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ORIGINAL PAPER

ROLC strawberry plant adaptability, productivity, and tolerance to soil-borne disease and mycorrhizal interactions

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Abstract The potential to improve strawberry cultivation was assessed regarding the use the *rolC* genes from *Agrobacterium rhizogenes* that can confer higher levels of free cytokinins. Strawberry (cv. Calypso) *rolC* lines were produced by genetic transformation of *Agrobacterium tumefaciens*. Yield and fruit quality of the control and transgenic lines were measured under open-field conditions. The effects of the transgenic *rolC* lines depended on gene copy number: *rolC* lines with one (Line A) or two gene (Line B) copies showed 30% greater yields than controls, due to 20% more fruit per plant and an increased fruit weight. Line A also differed in terms of the highest fruit quality, due to 10.5% increased soluble solids and 12.7% higher acidity. Moreover, cv. Calypso *rolC* lines A and B had increased tolerance to greenhouse infection by *Phytophthora cactorum*. Conversely, for all of these characters, Line F (five *rolC* copies) was not significantly different from the control line. The same lines were also used to examine their symbiosis with root arbuscular mycorrhizal fungi (AMF) using vital and non-vital staining of roots collected at different stages of plant growth. Control and *rolC* plants showed similar intensities of AMF infection according to plant phenology and/or physiology. Furthermore, possible horizontal gene transfer of the *rolC* gene was tested for the AMF spores by PCR, with all AMF samples negative using *rolC* primers. The use of the *rolC* gene should be considered for the improvements provided in productivity, fruit quality and disease resistance of cultivated strawberry that show no effects on soil microorganisms.

Keywords *F. x ananassa* · *A. rhizogenes* · Genetic transformation · Plant phenotype · *P. cactorum* · Mycorrhizae

Introduction

The *rol* genes, and in particular *rolC*, have been used to improve important characteristics of horticultural crops. The insertion of *rolC* from *Agrobacterium rhizogenes* in ornamental plants, such as carnation and petunia, led to the expression of advantageous traits (Casanova et al. 2003), i.e., increased branching, better rooting of stem cuttings, and reduction in time to flowering (Winefield et al. 1999; Zuker et al. 2001). Transgene expression of the *rolC* gene increased the rooting rate of trifoliate orange and Japanese persimmon cuttings, enhancing their

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potential as dwarfing rootstock. Cells from ginseng transformed with *rolC* expressed more pathogenesis-related β-1,3-glucanase (Kaneyoshi and Kobayashi 1999; Koshita et al. 2002; Kiselev et al. 2006). *Rol* genes have also had positive effects in increasing tomato plant tolerance to specific diseases (Bettini et al. 2003).

The *rolC* gene effects on plant development are related to their ability to show both cytokinin-like and auxin-like activities, although this response does not appear to be related to increased indole-3-acetic acid (IAA) levels in *rolC* tissues (Schmülling et al. 1993; Casanova et al. 2003); however, it might be caused by an increase in auxin sensitivity (Maurel et al. 1991).

Strawberry is perceived as an interesting fruit crop for the use of the most advanced biotechnological applications, including gene cloning and recombinant technology, because of its large-scale production that is based on a quite limited varietal diversity (almost 60% of the world production is of one variety), and because it is grown as an annual fruit (Qin et al. 2008). It is important to understand the effects of changing plant hormonal regulation on plant development and fruiting for the improvement of agronomic performance of the strawberry, including plant adaptability and resistance to disease. Benefits can be easily achieved through the introduction of genes that can modify endogenous plant growth regulators.

Cultivated strawberry plants (*Fragaria x ananassa* Duch.) are mostly susceptible to attack by *Phytophthora cactorum* (Orsomando et al. 2001), a pathogenic oomycete that can attack many herbaceous and woody plants and has a wide host range. This pathogen causes "leather rot" and "root rot" diseases in strawberry plants, the morphological symptoms of which are recognized by rotting of root, crown, and fruit tissues (Pitrat and Rissel 1997). *P. cactorum* is an important organism that needs to be considered when studying the effects of *rolC* on increasing strawberry resistance to pathogens.

The acceptability of transgenic crops is linked to an appropriate evaluation of their potential risks for the environment (gene flow and gene impact on micro-organisms) and of their benefits in agricultural practice (reduced inputs, improved yield, quality and nutritional value). One of the least understood areas in environmental risk assessment of genetically modified crops is their impact on soil- and plant-associated microbial communities (Dunfield and Germida 2004).

The interaction between transgenic plant roots, plant residues and the soil microbial community is not well understood and needs a 'case by case' study approach (Gebhard and Smalla 1999; Hopkins and Gregorich 2005). Arbuscular mycorrhizal fungi (AMF) are an important group of non-target beneficial microorganisms that are fundamental for soil fertility and plant nutrition. They establish mutualistic symbioses with the roots of most plant species, and represent a crucial link between the root system and the soil, enhancing plant nutrition and water acquisition, and also resistance against parasites and other stress factors (Smith and Read 1997).

The mycorrhizae have key roles in ecosystem functionality. They are sensitive to cultural practices, as well as to fertilizer and pesticide application (Giovannetti and Avio 2002). Normal development of AMF has been reported for different transgenic tobacco plants and soybeans cultivated under controlled conditions (Vierheilig et al. 1995; Gianinazzi-Pearson et al. 2000; Powell et al. 2007).

Similarly, when the field development and community structure of the mycorrhizal fungi of transgenic host plants was investigated in *rolC* aspen plants fully infected by ectomycorrhizal fungi, there were no differences detected in the degree of mycorrhizal colonization between the control and transgenic plants (Kaldorf et al. 2002). With their intimate contact with root cells, the mycorrhizae can also be considered as suitable non-target organisms for analyzing horizontal gene transfer. Using selective agar plates methods, however, Zhang et al. (2006) showed no horizontal gene transfer from herbicide-resistant transgenic poplar plants containing the *bar* gene to the ectomycorrhizal fungus *Amanita muscaria*.

The present study describes the types of modifications that are induced by over-expressing the *rolC* gene in strawberry for traits of economic interest, such as increased plant adaptability, productivity and tolerance to soil-borne disease, as well as for fruit quality. *RolC* improves important agronomic characteristics of strawberry, while not affecting the symbiotic *rolC*–strawberry root–mycorrhiza interactions and showing no evidence for *rolC* DNA transfer between plants and the mycorrhizal spore population.

Materials and methods

Plant material

Genetic transformations in strawberry were performed using the LBA4404 (Ooms et al. 1985) strain of *Agrobacterium tumefaciens* containing the pAL4404 plasmid, and the binary vector pBI121 bearing the *NOS/NPTII* gene for kanamycin resistance and the *rolC* gene from *A. rhizogenes* under the control of the natural promoter (Slightom et al. 1986). Leaf tissue from in vitro proliferating shoots of cv. Calypso, an ever-bearing strawberry (*Fragaria x ananassa*), were used for the genetic transformation experiments (James et al. 1990). Four transgenic lines of Calypso were first evaluated in vitro and then in vivo to test the effects of the *rolC* gene in strawberry (Mezzetti et al. 2004a). Three lines (A, B and F) were selected and used for the present study.

RolC genomic characterization

'Calypso *RolC*' lines were characterized according to their copy number for the *rolC* and *nptII* genes by real-time PCR, using SYBR Green technology (Hernandez et al. 2004). We applied the Delta-DeltaCt comparative method for quantification (Ingham et al. 2001), using as external references two *Fragaria x ananassa* octoploid transgenic strawberry lines with one and three copies of the *nptII* and *DefH9-iaaM* genes, respectively, analyzed by Southern blotting (Mezzetti et al. 2004b). The copy numbers in the '*rolC* Calypso' lines were determined according to the equation: $10^{\frac{(C_t \text{ reference} - C_t \text{ GM target})}{\text{slope}}}$ where C_t is the threshold cycle and reflects the cycle number at which the fluorescence generated within the reaction for every sample crosses the threshold; the calibrator is a sample used as the basis for comparative results; the transgenes target is the 'Calypso *rolC*' lines and the slope is calculated according to the standard curve, which is specific for each gene, and which reflects the amplification efficiency.

Primers designed

The genes were detected using three primer sets that were designed with the Primer3 software (Rozen and Skaletsky 2000): *rolC* gene (RCf5/RCr498 5′-catg gctgaagacgacctgtc-3′; 5′-gaacctaagctgggtgctg-3′; fragment product, 493 bp); *nptII* gene (NH59515′-gccc tgaatgaactgcaggacgaggc-3′, NH59525′-gcaggcatggcc atgggtcacgacga-3′; fragment product, 410 bp); and *DefH9-iaaM* gene (Def2f 5′-aacaagcttcccaccaccatc-cag-3′, Def2r 5′-gcatgctcttttcacccgtattag-3′; fragment product, 488 bp).

Experimental design

The DNA of 'Calypso *rolC*' and the reference lines were quantified with the VersaFluor™ Fluorometer System (Bio-Rad). 10 ng of each sample were used for the analyses, which were performed using SYBR Green technology on an iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad). All real-time PCR runs were performed in duplicate and experiments were repeated three times for each gene. The reaction mixture was prepared with a final volume of 22 µl, including 11 µl of 2x SYBR Green I (Bio-Rad), 1 µl of forward and reverse primers (0.1 µM each), and 9 µl of DNA. Both target and calibrator DNA lines were analyzed in the same plate. The *nptII* gene was analyzed according to a homologous standard curve. The following thermal cycling protocols were used: one cycle at 95°C for 8 min, following by 40 cycles at 95°C for 50 s, 64°C for 50 s, and 72°C for 30 s. The *rolC* gene copies were analyzed according to standard curves of *rolC* and *DefH9-iaaM*. The PCR cycle was at 95°C for 8 min, followed by 40 cycles at 95°C for 50 s, 55°C for 50 s, and 72°C for 30 s. The specificity of the amplicons for all of the PCR runs was verified by melting curves. This cycle was performed at temperature increases of 0.05°C/s between 55 and 95°C.

Construction of standard curve

The standard curves were constructed using the PCR products obtained for *nptII* and *rolC* (target genes) and *DefH9-IaaM* (reference gene). The PCR products were purified with the Mini elute PCR Purification Kit (QIAGEN), and quantified with the VersaFluor™ Fluorometer System (Bio-Rad). Five-fold serial dilutions, ranging from 10^{-5} to 10^{-9} ng, were used to create the standard curves for all gene fragments. The standard curve efficiencies were 97.8, 99.0 and 99.6% for the *nptII, rolC* and *DefH9-IaaM* genes,

respectively. The slopes of the standard curves for *nptII, rolC* and *DefH9-IaaM* were −3.376, −3.346 and −3.331, respectively.

Calypso *rolC* plant productivity and quality

RolC and control plants were produced by standard micropropagation techniques, acclimatized in the greenhouse, and after in vivo propagation by runners, transferred to the open field (Mezzetti et al. 2004a). Plant productivity and fruit quality were studied under open-field cultivation conditions for three subsequent production cycles (i.e., over 3 years). Open-field plantations were renewed each year (early August) using fresh plants propagated by runners and grown under standard cultivation conditions. At each cycle, 40 plants of each clone were arranged in a completely randomized block design, with four plots of ten plants. Plant productivity was evaluated by recording the total number of fruits per plant, the average fruit weights, and the total plant fruit production. Fruit quality was evaluated by detecting soluble solid content, determined by a portable refractometer (Atago model N-1E), and total acidity, detected by titrating 10 g of fruit juice with 0.1 N NaOH, using bromothymol blue as the titration indicator (variation range, yellow–blue at pH 6.0–7.6), and expressed as meq NaOH/100 g fruit.

Interactions of *rolC* Calypso with *Phytophthora cactorum*

'Calypso' *rolC* clones were studied for their phenotypic behavior and response to *P. cactorum*. The present study was performed on plants growing in pots placed in a greenhouse for the control and *rolC* lines, and inoculated with the virulent *P. cactorum* strain Ph381 (Orsomando et al. 2001). The inoculation method consisted of placing a slice of agar with in vitro-cultivated mycelia on the wounded crown, which was then covered with Parafilm (Pitrat and Rissel 1997). This inoculation was repeated three times, twice in late spring (early and late May), and once in late summer (September), using ten plants (for each clone/ repetition) grown in pots (diameter, 20 cm height, 18 cm) under greenhouse conditions. Ten uninoculated plants (for each clone/ repetition) grown under the same conditions were also studied as controls, to better characterize the *rolC* phenotype. The number of wilted/ brown leaves, new green leaves and shoots on each plant was measured at and 30 days. The number of leaves and shoots were also monitored on the uninoculated plants.

Interactions of *rolC* Calypso with arbuscular mycorrhizal fungi

Sample collection

Roots of the control and *rolC* lines were analyzed at four different times: twice during the vegetative phase (December and February) and twice during the productive phase (April and June). In each phase, 60 roots samples were collected from four different plants identified from three experimental blocks in the field, for the control and *rolC* lines (12 blocks in total).

Symbiotic development

To estimate the symbiosis development of the strawberry root system, at four time-points, 30 × 1 cm-long pieces were randomly chosen and cut from each of six plant root systems. A total of 180 root fragments (from six plants) were collected of each line and were carefully washed and divided into two batches: 90 were stained by normal non-vital trypan blue (TB) staining of all fungal tissues (Phillips and Hayman 1970), and 90 were used for histochemical staining (for succinate dehydrogenase [SDH] activities) of the roots. This method made it possible to directly compare the total amount of fungal tissue in mycorrhizal root systems (TB staining) and the proportion that was alive (SDH staining). After cleaning the root samples in 10% KOH for 20 min at 50°C, the roots were washed in water and stained with 0.05% (w/v) trypan blue in lactoglycerol (lactic acid:glycerol:H_2O, 1:1:1, v/v/v) for 30 min at 50°C. SDH activity was measured according to the procedure described by Smith and Gianinazzi-Pearson (1990). Briefly, after digestion with 15 U/ml cellulase and 15 U/ml pectinase (Sigma–Aldrich) for 2 h at room temperature, the roots were immersed in a freshly made solution containing 0.2 M Tris-HCl, pH 7.0, 2.5 M sodium succinate 6-hydrate, 4 mg/ml nitroblue tetrazolium, 5 mM $MgCl_2$. Root fragments were stained overnight at room temperature and then rinsed for 15–20 min in a 3% active chlorine solution

of sodium hypochlorite. Mycorrhizal development was evaluated microscopically after non-vital and vital staining by the method proposed by Trouvelot et al. (1986). The degree of AMF colonization was evaluated by estimating the amounts of root cortex mycorrhization. This evaluation is reported as M%, corresponding to the colonization intensity of cortical cells occupied by AMF structures, and A%, corresponding to the estimation of arbuscule richness in the root system.

AMF spore isolation, DNA extraction and analyses

Mycorrhizal spores present in soil of 12 blocks of Calypso control and *rolC* A, B and F lines were isolated to study horizontal root-AMF *rolC* gene transfer, from the field-trial soil samples that were collected in June and December 2004 and in March 2005, after 11, 17 and 20 months, respectively, of establishment of the strawberry plantation. A total of 62 soil samples (500 g each) were analyzed and the spores were extracted by wet sieving and sucrose density gradient centrifugation (Daniels and Skipper 1982). The soil samples were sieved through 800, 425, 355, 300, 100, 63, and 32 μm sieves (Endecotts. Ltd., London). The contents of the 100, 63, and 32 μM samples were washed in water and centrifuged at 2,300×*g* for 10 min. The pellets were layered onto 72% water-sucrose solution (w/v), 20 g/l Calgon gradients and centrifuged at 800×*g* for 10 min. The resulting supernatants were passed through the 25 μm sieve under vacuum filtration, washed with tap water, and transferred to Petri dishes. The abundance of spores was determined for each sample under a Nikon stereomicroscope.

After grinding in liquid nitrogen, the spores were mixed with 1 ml extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% cetyl-trimethylammonium bromide [CTAB], 0.4% *β*-mercaptoethanol), sonicated for 10 min, and incubated at 65°C, for 15 min. The samples were extracted with an equal volume of chloroform-octanol (24:1) and centrifuged at 3,500×*g* for 5 min. Their RNA was digested by RNaseA at 37°C for 30 min. After a new chloroform-octanol (24:1) extraction, the supernatants were transferred to fresh tubes and 0.6 vol isopropanol added. After centrifugation (13,000×*g* for 10 min), the supernatants were discarded and the DNA pellets were washed in 500 μl 70% ethanol. The final pellets were dissolved in 100 μl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The DNA was purified by Sepharose CL-6B (Sigma) spin columns (Bramwell et al. 1995).

The DNA of the AMF spores was analyzed with specific primers for the *rolC* gene, as described above. PCR amplification was carried out in a 20 μl total volume, containing 30 ng DNA, 0.2 μM of each primer, 10 μl of 2x Master Mix QIAGEN. PCR was performed on a Bio-Rad iQ-Cycler™ under the same conditions as described above. The quality of the DNA was confirmed using universal ITS1/4 fungal primers (White et al. 1990).

Statistical analyses

Data from field evaluations on plant productivity, fruit quality and AMF symbiosis were subjected to one-way ANOVA for means comparisons, and significant differences were calculated according to Duncan's Multiple Range Test, $P \leq 0.01$. The arcsin square-root transformation was used to normalize the percentage ratios data prior to statistical analysis, while square-root transformation was used to normalize productivity and quality means.

Results

rolC strawberry genomic characterization

The Calypso strawberry lines showed a mean of one copy of the *nptII* and *rolC* genes in Line A, two copies of the *nptII* and *rolC* genes in Line B, and five copies of the *nptII* and *rolC* genes in Line F (Supplementary Table 1). The comparative quantification using real-time PCR technology was an efficient method for genomic characterization of the transgenic lines.

Plant productivity and quality

From field trial, the Calypso *rolC* Lines A and B had more than 20% more fruit per plant than the controls, while Line F produced the same number of fruits as the control (Table 1). Differences in fruit weight were observed only for Line A, with a 12% increase in comparison to the controls. The *rolC* effects on increasing fruit number per plant and fruit size

resulted in a significant increase (up to 37%) in total yield of the two lines with the lower gene-copy numbers. The increased productivity of the *rolC* Line A corresponded also to significantly higher values of fruit total sugar (+10.5%) and acid (12.7%) contents, although only for Line A. The lack of improvement in Line F for both production and quality parameters can be related to gene co-suppression, due to the five *rolC* gene copies, as opposed to one and two in Lines A and B (Supplementary Table 1).

Interactions of *rolC* Calypso with *P. cactorum*

The plant responses to *P. cactorum* inoculation resulted in a reduced number of leaves with symptoms in the plants of the Calypso *rolC* Lines A and B, as compared to the control lines. This effect was stable at the three inoculation treatments (Supplementary Table 2). The plants of Lines A and B differed from the controls and the F line also in their ability to produce a higher number of new shoots and leaves (Table 2), with this again after the three inoculation treatments and as the total means. Uninoculated plants of the same lines performed with a more vigorous habitus differed in a higher number of shoots and leaves (Fig. 1; Supplementary Table 3). This different behavior corresponded to the *rolC* phenotype described in other plants (Casanova et al. 2003; Zuker et al. 2001), and it can be considered as *rolC* effect on increasing strawberry tolerance to *P. cactorum*.

Interactions of *rolC* Calypso with arbuscular mycorrhizal fungi

Symbiotic development

Indigenous AMF infection was detected in all samples of Calypso control and *rolC* lines. Non-vital staining revealed an annual average colonization (*M*) that ranged from 27.4% (Line A) to 35.3% (Line F), and the presence of arbuscule (*A*) ranged from 12.2% (Line A) to 16.7 % (Line B). The only significant differences on mycorrhizal infection were seen between the Calypso control and *rolC* Line A in the sample collected in February (Table 3). The SDH vital staining showed a similar trend (data not shown). The annual average colonization (*M*) ranged

Table 1 Strawberry 'Calypso' control (CT) and rolC Lines (A, B and F): total number of fruits per plant, fruit weight, total plant yield, total sugar content (SS) and titratable acidity (TA)

	Total no. fruit/plant	Fruit weight (g)	Total yield (g)	SS (Brix)	TA (NaOH-ml)
'Calypso' CT	74.9 a	10.8 a	808.7 a	7.6 a	11.0 a
rolC Line A	91.5 b	12.1 b	1107.4 b	8.4 b	12.4 b
rolC Line B	96.6 b	11.1 ab	1072.3 b	8.1 b	11.2 a
rolC Line F	73.2 a	10.1 a	738.9 a	7.5 a	10.8 a

For each trait, means followed by at least one common letter are not significantly different, according to Duncan's Multiple Range Test ($P \leq 0.01$)

Table 2 Strawberry 'Calypso' (CT) control and *rolC* Lines (A, B and F) responses to *P. cactorum* inoculation of greenhouse plants, expressed as number of new shoots and green leaves after 30 days from the inoculations

Lines	No. shoots				No. green leaves			
	Early May	Late May	Late September	Means	Early May	Late May	Late September	Means
'Calypso' CT	3.6 c	3.5 a	3.2 c	3.4 b	3.5 c	3.5 b	3.4 c	3.5 b
rolC Line A	5.1 a	4.6 b	4.2 ab	4.6 a	4.8 a	4.7 a	4.6 ab	4.7 a
rolC Line B	4.7 ab	4.9 b	4.5 a	4.7 a	4.2 b	4.2 a	4.2 b	4.2 ab
rolC Line F	4.5 b	4.5 b	3.8 b	4.3 ab	3.5 c	3.4 b	3.7 cb	3.5 b

Mean values detected from ten plants of 'Calypso' (CT) and '*rolC* lines (A, B and F)' after three inoculation treatments: early and late May, and early September

For each trait, means followed by at least one common letter are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.01$)

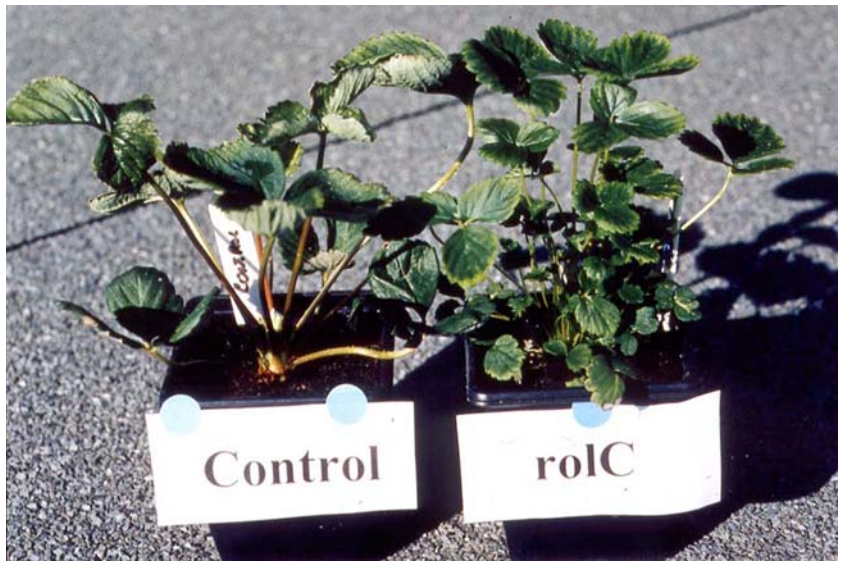

Fig. 1 Plant phenotype induced by the *rolC* expression in Calypso *rolC* Line B, differing from the control for the highest vigorous and number of shoots

Table 3 AMF colonization (M%) and arbuscular density (A%) detected by non-vital staining (TB) from roots of 'Calypso' control and *rolC* transgenic lines, sampled in different months of 2003 and 2004 in field production cycles

	December 2003		February 2004		April 2004		June 2004		Annual means	
	M%	A%	M%	A%	M%	A%	M%	A%	M%	A%
'Calypso' CT	35.7 a	17.4 a	46.1 a	22.1 a	35.0 a	11.8 a	22.9 a	9.6 a	34.9 a	15.2 a
rolC Line A	24.5 a	11.8 a	23.4 b	8.9 a	27.1 a	8.4 a	34.6 a	19.5 a	27.4 a	12.2 a
rolC Line B	25.9 a	14.4 a	34.0 ab	15.7 a	29.7 a	15.3 a	41.5 a	21.5 a	32.7 a	16.7 a
rolC Line F	36.8 a	22.3 a	34.6 ab	16.2 a	36.2 a	12.9 a	33.6 a	10.9 a	35.3 a	15.5 a

For each trait, means followed by at least one common letter are not significantly different, according to Duncan's Multiple Range Test ($P \leq 0.01$). The data were normalized according to the arcsine square root

from 17.1% (Line A) to 22.8% (control), and the presence of arbuscule (*A*) ranged from 4.3% (Line A) to 9.9% (Line F); the annual mean colonization showed a similar trend. In general, the roots from the Calypso control plants collected in December and February showed the highest M and A% values with both staining methods (TB and SDH), while lower values were seen for roots sampled in April and June. In contrast, the *rolC* lines, and particularly Lines A and B, showed more constant mycorrhizal infection values across all of the seasons.

Genetically modified root–AMF gene flow

Purified AMF spores isolated from soil samples near the plants in different plots of the *rolC* experimental trial were analyzed by PCR for detection of the *rolC* gene. The number of samples and the purification and DNA extraction procedures allowed an analysis that revealed an elevated number of AMF spores (approximately from 10.6×10^5 to 23.0×10^5). None of the AMF samples were positive for the PCR analyses with *rolC* primers.

Discussion

This is the first report on the use of the *rolC* gene in a fruit plant. The *rolC* gene in strawberry increased the yield by up to 37%, as well as increasing the total soluble sugar content and total acidity (Table 1). It also increased other nutritional attributes, such as fruit total antioxidant capacity and polyphenol contents (Scalzo et al. 2005). These results were linked to the low *rolC* gene copy numbers. The low-copy-number transgenic lines were also tolerant to *P. cactorum*

infection, while the control was susceptible. Also in this case, Line F (five copies of the gene) performed more similarly to the control. The effect of the *A. rhizogenes* gene for increasing plant tolerance has already been seen in tomato lines genetically modified using the *rolD* gene (Bettini et al. 2003). Thus the *rolC* and *rolD* genes have similar effects on endogenous plant growth regulator metabolism, and they both increase plant disease tolerance.

The aim of this study was also to determine whether the roots of the Calypso *rolC* lines affect the soil non-target AMF community in a different way to that of control Calypso plants. The results showed no significant reductions in AMF between the controls and the *rolC* Calypso plants, and thus the positive effects of AMF demonstrated in strawberry may be preserved (Koomen et al. 1987; Niemi and Vestberg 1992; de Silva et al. 1996; Taylor and Harrier 2001). The stability of the *rolC* roots–AMF interaction was also seen in the first study on mycorrhizal colonisation carried out on *rolC* aspen (Kaldorf et al. 2002).

The constant seasonal levels of AMF infection, as well as the increased tolerance to *P. cactorum* shown by *rolC* Lines A and B, confirmed the importance of enhanced plant growth metabolism on symbiosis effects and/or triggering plant defence reactions against some soil-borne fungal pathogens (Azcón-Aguilar and Barea 1996; Whipps 2004). Horizontal gene transfer from transgenic plants is considered an important biosafety issue, although there have still been few scientific approaches applied with this aim. Fungi often grow in intimate contact with plants, and for mycorrhizzal symbioses, even within the plants. The in vitro cultivatable systems now available (Silvani et al. 2008) probably cannot be easily applied to this type of biosafety study. The approach followed in this study was based on DNA purification of AMF spores sampled from soil collected near to plants of the *rolC* lines, and PCR analyses with specific primers for the *rolC* gene sequences. This method allowed us to analyze a very high number of bulked AMF spores (approximately from 10.6×10^6 to 23.0×10^6), which were finally all negative in the PCR analyses, thus confirming the absence of root–AMF horizontal gene transfer of *rolC*.

The present study provides evidence of a pleiotropic effect of the *rolC* gene on important strawberry traits, including plant productivity and fruit quality. These increases in productivity and fruit quality that were detected for the two low-copy-number *rolC* lines is proof of the usefulness of this gene for improving important characters in strawberry varieties. The study of non-target organisms also showed effects on increased tolerance to *P. cactorum*, an important soil-borne disease of strawberry, of the lines performing with the *rolC* phenotype. These improvements are an example of the benefits that can be expected for the use of the *rolC* gene in the genetic improvement of strawberry.

These agronomic benefits are probably also related to the stable AMF symbiotic interaction that was detected during the cultivation period for the roots of the Calypso *rolC* lines. However, the roots from the *rolC* lines differed in their greater symbiosis intensity during the cultivation cycle, while the control plants showed a seasonal variation in symbiosis intensity according to phenology and physiology of the plants, as is well known for terrestrial ecosystems (Bohrer et al. 2004). This AMF spore DNA study represents the first study carried out on genetically modified strawberry grown in the open field, and the negative PCR analyses can be considered as an important result that excludes the possibility of *rolC* gene transfer from the cultivated transgenic strawberry to the soil AMF. Thus the stability of the root–AMF interactions and the absence of *rolC*-positive samples can be considered as important achievements that exclude negative environmental impacts of the cultivation of the *rolC* genetically modified strawberry.

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Author contributions B.M. first conceived the idea of testing the *rolC* gene in strawberry. L.L., F.C. and B.M designed the field experimental trial and defined the evaluation parameters. E.C. and F.C. followed the field trial and collected the field data. L.L. and E.C. performed the AMF analysis. All authors discussed the results and commented on the manuscript, which was written by B.M and L.L.

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