ORIGINAL ARTICLE

# Agrobacterium rhizogenes rolA gene promotes tolerance to Fusarium oxysporum f. sp. lycopersici in transgenic tomato plants (Solanum lycopersicum L.)

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**Abstract** In order to assess the role of *Agrobacterium rhizogenes rol* genes on the defence response of plants to pathogens, tomato plants (*Solanum lycopersicum* L.) were transformed with the *rolA* gene. Consistently with previous descriptions of *rolA*-induced phenotype, insertion of this gene had a pleiotropic effect determining highly aberrant plants, with wrinkled, intensely green leaves, thick stems and small fruits often lacking seeds. Infection of transgenic plants with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* showed the acquirement of resistance/tolerance to the pathogen as evaluated both on the primary transformants by electrolyte leakage and on the transgenic progenies by direct infection. Determination of the endogenous levels of indole-3-acetic acid (IAA) and abscisic acid (ABA) showed a 30–35 % decrease of both phytohormones in *rolA* plants harbouring three copies of the transgene compared to the controls, while a significantly lower level of ABA was observed in plants with one copy of the transgene. This is the first demonstration of the direct involvement of *rolA* gene in plant pathogen tolerance acquisition.

**Keywords** ABA · IAA · Defence response · *Fusarium oxysporum* · *rolA* gene · Tomato

**Abbreviations**

| | |
|---|---|
| ABA | abscisic acid |
| IAA | indole-3-acetic acid |
| PCR | polymerase chain reaction |
| RT-PCR | reverse transcription polymerase chain reaction |

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## Introduction

The soil bacterium *Agrobacterium rhizogenes* is the causal agent of hairy root disease in Dicotyledons. A central role in pathogenesis is ascribed to the *rol* oncogenes (*rolA, B, C* and *D*) carried on the Ri plasmid, which are transferred to the plant cell and are essential for hairy root production (Costantino et al. 1994; Nilsson and Olsson 1997).

The *rolD* gene encodes the enzyme ornithine cyclodeaminase involved in the biosynthesis of proline (Trovato et al. 2001); the product of the *rolB* gene has tyrosine phosphatase activity (Filippini et al. 1996), while that of *rolC* has been proposed to act as a non-specific glucosidase hydrolyzing plant cell walls and releasing oligosaccharides (Faiss et al. 1996). No clear indication is yet available concerning the possible role of the *rolA* gene, encoding a small (11.4 kDa) basic protein with no significant sequence homology to known proteins (Vilaine et al. 1998; Ridgen and Carneiro

1999). Based on the currently available experimental evidence more functions can be proposed for the *rolA* gene. Its biochemical characteristics initially led to the hypothesis that RolA could regulate gene expression in response to auxins (Vansuyt et al. 1992). In accordance with these results, Ridgen and Carneiro (1999) using comparative protein modelling found that the RolA protein sequence adopted the same folding as the bovine papillomavirus-1 E2 DNA-binding domain, and in preliminary in vitro experiments observed binding of a *rolA*-GST fusion protein to DNA. On the other hand, in tobacco plants expressing a rolA::GUS translational fusion GUS activity was associated with the plasmalemma fraction, suggesting that RolA could be be a non-integral, plasmalemma-associated protein (Vilaine et al. 1998). Subsequently, Barros et al. (2003) reported a 50-fold increase of beta-glucoronidase (GUS) activity in transformed tobacco leaf extract expressing GUS and RolA as a fusion protein. The study revealed that RolA could have a role in protection from proteolysis and proposed that it interfered with the protein degradation pathway. The resulting increase in protein stability could extend the duration of RolA effect in the plant.

Transformation with *rolA* of different plant species produces a pleiotropic phenotype characterized by wrinkled, intensely green leaves, reduced height and root system, decreased flower number and sterility (van Altvorst et al. 1992; Schmülling et al. 1993; Holefors et al. 1998; Zia et al. 2010). *rolA*, even if in a less severe form than *rolB*, also induces in vitro root formation in some plant cells and tissues (Spena et al. 1987; Vilaine et al. 1998).

The observed phenotypic modifications suggest alterations in metabolism and/or sensitivity to different phytoregulators. *rolA*-transgenic plants and tissues have in fact shown enhanced sensitivity to auxins (Maurel et al. 1991; van Altvorst et al. 1992), altered gibberellin content (Dehio et al. 1993; Schmülling et al. 1993; Moritz and Schmülling 1998) and polyamines metabolism (Sun et al. 1991; Altabella et al. 1995; Ben-Hayyim et al. 1996). The *rolA* gene also appears to influence secondary metabolism: in transgenic tobacco root cultures nicotine production is increased (Palazón et al. 1997), and in transgenic *Rubia cordifolia* calli a higher anthraquinone production with respect to control has been observed (Shkryl et al. 2008).

*rol* genes have been investigated concerning their role in pathogen response. In tomato the insertion of the *rolD* gene led to an increased competence for active defence, as shown by the acquirement of *Fusarium* toxin tolerance and the enhanced expression of the systemic acquired resistance marker gene PR-1 (Bettini et al. 2003). The *rolB* and *rolC* genes have been proposed to activate plant defence reactions by modulating intracellular ROS levels, stress resistance of transformed cells and inhibition of programmed cell death (Bulgakov et al. 2013). Arshad et al. (2014) have recently shown that tomato plants transformed with the *rolB* gene display foliar resistance to the fungal pathogens *Fusarium oxysporum* and *Alternaria*

*solani* that could be due to an increase in the content of secondary metabolites, especially phenolics.

The present paper describes the effect of *rolA* on the interaction between tomato (*Solanum lycopersicum* L.) and the phytopathogenic fungus *F. oxysporum* f. sp. *lycopersici* race 0. *Fusarium* wilt is one of the most destructive diseases of tomato, causing severe damage and crop losses (Agrios 2005). The fungus lives in soil and infects the plant through the root system. The growth of the mycelium inside the vascular system leads to plant colonization and determines the typical wilting symptoms. *Fusarium* also produces a non specific toxin, fusaric acid, whose effects on plant cells and tissues include alteration of membrane permeability, increase of electrolyte leakage, production of reactive oxygen species and induction of phytoalexin synthesis (Marrè et al. 1993; Bouizgarne et al. 2006).

The tomato cultivar Tondino has been transformed with the *rolA* gene and the acquirement of resistance/tolerance to *F. oxysporum* has been evaluated both on the primary transformants by ion leakage and on the transgenic progenies by direct infection with the pathogen.

## Materials and methods

### Bacterial and fungal strains and media

*Agrobacterium tumefaciens* strain GV3101 harbouring the *Agrobacterium rhizogenes rolA* gene under the control of its own promoter was grown at 28 °C in YEB medium supplemented with 50 μg ml$^{-1}$ rifampicin and 100 μg ml$^{-1}$ kanamycin. *Fusarium oxysporum* f. sp. *lycopersici* race 0 isolate "Cesena" (kindly provided by SAIS, Cesena, Forlì, Italy) was grown on Potato Dextrose Agar medium (BD Difco™) at 23 ± 1 °C, photoperiod 12 h light-12 h dark.

### Plant material

Tomato plants of cultivar Tondino, susceptible to *F. oxysporum* f. sp. *lycopersici* (kindly provided by Petoseed Italy), transgenic for *rolA* gene were obtained as described in Bettini et al. (2003). Transgenic and control micropropagated plants were grown in vitro at 25 ± 1 °C with a 16 h light-8 h dark photoperiod. The first generation transgenic plants and the corresponding untransformed in vitro-regenerated controls were transferred to a containment greenhouse for morphological analysis and self-fertilization.

### Molecular analysis

DNA from plant leaves was extracted using the "Nucleospin Plant II" kit (Macherey-Nagel GmbH and Co. KG). The presence of the inserted gene was assessed by PCR with the following primers: *rolA* forward 5'-GTTTATCGCTCGTCTGTTCT-3'

and *rolA* reverse 5'-GTATTTGTCTATCTTTCTCGC-3'. An amplification with primers for a β-1,3-glucanase gene (GenBank Accession no. M80608) was always carried out in order to verify the correct amplification of the samples. The sequence of the primers was *glu* forward 5′-ATTGTTGGGTTTTTGAGGGAT-3′ and *glu* reverse 5′-TTTAGGTTGTATTTTGGCTGC-3′. Amplifications were carried out on 500 ng genomic DNA with 0.2 mM dNTPs, 0.5 μM primers and 1 unit of *Taq* polymerase (DreamTaq, Fermentas Thermo Scientific Molecular Biology Inc.) in a PTC-200 thermal cycler (Bio-Rad Laboratories Inc.). Copy number of the inserted gene was determined by inverse PCR (iPCR) as described by Does et al. (1991). In short, genomic DNA was digested with the restriction endonuclease *Taq*I, circularized by ligation and linearized again by digestion with three different enzymes, *Mst*II, *Ssp*I and *Sst*II. The junction fragments, i.e. the sequences of the plant genomic DNA flanking the known T-DNA sequences, were then amplified by PCR with primers designed on the sequence of the construct (nopaline synthase 5′ regulatory region and neomycin phosphotransferase II gene, respectively).

RNA extractions from plant leaves were carried out using the "Nucleospin RNA Plant" kit (Macherey-Nagel GmbH and Co. KG). RT-PCR for the analysis of transgene expression was performed with the "Titan One Tube RT-PCR System" (Roche Applied Science) with the following primers: *rolA1* forward 5′-TAAGCTTGTTAGGCGTGCAA-3′ and *rolA1* reverse 5′-AATCCCGTAGGTTTGTTTCG-3′. Control amplifications lacking the reverse transcriptase enzyme were always included to confirm the absence of contaminating DNA.

### Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out according to Salvianti et al. (2008). Five-hundred ng of total RNA were reverse transcribed (TaqMan Reverse Transcription Reagents, Applied Biosystems by Life Technologies) in a final volume of 25 μl and 2 μl of the reaction were then amplified with either *rolA1* or 18S rDNA gene primers. Primers for 18S amplification were as follows: 18S forward 5′-AATGATTAACAGGGACAGTCG-3′ and 18S reverse 5′-ACCTTGTTACGACTTCTCCTT-3′. Ten microliters aliquots of the PCR were finally run on an agarose gel and photographed (Gel Doc 2000, Bio-Rad Laboratories). ImageJ (http://imagej.nih.gov/ij/) analysis was performed to evaluate the amount of *rolA* transcript level by normalization with the 18S rDNA transcript. Statistical significance of the observed differences was evaluated with the Student's *t* test.

### Electrolyte leakage

Electrolyte leakage measurements were carried out as described (Storti et al. 1992), with minor modifications. One gram of leaf disks (7 mm diameter) from four leaves of four different Tondino plants, transgenic plants or untransformed regenerated controls (0.25 g/plant) was incubated in 25 ml of a 37 g $1^{-1}$ sucrose solution supplemented or not with 5 mM fusaric acid (Sigma Aldrich). Conductivity was measured with a digital conductivity meter (Top Tronic X74174) every 2 min for 60 min.

### Extraction and quantification of indole-3-acetic acid (IAA) and abscisic acid (ABA)

Determination of IAA and ABA levels in the primary rolA-2 and rolA-23 transformants and the corresponding controls was carried out as previously described (Baraldi et al. 1995; Bettini et al. 2010). Phytohormone amounts were determined from at least triplicate samples. Statistical significance of the observed differences was determined with the Tukey test.

### Infection with *F. oxysporum* f. sp. *lycopersici* race 0 and statistical analysis

Selected second-generation rolA-2 and rolA-23 transgenic plants, the corresponding controls and seed-derived cultivar Tondino were infected with *F. oxysporum* f. sp. *lycopersici* and screened for symptom development. Fifteen day-old cotyledonary plantlets (20/genotype) were infected under controlled conditions by immersion of the rooting apparatus into a fungal suspension ($1.5 \times 10^6$ conidia ml$^{-1}$), while between five and ten uninfected plants were used as a positive control. Before immersion into the conidial suspension, the roots were cut to facilitate fungal entry. Symptoms were evaluated 15, 30 and 40 days after inoculation by dividing the plants in three classes: healthy, with green cotyledons and true leaves; diseased, with yellow, wilting cotyledons; dead. A Disease Index (DI) was calculated by assigning a value of zero to healthy plants, one to diseased plants and two to dead plants; this value was then multiplied by the number of plants of each progeny having the same value. The resulting values were finally divided by the total number of plants of the progeny. All the infection experiments were performed in two replicates. Significance of the observed differences was evaluated by Tukey test with the PAST 3.x software (Hammer et al. 2001).

## Results

### Molecular and morpho-physiological analysis of primary transformants

Five-hundred eighty cotyledons of 11 day-old *Solanum lycopersicum* cv. Tondino plantlets were used for transformation with the *A. rhizogenes rolA* gene. Regeneration frequencies were 12.3 % for the transformation with *rolA* and 35.5 % for the untransformed controls. The primary regenerants obtained were micropropagated and the presence and the expression of the inserted gene assessed by PCR and RT-PCR,
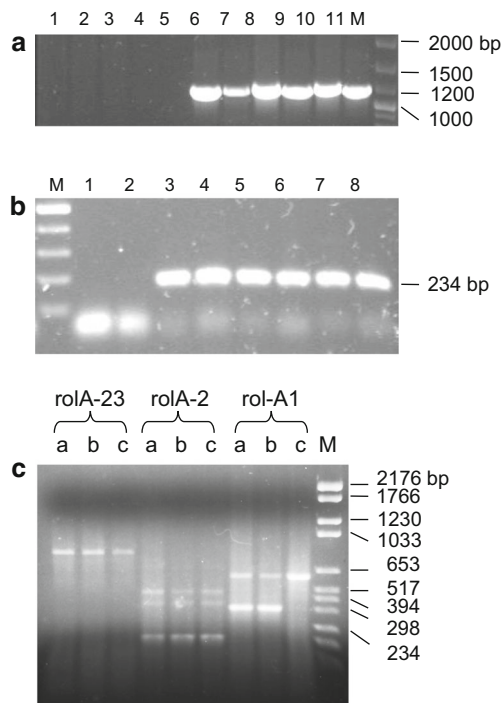
Fig. 1 Molecular analysis of the *rolA*-transgenic plants. **a** PCR amplification with *rolA*-specific primers. Lanes 1–5, controls; lanes 6–11, rolA-1, rolA-2, rolA-6, rolA-9, rolA-12, rolA-23; M, molecular weight marker (GeneRuler 100 bp DNA Ladder Plus, Fermentas Thermo Scientific Molecular Biology Inc.). **b** RT-PCR for *rolA* expression. Lanes 1–2, controls; lanes 3–8, rolA-1, rolA-2, rolA-6, rolA-9, rolA-12, rolA-23; M, molecular weight marker. **c** Copy number determination for the transgenic clones rolA-1, rolA-2 and rolA-23 by iPCR. For each clone the products of PCR on the restriction fragments derived from the digestions with *Sst*II (**a**), *Ssp*I (**b**) and *Mst*II (**c**) are shown. M, molecular weight marker VI (Roche Applied Science)

respectively. The transgene was found in all the clones analysed, but was expressed only in some of them possibly due to different landing sites in the tomato genome. Results obtained for 6 transgenic clones (rolA-1, rolA-2, rolA-6, rolA-9, rolA-12 and rolA-23) are reported in Fig. 1a, b. Further

analysis to determine transgene copy number was performed, showing that the transgenic plants had one to three *rolA* copies (Fig. 1c). The presence of only one amplification product on the *Mst*II fragment of the rolA-1 clone was probably due to the presence of a *Mst*II site between the genomic *Taq*I site and one of the copies of the integrated T-DNA. The transgenic clones rolA-1, rolA-2 and rolA-23 having different transgene copy number, and the corresponding untransformed, in vitro-regenerated controls (four clones) were multiplied by vegetative propagation. Ten plantlets from each transgenic clone, three for each of the untransformed controls and ten seed-derived plants of the cultivar Tondino were then transferred to a containment greenhouse for morphological analysis and self-fertilization.

Consistently with previous descriptions of *rolA*-transformed tomato plants (van Altvorst et al. 1992), all the transgenic plants were highly aberrant with wrinkled, intensely green leaves, thick stems and small fruits often lacking seeds (Fig. 2). Expression levels were evaluated by semi-quantitative RT-PCR on the rolA-23 and rolA-2 clones, having one and three copies of the transgene, respectively, and exhibiting the strongest phenotypic differences. Results (Fig. 3) showed a direct correlation among transgene copy number, increased expression level and plant severity.

## Electrolyte leakage

To investigate whether insertion of the *rolA* gene could affect the defence response to *F. oxysporum* f. sp. *lycopersici*, electrolyte leakage in the presence of fusaric acid was evaluated on the primary transformants rolA-1, rolA-2 and rolA-23, the corresponding control clones 1 and 9 and seed-derived Tondino plants. Results (Fig. 4) showed that all the transgenic clones exhibited a higher level of tolerance to the toxin when compared to the untransformed regenerated controls, whose susceptibility to fusaric acid was even higher than that of seed-
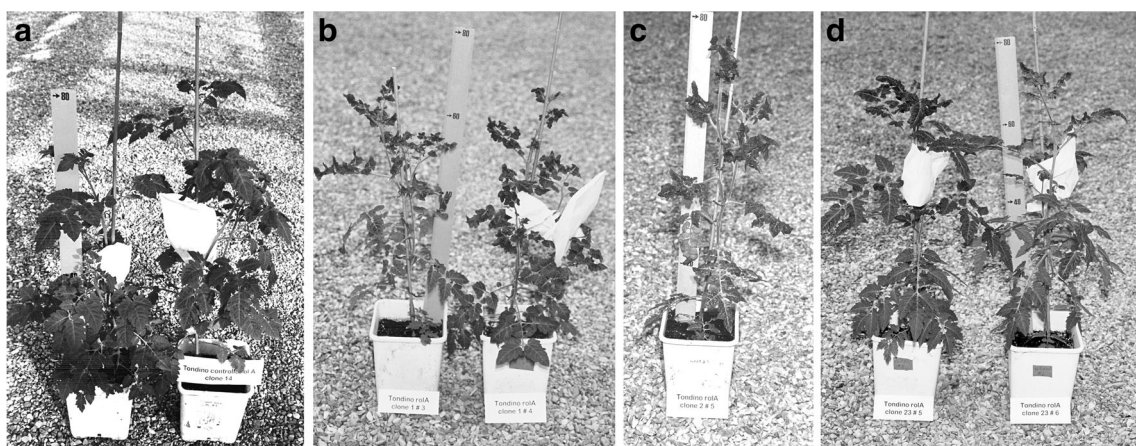


Fig. 2 Effect of the *rolA* gene on the phenotype of tomato plants of the cv. Tondino. **a** untransformed in vitro-regenerated controls; **b** clone rolA-1 (two copies of the transgene); **c** clone rolA-2 (three copies of the transgene); **d** clone rolA-23 (one copy of the transgene). Paper bags protect inflorescences for self-fertilization
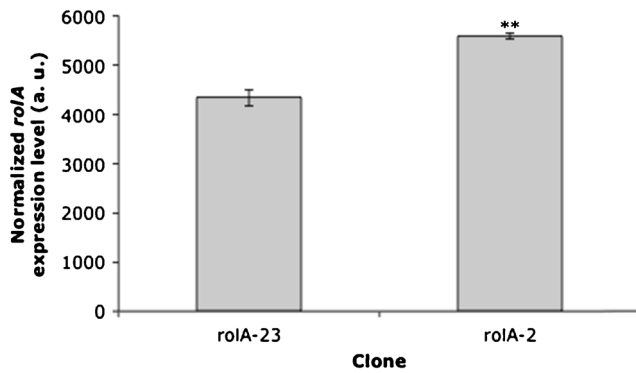
**Fig. 3** *rolA* gene expression levels in transgenic plants with different copy number. The amount of rolA-2 and rolA-23 transcript was normalized with 18S rDNA. Significance of the difference between the clones is indicated with two asterisks (Student's *t*, $P \leq 0.01$). Three replicates per sample were analysed. a. u., arbitrary units

derived Tondino plants. The rolA-23 clone appeared to be the most resistant to fusaric acid.

### Levels of endogenous IAA and ABA in the apical shoots

The clone rolA-2, harbouring three copies of the transgene, showed a significant 30–35 % decrease in the amount of both phytohormones compared to controls, while the clone rolA-23 (one copy) was characterized by only a significantly lower level of ABA (Table 1).

### Infection with *Fusarium oxysporum* f. sp. *lycopersici*

Our previous molecular PCR analyses had found a direct correlation between aberrant phenotype and presence of *rolA* insertion (data not shown). Consequently, transgenic plants

were identified in the progenies by selecting the ones with aberrant phenotype. These plants and untransformed regenerated controls were infected with *F. oxysporum* f. sp. *lycopersici* race 0 as described in Materials and Methods. Analysis was performed by calculating for each sample the average DI at different time points (15–30–40 d after infection) (Fig. 5). Up to 30 d after infection both rolA-23 and rolA-2 plants showed a level of tolerance to *Fusarium* significantly higher than the controls as the average DI 30 d after the infection was respectively 0.47 and 0.78 for the transgenic plants, being 1.02 for the untransformed control individuals. However, 40 d after the infection only rolA-23 maintained a higher tolerance to the fungus, as the DI for rolA-2 was not significantly different from that of the controls.

### Discussion

The insertion of the *rolA* gene in tomato plants increased the level of tolerance to *F. oxysporum* f. sp. *lycopersici* both on the primary transformants and on the transgenic progeny. In a preliminary screening by electrolyte leakage the primary transformants were found to be resistant to the toxic effect of fusaric acid, with the clone rolA-23 clearly more responsive (Fig. 4). In accordance, direct infection of the transgenic progeny showed increased tolerance to the pathogen: even if not completely, the introduction of *rolA* increased up to twice plant protection from *Fusarium* challenge (Fig. 5). To our knowledge, this is the first report describing a contribution of *rolA* gene in plant resistance by direct pathogen infection.

The present hypotheses on *rolA* function do not allow to explain the observed defense promotion. However, as



**Fig. 4** Time course of electrolyte leakage from leaf disks of seed-derived cv. Tondino plants, the untransformed in vitro-regenerated control clones 1 and 9, and the transgenic tomato clones rolA-1, rolA-2 and rolA-23 in the presence of 5 mM fusaric acid
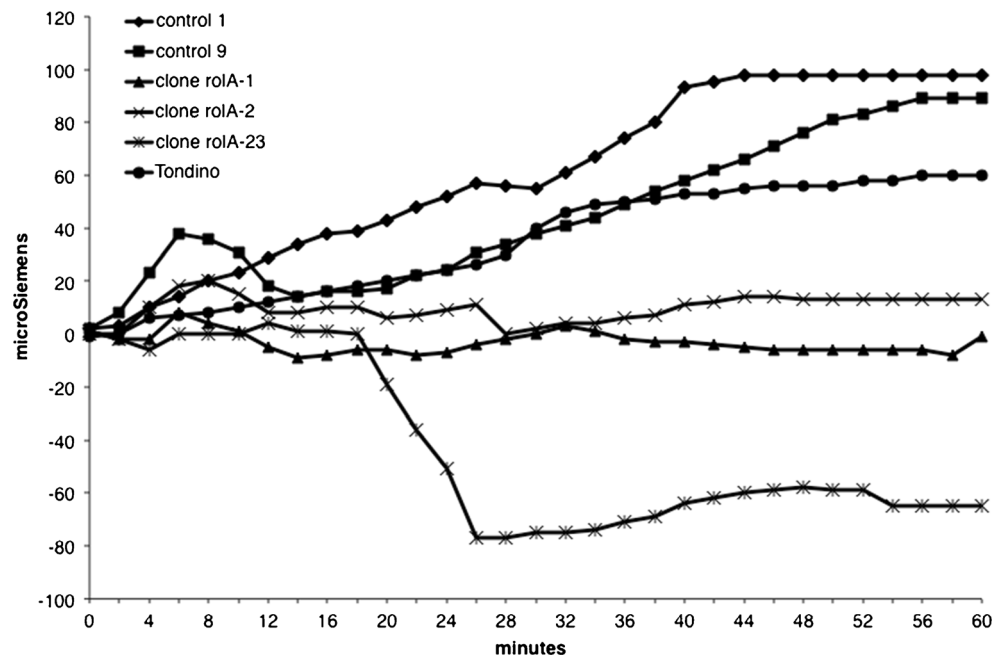
**Table 1.** Levels of the endogenous phytohormones indole-3-acetic acid (IAA) and abscisic acid (ABA) in the apical shoots of the transgenic clones rolA-2 and rolA-23 and of the untransformed in vitro-regenerated clones (controls). The free IAA and ABA concentrations were measured by GC-MS-SIM with stable isotopes as internal standards. For each phytohormone significant differences between the clones were assessed by Tukey test and are indicated with one or two asterisks ($P \le 0.1$ and $P \le 0.05$, respectively).

|  | ABA (ng g$^{-1}$ fw) | IAA (ng g$^{-1}$ fw) |
| --- | --- | --- |
| Untransformed controls | 56,14 ± 1,761 | 15,30 ± 0,762 |
| Clone rolA-23 | 49,21 ± 1,515* | 13,13 ± 2,038[n.s.] |
| Clone rolA-2 | 36,31 ± 4,983* | 10,83 ± 0,387** |

previously described in the Introduction, the other *rol* genes, *B*, *C* and *D* have already been known to be involved in defense mechanisms. For example, in potato tuber discs *rolC* promotes a reduction in the infection level of phytopathogenic fungi (Fladung and Gieffers 1993), and in tomato *rolB* induces fungal tolerance by increasing the amount of total phenolics (Arshad et al. 2014).

The study shows that in transgenic plants a direct correlation between gene copy number, expression level and severity of the phenotype could be established, while the same is not true when pathogen tolerance is taken into account. As high level expression of *rolA* in rolA-2 plants determined extreme severity showing a reduced fitness, so we can suggest that it could interfere with the complete deployment of defense response.

*Fusarium*-tolerant plants had a reduced amount of ABA with respect to controls (Table 1). Abscisic acid, known as a relevant player in abiotic stress tolerance (Tuteja 2007), in recent evidence also showed a prominent role in biotic stress where it acted as a negative regulator of disease resistance (Anderson et al. 2004; Robert-Seilaniantz et al. 2011). In particular, in tomato an increased concentration of ABA promoted fungal and bacterial infection (Audenaert et al. 2002; de Torres-Zabala et al. 2007; Asselbergh et al. 2008). These studies are in agreement with our results on *rolA* plants, and could have important agricultural implications because abiotic stress

induces ABA accumulation, which in turn may result in enhanced susceptibility to pathogens. Moreover, in tomato ABA-deficient mutants had reduced plant growth and fruit size (Nitsch et al. 2012), confirming that endogenous ABA influenced growth promotion (Sharp et al. 2000; Finkelstein et al. 2002). Thus, the phenotype of *rolA* tomato plants, having a reduced ABA concentration, was in agreement with that of ABA-deficient mutant plants of the above mentioned study.

Indole-3-acetic acid level, even if reduced in both clones, was significantly lower in rolA-2 plants which were less tolerant to *Fusarium* infection. In the banana-*F. oxysporum* f. sp. *cubense*, tomato-*F. oxysporum* f. sp. *lycopersici* and barley-*Fusarium culmorum* interactions the exogenous application of IAA increased plant resistance to the pathogen (Fernández-Falcón et al. 2003; Sharaf and Farrag 2004; Petti et al. 2012). A lower IAA content could therefore be one of the factors determining the decreased level of tolerance observed in rolA-2.

As previously reported by Schmülling et al. (1993) in tobacco, also our data showed indirect evidence that gibberellin was decreased in *rolA* plants. The germination percentage for the clone rolA-23 was in fact 5 % for untreated and 66 % for seeds treated with GA$_3$. Gibberellin partially works in limiting pathogen effects: genetic study showed a reduced fungal colonization in mutants that are impaired in gibberellin synthesis (Schäfer et al. 2009).

Phytohormones are known to exert their action on plant growth and development in a defined range of endogenous concentrations (Bradford and Trewavas 1994). The same could be true for their effect on the defense response, where an amount of phytoregulators above or below a given threshold could alter plant fitness creating a condition for the deployment or not of resistance.

*rol* genes also promote secondary metabolite production that, even if not essential for cell survival, is often involved and/or stimulated in defense processes (Walton 2001). In *rolA* plants the enhanced biosynthesis of secondary metabolites such as nicotine and anthraquinones, having known antimicrobial and insect-repellent activities (Godard et al. 2008;

**Fig. 5** Time course of disease progression expressed as average disease index (DI) values for the transgenic and the untransformed control plants. Tukey test was used to determine the statistical significance of the observed differences between rolA-23, rolA-2 and control plants at each time-point, same letters indicating values not significantly different at $P \le 0.05$ to $P \le 0.001$. a. u., arbitrary units
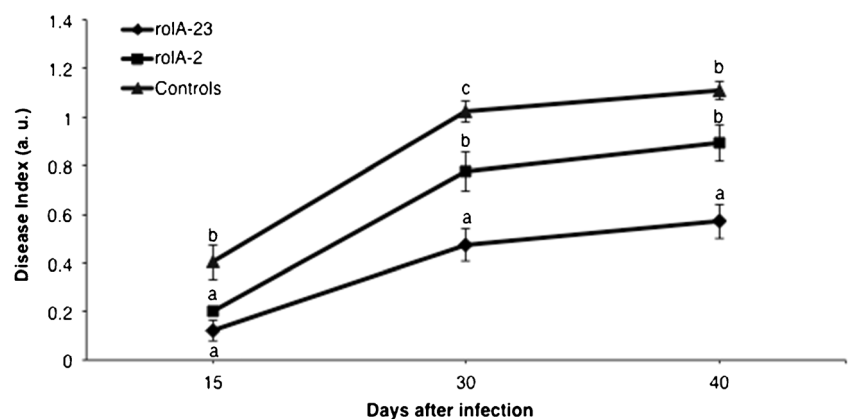
Kremer et al. 2012; Mithöfer and Boland 2012), could also contribute to the defence response.

Finally the *rolA* gene is also expressed in *A. rhizogenes*, driven by a specific prokaryotic promoter corresponding to a spliceosomal intron (Pandolfini et al. 2000). In the bacterium *rolA* is preferentially active during stationary growth, suggesting a *rolA* function on high cell density (Pandolfini et al. 2000). This condition is characterised by the transcription of genes involved in long-term survival and in the protection against pH, $H_2O_2$, high osmolarity, UV, drought, and nutrient starvation (Loewen and Hennge-Aronis 1994; Thorne and Williams 1997). The presence of *rolA* expression in bacteria in stressing growth status strengthens its role also in the plant defense response.

The study shows that the *rolA* gene, in addition to conferring pathogen tolerance, pleiotropically affects plant phenotype and phytohormone content of tomato plants. Further studies are therefore required to assess the possible correlations between one or more metabolic effects of *rolA* and pathogen tolerance, and if any of the observed modifications could affect other agronomically important traits.

**Author contribution statement** P. P. Bettini obtained the transgenic plants, carried out phenotypic analysis and self-fertilization, performed molecular analysis and ion leakage experiments, and wrote the paper. R. Baraldi and F. Rapparini carried out the determination of ABA and IAA levels and revised the manuscript. E. Santangelo, P. Mosconi and P. Crinò performed the infection experiment, collected and analyzed the data and revised the manuscript. M. L. Mauro obtained the construct with the *Agrobacterium rhizogenes rolA* gene in the appropriate *Agrobacterium tumefaciens* strain for transformation and wrote the paper.

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