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Pleiotropic effect of the insertion of the *Agrobacterium rhizogenes rolD* gene in tomato (*Lycopersicon esculentum* Mill.)

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Abstract The *Agrobacterium rhizogenes rolD* gene, coding for an ornithine cyclodeaminase involved in the biosynthesis of proline from ornithine, has been inserted in *Lycopersicon esculentum* cv Tondino with the aim of studying its effects on plant morphological characters including pathogen defense response. The analysis of plants transgenic for *rolD* did not show major morphological modifications. First generation transgenic plants however were found to flower earlier, and showed an increased number of inflorescences and higher fruit yield. Transformed plants were also analysed for parameters linked to pathogen defense response, i.e. ion leakage in the presence of the toxin produced by the fungus *Fusarium oxysporum* f. sp. *lycopersici*, and expression of the pathogenesis-related PR-1 gene. All the plants harbouring the *rolD* gene were shown to be more tolerant to the toxin in ion leakage experiments, with respect to the untransformed regenerated controls and the cv Tondino. PR-1 gene expression was quantitated by means of real-time PCR both at the basal level and after treatment with salicylic acid, an inducer of Systemic Acquired Resistance. In both cases the amount of PR-1 mRNA was higher in the transgenic plants. It seems therefore that the transformation of tomato plants with *rolD* could lead to an increased competence for defense response, as shown by toxin tolerance and increased expression of the Systemic Acquired Resistance marker gene PR-1. The results are finally discussed in view of their possible economic relevance.

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Introduction

rol A, B, C and *D* genes have been identified as the main determinants of the hairy root disease caused on dicotyledonous plants by the soil bacterium *Agrobacterium rhizogenes*.

When individual *rol* genes are inserted in plants, they have different phenotypic effects that can be at least in part ascribed to modifications in the endogenous hormone equilibrium and have shown in some cases to be potentially interesting also for economic purposes.

In particular, transformation with the *rolA* gene, whose function is up to now unknown, results in plants with a highly aberrant phenotype, characterized by wrinkled, intensely green leaves, long internodes, dwarfism or semi-dwarfism and retarded senescence (van Altvorst et al. 1992; Schmülling et al. 1993; our unpublished data). The pleiotropic alterations observed in tobacco and potato have led to the hypothesis of a functional imbalance in the auxin/cytokinin ratio in favour of cytokinins (Schmülling et al. 1993). In transgenic tobacco plants a reduction of gibberellic acid content has also been reported, suggesting the involvement of *rolA* in gibberellin metabolism (Dehio et al. 1993).

The *rolB* protein on the other hand, has been shown to have a tyrosine phosphatase activity and therefore a possible role in the auxin signal transduction pathway (Filippini et al. 1996). Tomato plants trasnsgenic for *rolB* have wider and shorter leaves and a reduced apical dominance than the wild-type (van Altvorst et al. 1992; our unpublished data). Moreover, in tobacco leaf-explants and thin cell-layers *rolB* promotes the formation of roots and root meristemoids, respectively, a response that is enhanced in the presence of exogenous auxin (Altamura et al. 1994).

Estruch et al. (1991) have demonstrated that *rolC* can be involved in the release of active cytokinins from their

inactive glucosides due to its cytokinin-β-glucosidase activity. This role is consistent with the observed phenotype of *rolC* -transgenic plants, characterized mainly by a reduction of apical dominance and plant height (Schmülling et al. 1993; Nilsson et al. 1993; our unpublished data). In ornamental plants such as carnation and *Petunia* the insertion of *rolC* leads to the expression of advantageous traits, i.e. increased axillary budbreak and development, better rooting ability of stem cuttings (Zuker et al. 2001), increased branching and reduction in time to flowering (Winefield et al. 1999).

Concerning the *rolD* gene, its function as an ornithine cyclodeaminase enzyme, catalysing the conversion of ornithine to proline, has recently been elucidated (Trovato et al. 2001). In transgenic tobacco plants, *rolD* induces a striking earliness in the induction of the flowering process and an increase in the number of flowers (Mauro et al. 1996), that have been related to an accumulation of proline or to a depletion of ornithine.

In this paper we report that the insertion of *rolD* in tomato (*Lycopersicon esculentum* Mill.) has a pleiotropic effect, affecting traits of economic interest such as plant productivity, as well as characters generally correlated with the defense response to pathogens.

Materials and methods

Bacterial strains and media

Agrobacterium tumefaciens strain GV3101 harbouring the *rolD* construct as described in Mauro et al. (1996) (a generous gift of Dr. M. L. Mauro and Prof. P. Costantino, Dipartimento di Genetica e Biologia molecolare, Università di Roma “La Sapienza”), was grown at 28 °C in YEB medium supplemented with 50 μg/ml of rifampicin and 100 μg/ml of kanamycin.

Plant material

rolD-transgenic tomato plants of the cv Tondino, susceptible to *Fusarium oxysporum* f.sp. *lycopersici* (kindly provided by Petoseed Italia, Parma, Italy), have been obtained following the protocol of Horsch et al. (1988) with some minor modifications. Eleven-day old cotyledons from tomato seedlings grown in axenic culture were cut at both ends and incubated overnight on regeneration medium consisting of LS salts (Linsmajer and Skoog 1965) supplemented with Gamborg’s vitamins and phytoregulators (IAA 0.4 mg/l and BAP 1 mg/l) (LS1G). Cotyledons were infected with *A. tumefaciens* (5×10^9 bacteria/ml), from an overnight culture with the addition of 11 mg/l of acetosyringone, for 30 min in liquid medium without phytohormones with gentle agitation, blotted on sterile filter paper and co-cultivation was carried out for 48 h in the dark. Explants were then transferred to LS1G medium containing 500 mg/l of carbenicillin and 100 mg/l of kanamycicn for the selection of transformants. An aliquot of the cotyledons was plated on non-selective medium to obtain the untransformed regenerated controls. Finally, regenerating shoots were transferred to hormone-free medium for root production and maintained by micropropagation.

Transgenic and control micropropagated plants were grown in vitro at 25 ± 1 °C with a 16 h light-8 h dark photoperiod. For morphological analysis and self-fertilization, plants were transferred to a containment greenhouse and observations were performed twice a week until the appearance of fruits.

Molecular analysis of the transgenic plants

DNA and RNA extractions from the transgenic plants and the regenerated controls were carried out using the Macherey-Nagel “Nucleospin Plant” and “Nucleospin RNA Plant” kits, respectively.

The presence of the inserted gene was assessed by PCR with *rolD*-specific primers, as described in Mauro et al. (1996). RT-PCR for the analysis of transgene expression was performed with the “Titan One tube RT-PCR System” (Roche Molecular Biochemicals), following the manufacturer’s instructions. Control amplifications lacking the reverse transcriptase enzyme were always included to confirm the absence of contaminating DNA.

Copy number of the *rolD* gene was determined by Southern hybridisation (Gene Images, Amersham Biosciences) with fluorescein-labelled vector sequences corresponding to *rolD* or *nos-nptII*. Ten micrograms of DNA double-digested with the restriction endonucleases *Bam*HI/*Bgl*II and *Pst*I/*Bgl*II were separated on 1% agarose gels, transferred to nylon membranes (Roche Molecular Biochemicals), and hybridisation was carried out as recommended by the manufacturer.

SA treatment and real-time PCR

Leaves from mature in vivo plants were detached and immersed in a solution of 1 mM SA. Samples were taken after 4, 24, 48 h and immediately frozen in liquid nitrogen for RNA extraction. Control leaves were immersed in water for 24 h or did not receive any treatment.

Real-time PCR for the quantitative analysis of PR-1 gene expression was performed with the *Taq*Man technology (Applied Biosystems Group) essentially as described by Pinzani et al. (2001). Firstly, 1 μg of total RNA was reverse transcribed with random hexamer primers (*Taq*Man Reverse Transcription Reagents). The PCR reaction mix consisted of 5 μl of the retrotranscription reaction, 6 μM of primers, 5 μM of *Taq*Man fluorogenic probe and 1× *Taq*Man Universal PCR Master Mix in a final volume of 25 μl. Primers for PR-1 amplification were 5′-CCGTGCAATTGTGGGT-GTC-3′ (forward) and 5′-GAGTTGCGCCAGACTACTTGAGT-3′ (reverse), and the sequence of the internal oligonucleotide probe was 5′-AGAGGCCAGACTATAACTACGCTACCAACCAAT-GT-3′.

For each *Taq*Man assay a reference calibration curve was prepared containing 10, 1, 0.1, 0.01, and 0.001 ng of retrotranscribed PR-1 plasmid (a generous gift of Prof. P.J.G.M. de Wit, Wageningen University, The Netherlands). Amplification and detection were performed with the ABI Prism 7700 system with the following profile: one cycle of 2′ at 50 °C, one cycle of 10′ at 95 °C, 50 cycles of 15″ at 95 °C and 1′ at 60 °C.

Ion leakage

Ion leakage measurements in the presence or absence of 5 mM fusaric acid (Sigma) were carried out on 1-g leaf disks from four leaves of different transgenic, control or Tondino plants (0.25 g/plant), as previously described (Storti et al. 1992).

Results

One thousand one hundred and sixty two cotyledons of *L. esculentum* cv Tondino (Petoseed Italia, Parma, Italy) were used for transformation with the *A. rhizogenes rolD* gene. A regeneration frequency of 6.5% was observed for the transformed cotyledons, with respect to 31.3% for the untransformed controls.

The putatively transformed plantlets were micropropagated on selective medium prior to molecular analysis.

Table 1 Student's *t*-test analysis for characters observed in the *rolD*-transgenic plants and the corresponding untransformed regenerated controls

Character	Groups	Mean ± SE	*df*	*t*	*P*-value
No. inflorescences/plant	rolD3	15.1 ± 1.8	21	0.6	n.s.
	Controls	14 ± 1			
	rolD4	17 ± 0.8	20	1.5	n.s.
	Controls	14 ± 1			
	rolD23	22.6 ± 2.5	20	3.7	< 0.01
	controls	14 ± 1			
No. fruits/plant	rolD3	40.5 ± 1.8	21	4.3	< 0.01
	Controls	23.6 ± 2.2			
	rolD4	37.6 ± 2.2	20	3.3	< 0.01
	Controls	23.6 ± 2.2			
	rolD23	37.6 ± 1.3	20	3.4	< 0.01
	Controls	23.6 ± 2.2			
Mean weight fruits/plant	rolD3	8.3 ± 0.3	21	2	n.s.
	Controls	9.2 ± 0.2			
	rolD4	10 ± 0.35	20	1.8	n.s.
	Controls	9.2 ± 0.2			
	rolD23	9.2 ± 0.7	20	0.012	n.s.
	Controls	9.2 ± 0.2			
Plant height (first inflorescence)	rolD3	16.5 ± 1.4	22	1.067	n.s.
	Controls	18 ± 0.6			
	rolD4	19.6 ± 2	21	0.9	n.s.
	Controls	18 ± 0.6			
	rolD23	21 ± 1.8	21	1.82	n.s.
	Controls	18 ± 0.6			
Plant height (first fruit)	rolD3	68 ± 4.4	21	1.6	n.s.
	Controls	59.5 ± 2.6			
	rolD4	66.2 ± 3.1	20	1.28	n.s.
	Controls	59.5 ± 2.6			
	rolD23	64.2 ± 0.6	20	0.9	n.s.
	Controls	59.5 ± 2.6			
No. days before flowering	rolD3	15.6 ± 0.8	22	3.5	< 0.01
	Controls	18.5 ± 0.4			
	rolD4	15.2 ± 0.9	21	3.8	< 0.01
	Controls	18.5 ± 0.4			
	rolD23	15.8 ± 0.7	21	3.3	< 0.01
	Controls	18.5 ± 0.4			

The presence of the transgene was assessed on ten different clones derived from ten plants by PCR with *rolD*-specific primers. A control PCR with primers for the β-1,3-glucanase gene was always performed in order to verify the correct amplification of the samples. All the clones analysed contained the transgene, no fragments being amplified in the control plants (data not shown). When *rolD* expression was analysed by RT-PCR, as described in Materials and methods, 3/5 clones showed amplification, thus demonstrating that not all the inserted genes were expressed, probably due to different landing sites in the tomato genome. Based on these data, the three clones harbouring and expressing the transgene (rolD3, rolD4 and rolD23) were chosen for further analysis.

The characterization of the transgenic clones was completed with the determination of transgene copy number, which was performed by Southern hybridization with fluorescein-labelled probes derived from either *rolD* or *nos-nptII* vector sequences. Results showed that rolD3 and rolD23 plants had only one copy of the transgene, while in rolD4 two copies had been inserted.

Six plants from each transgenic clone, three plants each for the untransformed regenerated controls, 1, 4, 5, 6, 9, 33 and ten seed-derived plants of the cv Tondino, were then transferred to the greenhouse for morphological analysis and self-fertilization. Observations were performed twice a week until the appearance of the first fruit, and the following characters were recorded: setting of the first inflorescence, plant height at the appearance of the first inflorescence and the first fully developed fruit, the total number of inflorescences and fruits, and fruit weight. The data obtained were subjected to statistical analysis using the Student's *t*-test (Table 1).

The overall aspect of mature *rolD* plants was not significantly different from both the untransformed regenerated controls and the cv Tondino, except for an increased branching that gave rise to a slightly "bushy" appearance. Concerning the analysed characters, transgenic plants showed a significant earliness in flowering (P-value < 0.01), while the average weight of fruits/plant and plant height were not significantly different from those of controls. The total number of inflorescences per plant was significantly increased (P-value < 0.01) only in the rolD23 clone. However, the most striking change observed regarded fruit number that was significantly higher in the transgenic plants (P-value < 0.01), with an increase of 58.3% in rolD3, and 62.8% in rolD4 and

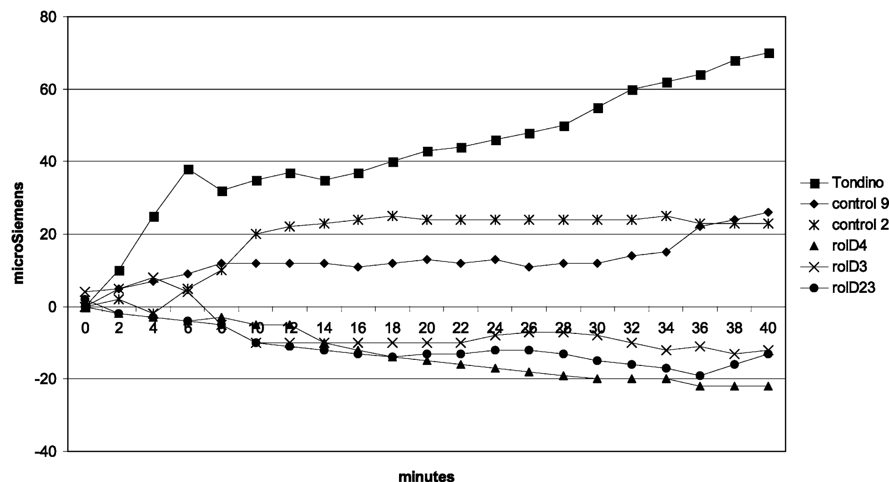

Fig. 1 Ion leakage curves for seed-derived cv Tondino plants, untransformed regenerated controls and *rolD*-transgenic tomato plants in the presence of 5-mM fusaric acid

Table 2 Basal level of expression of PR-1 in the *rolD*-transgenic clones, the untransformed regenerated control 6 and the seed-derived Tondino plants. Quantitation of the PR-1 mRNA was performed by real-time PCR as described in the text

Clone	Transgene copy no.	ng mRNA PR-1/μg RNA
Tondino		0.244
Control 6		15.82
rolD3	1	369.5
rolD4	2	53.6
rolD23	1	230.9

rolD23. Significance was higher when *rolD* plants were compared with seed-derived Tondino (data not shown).

To investigate whether the insertion of the *rolD* gene had an influence on the defense response to pathogens or not, two parameters that could be indicative of an increased "competence" for two different defense processes were evaluated, i.e. ion leakage in the presence of fusaric acid, the toxin produced by the phytopathogenic fungus *F. oxysporum* f. sp. *lycopersici*, and the expression of the pathogenesis-related PR-1 gene, considered to be a marker for Systemic Acquired Resistance (SAR).

Ion leakage experiments were carried out on leaves from four different greenhouse-grown plants for each clone, in the presence of 5 mM fusaric acid, as previously described. Results (Fig. 1) show that all the three transgenic clones exhibited a higher level of tolerance to the toxin when compared to the untransformed regenerated controls, the seed-derived Tondino showing the greatest susceptibility.

PR-1 basal expression was quantitatively analysed through real-time PCR on total RNA extracted from greenhouse-grown transgenic and control plants. The basal level of expression (Table 2) was again higher in all the transgenic clones with respect to the control. Finally, we have also quantitated the PR-1 mRNA after treatment with an inducer of SAR, salicylic acid (SA) on rolD4, the control clone 6 and Tondino (Fig. 2). Detached leaves were immersed in 10 mM of salicylic acid and samples taken after 4, 24 and 48 h, while controls were treated with water for 24 h, or received no treatment. Data show that salicylic acid was effective in all cases in inducing

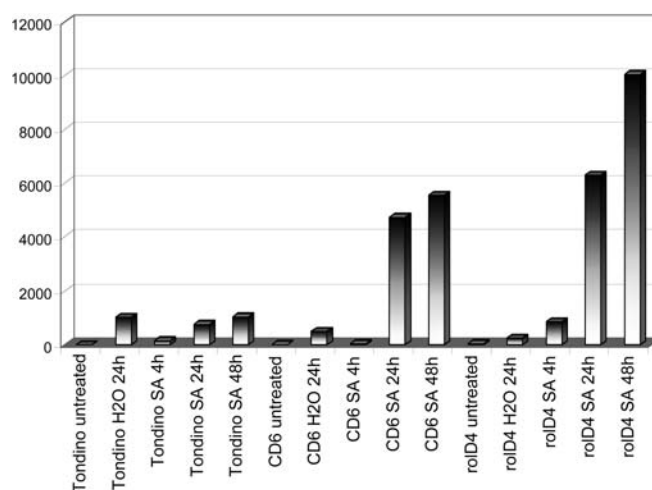

Fig. 2 Amount of PR-1 mRNA (ng/μg total RNA) determined by real-time PCR in the leaves of seed-derived plants of the cv Tondino, the untransformed regenerated clone 6 (CD6) and the transgenic clone rolD4 after treatment with 1 mM SA for 4, 24 and 48 h. Controls were treated with distilled water for 24 h or received no treatment (untreated)

PR-1 expression in a time-dependent manner, the highest amount of PR-1 mRNA/μg of RNA being found in the transgenic clone rolD4. It is worth noting that control regenerated plants were less susceptible to the toxin and more responsive to salicylic acid than Tondino seed-derived ones.

Discussion

In this paper we show that the insertion of the *A. rhizogenes rolD* gene in the tomato has a pleiotropic effect on characters of potential economic interest, such as plant productivity and response to a pathogen.

The most-striking phenotypic effect of transgenosis was in fact a highly significant increase in the number of fruits/plant produced by the three transgenic clones analysed with respect to both the untransformed regenerated controls and the cv Tondino, while fruit weight did

not vary (Table 1). The number of inflorescences/plant on the other hand was significantly higher when compared to controls only in the clone rolD23, although transgenic plants always showed more fruits. This finding is only apparently in contradiction with the higher number of fruits produced by the transgenic plants, as in this case the increase could be due either to a higher number of flowers/inflorescence, or to a higher rate of transition from flowers to fruits.

This effect of *rolD* is completely new, as it has not been reported in earlier work on plants transformed with the same gene (Mauro et al. 1996; Trovato et al. 1997), and has the potential, if confirmed in segregating progenies, to be exploited for economic purposes. In fact, the insertion of *rolD* in tomato did not induce any morphological aberration, and fruits were perfectly normal and set fertile seeds. On the other hand, our observation of a significant earliness in flower setting of *rolD* plants, even if not as striking as in the case of tobacco, was consistent with previous findings (Mauro et al. 1996).

In order to test if the transformation with *rolD* had an influence on defence from pathogens, we analysed two parameters known to be indicative of active defence, ion leakage in the presence of fungal toxins and expression of the pathogenesis-related gene PR-1.

All the transgenic clones were shown to be tolerant to the toxic effect of fusaric acid in ion-leakage experiments, while both the regenerated untransformed controls and the cv Tondino were susceptible (Fig. 1). Ion leakage in the presence of purified fungal toxins and/or culture filtrates has been shown to be positively correlated with in vivo resistance in several plant-parasite interactions (Buiatti and Ingram 1991; Storti et al. 1992), and can therefore be considered as a marker for pathogen tolerance.

Concerning the expression of the pathogenesis-related gene PR-1, the level of the corresponding mRNA was higher in the transgenic clones both constitutively and after SA treatment (Table 2 and Fig. 2). SA is known to induce pathogenesis-related proteins, and in fact an increase in gene expression was observed also in the regenerated controls and to a much lesser extent in the cv Tondino. However induction of PR-1 expression was observed already at 4 h after treatment in the *rolD* plants, and at 48 h was 2-fold higher than in the untransformed control and 10-fold than in the Tondino seed-derived plants.

No clear explanation is as yet available for the role of an accumulation of proline or a decrease of ornithine in the defence response. However, proline has been shown to be involved in the response of plants to diverse environmental stresses, and in particular to drought and high salinity where it is supposed to act as an osmoprotectant (Hare and Cress 1997; Nanjo et al. 1999). The putative involvement of proline in the plant defence response is mainly attributed to the fact that it is a constituent of hydroxyproline-rich glycoproteins (HRGPs), a major class of cell-wall structural components. The expression of genes coding for HRGPs increases after fungal infection or wounding (Corbin et al. 1987), and the oxidative cross-linking of (hydroxy)proline-rich cell-wall proteins contributes to the toughening of the cell wall during the hypersensitive response (Bradley et al. 1992).

An interesting point with respect to our data is the finding (Hoyos and Zhang 2000) that exposure to high osmotic potentials by treatment with NaCl, proline or sorbitol induces in tobacco the activation of a salicylate-induced protein kinase (SIPK). SIPK is also induced by pathogens and elicitors, and is involved in the activation of defense-related genes (Zhang and Klessig 1997; Zhang et al. 1998). A relationship between protein kinase-mediated signal transduction in the defense response and the activation of pathogenesis-related gene expression has been clearly established in several instances (Lee et al. 2001; Murillo et al. 2001); therefore it is tempting to speculate that in the transgenic plants the increase in proline content due to the presence of *rolD* could induce SIPK, starting a signal transduction cascade whose final outcome was PR-1 gene expression.

Ornithine, on the other hand, could not be of importance per se but rather as a precursor of polyamines, small molecules involved in plant growth, development and stress responses (Martin-Tanguy 2001). However, even if there is increasing evidence for a role of polyamines in the plant defense response (Cowley and Walters 2002; Rabiti et al. 1998; Edreva 1997; Yamakawa et al. 1998), it is not possible so far to find a link between a decrease in polyamine content due to the depletion of ornithine or a stimulation of polyamine biosynthesis following SA treatment, and a stimulation of the synthesis of pathogenesis-related proteins.

In conclusion, available data seem to favour the possibility that the observed effect on parameters linked to pathogen defense response could be due to an increase in proline content in the plants bearing the ornithine cyclodeaminase *rolD* gene. Further investigations are in progress in order to demonstrate the validity of our hypothesis.

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