and the experiment was repeated once. In the fourth experiment, timentin was compared with a combination of carbenicillin and cefatoxime (Table 1). The infected and noninfected tissues were subcultured on the same medium three times, each with a 2-week interval, and then transferred to antibiotic-free medium for 10 days to evaluate *Agrobacterium* growth. Each treatment had 2 plates, each with six leaf segments, and the experiment was repeated once.

The stability and lifetime of timentin were tested, since the manufacturer recommends that a timentin solution be made from ticarcillin and clavulanic acid and used the same day of its preparation and that solid medium plates with timentin should not be stored for more than 1 week. In the first experiment, a total of 27 petri plates containing MS1 medium with 250 mg/l timentin at ratios of 30:1, 50:1 and 100:1 were placed on an illuminated shelf. After 20, 30 and 70 days, tobacco leaf segments infected with *A. tumefaciens* LBA4404/pBI121 were transferred to 9 of these plates (3 plates per ratio) for an effectiveness assay. In the second experiment, ticarcillin and clavulanic acid stock solutions either separately or mixed were stored at –20°C or –80°C for 4 weeks and then used to make MS1 media containing 250 mg/l timentin at three ratios. Tobacco leaf segments infected with LBA4404/pBI121 were cultured on these media for 3 weeks before *Agrobacterium* growth was evaluated visually. There were 4 plates per treatment with six leaf segments per plate. Bacterial growth was recorded either as negative or positive, indicating *A. tumefaciens* growing on none or at least one leaf segment, respectively.

Results and discussion

Streaking *A. tumefaciens* on medium containing timentin indicated that 250 mg/l of timentin at ratios of 30, 50 and 100:1 strongly inhibited the growth of strains LBA4404, EHA105, and C58. Visual growth was not observed with all three strains on any of the media.

Growth and regeneration of tobacco leaf tissues were affected minimally by timentin at concentrations of 50, 100, 150, 200, and 250 mg/l at ratios of 30:1, 50:1 and 100:1. The only minor effect on tissue growth and regeneration was observed on medium containing either 200 or 250 mg/l timentin at 30:1, or on media containing 1000–5000 mg/l timentin at 100:1. In these cases, the callus turned light yellowish, and shoot regeneration was delayed for about 1 week (data not shown).

The percentage of explants regenerating shoots from Siberian elm leaf tissues was not affected by timentin except at a concentration of 400 mg/l at 100:1 (Table 1). The number of shoots regenerated from leaf explants was not affected (Table 1).

In the first experiment to test the effectiveness of timentin on the suppression of *A. tumefaciens*, extensive growth of *A. tumefaciens* was observed from the infected tobacco tissues on the medium without timentin after 3 weeks but was not detected on media with 250 mg/l of timentin at 30, 50, and 100:1 of ticarcillin to clavulanic acid. This was confirmed in the second experiment where *A. tumefaciens* resumed growth on the medium with 50, 100, and 150 mg/l of timentin but did not show growth on media with either 200 or 250 mg/l of timentin at the same three ratios tested in the first experiment. However, when the tissues that showed no growth of *A. tumefaciens* were transferred to medium free of timentin, *A. tumefaciens* growth recurred. This resumption of growth was also clearly visible when tissues on media with 500, 1000, 2500, and 5000 mg/l of timentin at 100:1 of ticarcillin to clavulanic acid were transferred to timentin-free medium (data not shown). This suggests that timentin was effective as a bacterial static agent but not as a bactericidal agent.

When *A. tumefaciens*-infected tissues were subcultured three times for a total period of 6 weeks, *Agrobacterium* growth was visually detected from only 1 out of 18 tissues on medium with 500 mg/l timentin, and from 3 out of 18 tissues on medium with 500 mg/l carbenicillin and 250 mg/l cefotaxime (Table 2). *Agrobacterium tumefaciens* did not grow on medium with a combination of timentin (500 mg/l) and carbenicillin (250 mg/l) (Table 2). These results indicate that timentin was as effective as a combination of carbenicillin and cefotaxime.

Timentin (250 mg/l), at the three ratios tested, was stable in solid medium for up to 70 days, since growth of *A. tumefaciens* was not observed when infected tissues were inoculated on these media (data not shown). However, timentin stock solutions, either separated or mixed, appeared to be unstable after storage at –20°C or –80°C for 4 weeks. When these stock solutions were used to make media containing 250 mg/l timentin at 30, 50, and 100:1 of ticarcillin and clavulanic acid, *A. tumefaciens* was suppressed only on media with timentin at 30:1 and 50:1 from separated stock solutions which were stored at –20°C and on medium with timentin at 50:1 from a mixed stock solution that was stored at –80°C.

Table 2 Inhibition of *A. tumefaciens* by timentin and carbenicillin/cefatoxime after 3 subcultures[a]

Agrobacterium infection	Antibiotics (mg/l)	Number of segments showing *Agrobacterium* growth after three subcultures/ total inoculated
No	–	0/24
No	Timentin 500 mg/l	0/24
Yes	Timentin 500 mg/l	1/24
Yes	Carbenicillin (500 mg/l) Cefatoxime (250 mg/l)	3/24
Yes	Carbenicillin (250 mg/l) Timentin (500 mg/l)	0/24

[a] Subculture was done every 2 weeks. Timentin consisted of 100:1 (w/w) ticarcillin:clavulanic acid. The growth of *A. tumefaciens* was evaluated visually 10 days after transfer to antibiotic-free medium

An ideal antibiotic for inhibiting *A. tumefaciens* or *A. rhizogenes* in genetic transformation should be highly effective, have no negative effect on plant growth and regeneration, be stable in culture, and be inexpensive. Currently, carbenicillin and cefotaxime are the most commonly used antibiotics for such a purpose. However, tissues need to be subcultured on the antibiotic-containing medium at least two to three times to maintain bacterial suppression, and often it is very difficult to completely eliminate *Agrobacterium* from the tissues of some species (Hammerschlag et al. 1995; Shackelford and Chlan 1996). The high cost of carbenicillin and cefotaxime makes the genetic transformation very costly, especially when developing an optimized transformation system for recalcitrant species (Colby et al. 1991; De Bondt et al. 1994; James et al. 1993). Our results have demonstrated that timentin, a mixture of ticarcillin and clavulanic acid, may be an effective, lower cost alternative antibiotic. Timentin was as effective as carbenicillin and cefotaxime in suppressing *A. tumefaciens*. The compound had little negative effect on tissue growth and regeneration on tobacco and Siberian elm. Timentin can also be an alternative antibiotic for species where the regeneration potential is negatively affected by carbenicillin and cefatoxime. Timentin is stable in culture medium for at least 10 weeks, but is not stable in solutions stored at –20°C or –80°C. On the basis of these results, timentin should be used at 200–500 mg/l at ratios of 50:1 or 100:1 (w/w) ticarcillin:clavulanic acid. An alternative is to use timentin in combination with lower concentrations of carbenicillin. Tissues should be subcultured on timentin-containing medium three times. The use of timentin on callus formation and plant regeneration should be tested for each species.

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