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## THE INDUCTION OF LINCOMYCIN RESISTANCE IN *LYCOPERSICON PERUVIANUM* AND *LYCOPERSICON ESCULENTUM*

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Lincomycin-resistant calli were induced from both *Lycopersicon esculentum* and *Lycopersicon peruvianum* using *N*-nitroso-*N*-methylurea (NMU) mutagenesis. From these calli lincomycin-resistant plants were regenerated. For *L. peruvianum* it was shown that the resistant plants could be divided in two classes with respect to their resistance to lincomycin and its derivative clindamycin. The first class comprised plants which were resistant to 500 mg/l lincomycin and showed no shoot or root formation in the presence of clindamycin; the second class consisted of plants resistant to 2000 mg/l lincomycin and these plants were able to form shoots and roots on clindamycin containing media. Lincomycin is an inhibitor of peptidyltransferase; chloroplast encoded parts of this enzymatic function are sensitive for this antibiotic. Reciprocal crosses between our lincomycin resistant and wild type *L. peruvianum* plants indicated a maternal inheritance of the mutation.

**Key words:** *Lycopersicon*; mutagenesis; protoplasts; in vitro selection; lincomycin resistance; maternal inheritance.

### Introduction

In most plant species chloroplasts are transferred unidirectional in sexual crosses, that is, only cell organelles of the maternal plant appear in the offspring [1]. This implies that only one type of chloroplasts will be present in the progeny. This is in contrast with a bidirectional transfer of organelles as observed in e.g. *Pelargonium* and *Oenothera* [2,3].

Several traits, encoded by chloroplast genomes, are of fundamental and/or agronomic interest. Among these traits are chlorophyll deficiency [4,5], resistance to antibiotics [4,6], resistance against herbicides [7,8] and resistance to toxins produced by plant pathogens [9,10].

An unidirectional transfer of cell organelles can pose the problem that it is not possible to obtain desired nucleus-organelle combinations

since reciprocal crosses may be impossible due to natural barriers (e.g. *L. peruvianum* × *L. esculentum* [11]). Fusion of somatic cells offers the possibility to deliver chloroplasts and mitochondria from donor cells into recipient cells [12,13]. This permits the transfer of cell organelles where this transmission is not possible in a sexual way.

As a plant species to study nucleus-organelle interaction we chose the molecularly and genetically well characterized tomato, *L. esculentum*, an important horticultural crop plant, and its wild relative *L. peruvianum*. In both *L. esculentum* and *L. peruvianum* organelles are inherited unidirectionally, i.e., organelles are only transferred maternally in sexual reproduction [1].

One of the first reported chloroplast encoded markers was chlorophyll deficiency in *Nicotiana tabacum* [5]. Resistance to antibiotics, encoded by chloroplasts, have also been described. In *N. tabacum* SR-1, streptomycin resistance is inherited maternally [6,14]; this resistance is caused by single basepair substi-

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NAA,  $\alpha$ -naphthalene acetic acid; NMU, *N*-nitroso *N*-methylurea.

tutions in the chloroplast 16S rRNA [15,16], or in the chloroplast gene encoding ribosomal protein S12 [17]. Recently, in *L. peruvianum* chloroplast encoded streptomycin resistance has been induced [18]. In *N. plumbaginifolia* [19,20] and *N. tabacum* [21] chloroplast encoded lincomycin resistance has been induced. Lincomycin is an inhibitor of protein synthesis on 70S ribosomes in chloroplasts (but not of the cytoplasmic 80S ribosomes) and inhibits thylakoid membrane formation (and therefore chlorophyll formation) in developing chloroplasts [22]. For *N. plumbaginifolia*, it is shown that the induced resistance depends on a single base pair substitution in a narrow domain of the chloroplast 23S rRNA gene [23]. Somatic cell fusion as a means to transfer cell organelles to recipient plants is a feasible method to study the transfer of new cytoplasmic information into the tomato, using selectable chloroplast markers as described above. Relatively large numbers of hybrid or cybrid cells can be selected for, using a positive selection system. Without these markers only limited numbers of fused cells can be analyzed, e.g. by performing a RFLP analysis at the DNA level, provided a suitable RFLP is available.

To obtain chloroplast encoded antibiotic resistance markers, protoplasts are treated with the mutagen NMU. This mutagen induces point mutations by alkylating nucleotides [24]. Furthermore, NMU is reported to induce plasmome mutations efficiently [25]. As described by Cseplö et al. [23] point mutations in the chloroplast 23S rRNA are associated with lincomycin resistance in *N. plumbaginifolia*.

In this work we describe: (1) the sensitivity of *L. esculentum* and *L. peruvianum* to lincomycin at several developmental stages; (2) the induction of lincomycin resistance in *L. esculentum* and *L. peruvianum*; (3) a partial characterization of the lincomycin resistant *L. peruvianum* variants and (4) inheritance of the lincomycin resistance trait in *L. peruvianum*.

## Materials and Methods

### Chemicals

Lincomycin, clindamycin and streptomycin

were purchased from Duchefa (Haarlem, The Netherlands). *N*-Nitroso-*N*-methylurea (NMU) was purchased from Serva (Heidelberg, F.R.G.). SeaPlaque Agarose was purchased from FMC BioProducts (Rockland, U.S.A.). Plant hormones were purchased from Sigma (St. Louis, U.S.A.)

### Plant material

Seeds of *L. peruvianum* were obtained from Dr. W.H. Lindhout, Centre for Plant Breeding Research (CPO) (Wageningen, The Netherlands). Seeds of *L. esculentum* cv. Moneymaker were obtained from Rijk Zwaan (De Lier, The Netherlands). Seeds were surface sterilized and grown in solidified MS medium [26] supplemented with 1.5% (w/v) sucrose.

The *L. esculentum* genotype MsK9 [27, 28] was obtained from Dr. M. Koornneef (Department of Genetics, Agricultural University, Wageningen, The Netherlands) and was transformed with plasmid pJW3 to introduce a kanamycin resistance gene into the nuclear genome. Plasmid pJW3 is analogous to the kanamycin resistance conferring plasmid pAGS112 used by Van den Elzen et al. [29].

### Plant growth conditions

All plant material was grown in vitro at 25°C; 1500 lux; relative humidity 60% and 16-h photoperiod.

### Protoplast isolation

Protoplasts were isolated as described by Koornneef et al. [28] with some modifications. For both *L. peruvianum* and *L. esculentum* pre-incubation medium and incubation medium CPW salts [30] supplemented with 7.3% (w/v) mannitol + 3 mM MES (pH 5.8) were used. Enzymes for *L. peruvianum* consisted of 0.6% (w/v) cellulysine + 0.1% (w/v) macerace; for *L. esculentum* 1% (w/v) cellulysine + 0.25% (w/v) macerace was used. *L. esculentum* protoplasts were washed with W5 medium [31]. *L. peruvianum* protoplasts were washed in CPW salts + 2% (w/v) KCl + 3 mM MES (pH 5.8). Protoplasts were used at a density of  $2 \times 10^5$  per ml for mutagenesis experiments or  $1 \times 10^5$  per ml for other purposes.

*L. peruvianum* protoplasts were cultured in 1/2V-KM medium [32] supplemented with 1 mg/l NAA + 0.5 mg/l BAP + 0.2 mg/l 2,4-D. *L. esculentum* protoplasts were grown in TM 2 medium [33] supplemented with 34.2 g/l sucrose, 1 mg/l NAA, 0.5 mg/l BAP, 0.2 mg/l 2,4-D. After 7 days of culture *L. esculentum* protoplasts were cultured in TM 2 medium without extra sucrose and in both TM 2 and 1/2V-KM media hormones were replaced by 0.75 mg/l BAP. Plating efficiency was determined as number of calli divided by number of protoplasts.

#### *Callus induction*

Leaf explants were placed on callus inducing medium (MS medium + 2% (w/v) sucrose, 2 mg/l BAP and 0.2 mg/l NAA). Petridishes were placed in the light at 25 °C; every 2 weeks callus was transferred to fresh callus inducing medium.

#### *Shoot induction*

Leaf explants were first placed on callus inducing medium and incubated in the dark. Upon development of a rim of white callus, explants were transferred to shoot inducing medium (solid MS medium + 2% (w/v) sucrose, 2 mg/l zeatin and 0.2 mg/l IAA) and placed in the light. Callus was induced as described; pieces of about 5 mm in diameter were placed on shoot inducing medium and incubated in light.

#### *Mutagenesis of protoplasts*

A 20 mM NMU solution in 0.05 M citric acid/0.1 M Na<sub>2</sub>HPO<sub>4</sub> buffer of pH 5.0 [25] was freshly prepared prior to use. From this stock, NMU was added to protoplasts in a final concentration of 0.03 mM.

#### *Selection for lincomycin resistance*

Calli of 0.5–1 mm were embedded in 0.8% (w/v) SeaPlaque Agarose and transferred to Greening Medium supplemented with 500 mg/l lincomycin. Greening Medium consisted of B5 macro- and micronutrients [34] (minus NH<sub>4</sub>NO<sub>3</sub>), Nitsch vitamins [35], 0.2 M mannitol, 7.3 mM sucrose, 0.55 mM myo-inositol, 0.027 mM glycine, 1 g/l caseinhydrolysate, 0.5 mg/l BAP and 0.05 mg/l NAA.

#### *Shoot and root induction*

Green colonies of about 8 mm diameter were transferred to shoot inducing medium supplemented with 500 mg/l lincomycin. Shoots of about 2 cm tall were transferred to rooting medium (solid MS 20 medium) + 500 mg/l lincomycin.

#### *Test for cross resistance*

Tests for cross resistance against streptomycin and clindamycin (a lincomycin derivative [36]) were performed either by placing leaf explants on shoot inducing medium or placing shoots in root-inducing medium, both containing 500 mg/l antibiotics.

#### *Test for resistance against kanamycin*

The isolated lincomycin resistant *L. esculentum* MsK9 was tested for kanamycin resistance by transferring shoots to medium containing 100 mg/l kanamycin and 500 mg/l lincomycin.

#### *Level of resistance to lincomycin*

This was determined by placing leaf explants from lincomycin resistant *L. peruvianum* plants on shoot inducing medium supplemented with 500, 1000 and 2000 mg/l lincomycin, respectively.

#### *Ploidy level*

Leaf epidermal strips from in vivo or in vitro grown plants were treated with a KI/I<sub>2</sub> solution to stain starch. The number of chloroplasts per guard cell pair was counted using a bright field microscope. The average of 10 guard cell pairs was taken as a measure for ploidy level [37].

#### *Reciprocal crosses*

Reciprocal crosses between lincomycin resistant and wild type *L. peruvianum* plants were performed; seeds were harvested and tested for their ability to germinate on MS20 medium supplemented with 500 mg/l lincomycin. Green plants were re-tested on the same medium before being considered resistant to lincomycin.

**Table I.** The effect of lincomycin on several developmental stages of *L. esculentum* and *L. peruvianum*. Lincomycin was added to the media in concentrations ranging from 0 to 2000 mg/l.

Tissue	Effect observed	Minimal concentration of lincomycin necessary for the observed effect
Seedlings	Reduction in shoot and root length	100 mg/l
Rooting shoots	Reduction in growth and absence of root formation	250 mg/l
Secondary callus growth	Reduction of growth and yellowing	50 mg/l
Greening of callus	No green colour developed	50 mg/l
Shoot formation on leaf discs	Absence of shoots	100 mg/l
Shoot formation on callus	Absence of shoots	50 mg/l
Greening of minicalli	Reduction of greening and callus-size	25 mg/l

## Results

### *Sensitivity of L. esculentum and L. peruvianum to lincomycin*

Plant material from several developmental stages of *L. esculentum* and *L. peruvianum* was tested for sensitivity at concentrations up to 2000 mg lincomycin per litre. For *L. esculentum*, all experiments were performed with genotype MsK9 except for the germination of seeds which was done with *L. esculentum* cv. Moneymaker since the geno-

type MsK9 is not true breeding. The results for *L. esculentum* and *L. peruvianum* were similar (Table I). Germinating seeds developed seedlings which showed reduction in shoot and root length at concentrations from 100 mg lincomycin per litre upward (Fig. 1). Shoots, placed in lincomycin containing medium did not form roots at a concentration of 250 mg lincomycin per litre; growth was retarded at higher lincomycin concentrations. The greening of secondary callus was inhibited by 50 mg lincomycin per litre. On leaf discs shoot formation was



**Fig. 1.** Influence of lincomycin on the growth of seedlings of *L. peruvianum*. Seeds were germinated on MS20 medium, supplemented with (from left to right) 0, 50, 100, 250, 500, 1000 and 2000 mg lincomycin per litre respectively.

prevented at concentrations of 100 mg lincomycin per litre. Shoot formation induced on secondary callus was absent at 50 mg lincomycin per litre; greening of isolated protoplasts was prevented at 25 mg lincomycin per litre, while growth was inhibited at concentrations of 250 mg/l and higher.

#### *Induction of resistance to lincomycin in L. peruvianum*

Protoplasts ( $10^7$ ) of *L. peruvianum* were isolated and cultured into minicalli; the plating efficiency was 6.7%. The  $6.7 \times 10^5$  resulting minicalli (having an average cell number of 20) were selected for greening in the presence of 500 mg/l lincomycin. However, this did not result in green calli showing a resistant phenotype. Therefore, the spontaneous frequency of lincomycin resistance in *L. peruvianum* was lower than  $1.5 \times 10^{-6}$  as compared to the number of minicalli. To increase the frequency of mutation,  $5.4 \times 10^6$  protoplasts were treated with 0.03 mM NMU; after culturing these protoplasts into minicalli, selection was performed as described. Plating efficiency was about 6.2%. In total, 24 green calli from *L. peruvianum* were isolated which remained green after two successive transfers to selective medium, corresponding to a frequency of  $7.2 \times 10^{-5}$ . Twenty-three calli were able to form shoots in the presence of lincomycin. From these 23 calli, shoots were placed in MS20 + 500 mg/l lincomycin; several shoots from 13 independently isolated green calli developed roots in this medium. These shoots were rooted on the same medium twice over before considering the obtained plants as lincomycin-resistant. The other shoots showed no root development.

#### *Induction of lincomycin resistance in L. esculentum*

For *L. esculentum* the plating efficiency was determined to be 2.2%. Among  $2.2 \times 10^5$  minicalli selected for lincomycin resistance no green calli were isolated, therefore the spontaneous frequency of lincomycin resistance in *L. esculentum* was lower than  $4.5 \times 10^{-6}$ . In total  $17.5 \times 10^6$  protoplasts were isolated and mutagen-

ized; plating efficiency was 1.5%. On selective medium, 5 green calli were isolated corresponding to a frequency of  $1.9 \times 10^{-5}$ . From these five calli, four developed shoots from which one also developed roots under simultaneous selective pressure of kanamycin and lincomycin.

#### *Characterization of lincomycin resistant L. peruvianum plants*

*Cross resistance to streptomycin and clindamycin, level of resistance to lincomycin.* In the regenerated lincomycin resistant *L. peruvianum* plants, resistance against streptomycin and clindamycin was tested on root formation by shoots and shoot formation by leaf explants in presence of lincomycin, streptomycin and clindamycin. All tested plants were resistant to lincomycin but sensitive to streptomycin, both as shoots and as leaf explants (Fig. 2).

With respect to clindamycin, differences between independently isolated plants could be observed. Shoots transferred to clindamycin containing medium were able to form roots, but differences existed with respect to root and shoot length. Shoot formation on leaf explants could only be observed in 4 out of 13 plants. Testing the isolated *L. peruvianum* plants for their level of resistance to lincomycin showed that also in this respect differences existed between distinct plants: maximum resistance, as judged by development of shoots on leaf explants in the presence of lincomycin ranged from 500 mg/l to at least 2000 mg/l. Two classes of lincomycin resistant *L. peruvianum* plants were distinguished:

Class I: plants resistant to up to 500 mg/l lincomycin; showing no or restricted root formation on shoots and no shoot formation on leaf explants on clindamycin containing media (isolates LRP 5, -7, -9, -11, -12, -13, -15, -18, -20);

Class II: plants resistant to 1000 or 2000 mg/l lincomycin; developing roots on shoots and shoots on clindamycin containing medium (isolates LRP 2, -25, -26, -27).

*Inheritance of the lincomycin resistance trait in L. peruvianum.* Eight different regenerated lincomycin resistant *L. peruvianum* plants were selected to use for reciprocal crosses. Six of these plants were normal with respect to size

**Table II.** Results of reciprocal crosses between lincomycin-resistant and wild type *L. peruvianum*. Seeds were harvested and tested for germination on medium containing 500 mg/l lincomycin.

Cross	No. of seeds harvested	No. of germinated seeds	No. of lincomycin resistant plants
LRP 2 × wt	3	3	3
LRP 5 × wt	40	40	40
LRP 11 × wt	63	54	54
LRP 25 × wt	10	10	10
wt × LRP 11	42	38	1
wt × LRP 25	5	4	0

and leaf shape; two were aberrant, showing a retarded growth and dark green, thick and lumpy leaves. At least two individuals from six variants formed flowers but only four variants developed fruits after fertilization. The ploidy level of regenerated *L. peruvianum* plants was determined by counting the number of chloroplasts per guard cell pair [37]. All six flowering variants showed no aberrant ploidy level compared to a diploid plant; only one (normal looking) plant, which did not form flowers, was determined to be tetraploid as was the case with the one regenerated lincomycin resistant *L. esculentum* variant.

Reciprocal crosses between flowering lincomycin-resistant and wild type *L. peruvianum* plants resulted in only a limited number of seed bearing fruits; in total 116 seeds were collected from crosses with mutant plants as female. From these seeds 107 germinated giving rise to lincomycin resistant plants only. From the reciprocal cross 47 seeds were collected, 42 seeds germinated from which all but one yielded lincomycin sensitive offspring (Table II and Fig. 3).

## Discussion

Chloroplast encoded resistance to lincomycin can be induced by a single basepair mutation [19,20,23]. For *N. plumbaginifolia*, it has been shown that lincomycin-resistant mutants possess basepair transitions in the chloroplast 23S rRNA genes [23]. The site for resistance to lincomycin was demonstrated to be the peptidyltransferase region of the 50S ribosome subunit, which is blocked by lincomycin. The 23S rRNA is an important component of the reactive region [38]. Because experiments to obtain lincomycin resistance by somaclonal variation were not successful, we concluded that the frequency of spontaneous lincomycin resistance in *L. peruvianum* and *L. esculentum* was lower than approximately  $10^{-6}$ . In our laboratory, Jansen et al. [18] found that the mutation frequency for inducing antibiotic resistance could be increased by two orders of magnitude, using the specific mutagen NMU at a concentration of 0.1 mM. For this reason we treated protoplasts of *L. esculentum* and *L. peruvianum* with NMU.

From the mutagenized protoplasts of both *L. esculentum* and *L. peruvianum*, we isolated lincomycin-resistant calli, which were green in a background of white-brown, lincomycin-sensitive calli. The observed frequency with which the lincomycin resistant calli occurred ( $1.9-7.2 \times 10^{-5}$  as compared to the total amount of microcalli) was somewhat lower than the mutation frequencies as determined by Cseplö et al. for lincomycin resistance in diploid *N. plumbaginifolia* ( $5.8-7.2 \times 10^{-4}$  [20]). This could be due to the lower concentration of NMU used in our experiments (0.03 mM vs. 0.1–0.3 mM). The difference between the frequency of induced resistance and spontaneous resistance is in agreement with the results of Jansen et al. [18]. From resistant calli, shoots were successfully induced under selective pressure for both *L. esculentum* and *L. peruvianum*.

**Fig. 2.** The effect of lincomycin, clindamycin and streptomycin on the growth and rooting of lincomycin resistant shoots of *L. peruvianum* variant LRP 2. The medium contained (from left to right, respectively) 500 mg/l lincomycin, clindamycin or streptomycin.

**Fig. 3.** Offspring obtained from crosses between lincomycin resistant *L. peruvianum* is lincomycin resistant. Germination on MS20 medium containing 500 mg/l lincomycin of seeds from (from left to right): *L. peruvianum* wild type, *L. peruvianum* isolate LRP 2 × *L. peruvianum* wild type and *L. peruvianum* isolate LRP 25 × *L. peruvianum* wild type.







The isolated *L. peruvianum* and *L. esculentum* plants were sensitive to streptomycin (meaning no cross-resistance to streptomycin had been induced) and resistant to the lincomycin derivative clindamycin (7-deoxy-7-chlorolincomycin. In this respect the isolated lincomycin-resistant plants may be similar to the lincomycin resistant mutants of *N. plumbaginifolia* [20]. For *L. peruvianum* differences in resistance to clindamycin were noticed as reflected by the response to root and shoot induction. Clindamycin has a comparable activity to lincomycin [36] and is used to re-test for lincomycin resistance. No plants were found which were resistant to lincomycin but sensitive to clindamycin.

Testing the *L. peruvianum* mutants for their level of resistance to lincomycin and taking into account the results from the tests for clindamycin resistance, we were able to distinguish two classes of *L. peruvianum* mutants. These two classes may be regarded as 'low' and 'high' resistant to lincomycin. To which extent this difference in level of resistance is correlated to a difference in the nature of the induced mutations is not known, but it can be envisaged that these differences are connected to different sites of mutation, as is the case with chloroplast-encoded streptomycin resistances [15,16].

The majority (12 out of 13) of the regenerated *L. peruvianum* plants was normal with regard to ploidy level as judged by chloroplast counting in guard cells. No correlation was found between the ability to flower, which was absent in several plants, and the ploidy level.

For *N. plumbaginifolia* it was shown that lincomycin resistance is inherited maternally [19]. Reciprocal crosses between lincomycin resistant and wild type *L. peruvianum* indicated that also lincomycin resistance in our plants is passed to the offspring as a maternal inheriting trait. Since the resistance is transmitted to the offspring, these plants can be considered to be lincomycin-resistant mutants.

The single lincomycin-resistant plant from the cross *L. peruvianum* wild type  $\times$  *L. peruvianum* LRP 11 is remarkable. This result cannot be explained in terms of Mendelian

genetics; in other words it is not likely that this plant has a nuclear-encoded lincomycin resistance since the reciprocal cross only yielded lincomycin resistant plants from 54 germinating seeds. Whether this result can be explained in terms of biparental inheritance of chloroplasts, which is reported to be a rare event in the genus *Lycopersicon* [39], has to be shown.

We have developed an efficient cell selection system for the cultivated tomato (*L. esculentum*). With the use of both nuclear and organellar encoded antibiotic resistance markers, cell fusion and the fate of cell organelles can be studied. The availability of the described lincomycin resistant *L. esculentum* variant and *L. peruvianum* mutants is part of the development of this system, as is the isolation of cytoplasmic encoded streptomycin resistance [18]. Suitable nuclear resistance markers are kanamycin resistance [40] and hygromycin B resistance [41] which can be introduced by *Agrobacterium* transformation. The intended study can give more insight in processes like cell organelle transfer, organellar DNA recombination [42] and the behaviour of organelles in various nuclear backgrounds. Since chloroplasts encode for several important agronomic traits [7–10,43], the study of their inheritance and transfer by means of cell fusion is also of practical importance for plant breeding.

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