Conferred resistance to *Botrytis cinerea* in *Lilium* by overexpression of the *RCH10* chitinase gene.

Francisco F. Núñez de Cáceres González [1,2], Michael R. Davey[1], Ester Cancho Sanchez[1] and Zoe A. Wilson[1*]

Corresponding author * Zoe A Wilson

[1] University of Nottingham, UK;

School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, United Kingdom.

Tel: +44 115 951 3235, Fax: +44 115 9516334

Zoe A Wilson: zoe.wilson@nottingham.ac.uk

[2] Universidad Autónoma del Estado de Hidalgo.

Ciudad del Conocimiento, Centro de Investigaciones Biológicas

Km. 4.5 Carr. Pachuca - Tulancingo

Pachuca, Hidalgo, 42184, México

Tel: +52 7717172000 Ext. 6657, Fax: +52 7717172112

Running Title:- *RCH10* chitinase-conferred resistance in Lily

Key Message: - Transgenic *Lilium* lines have been generated by *Agrobacterium*-mediated transformation that have enhanced resistance to *Botrytis cinerea* as a consequence of ectopic expression of a rice chitinase gene.

Abstract

The production of ornamentals is an important global industry, with *Lilium* being one of the six major bulb crops in the world. The international trade in ornamentals is in the order of £60-75 billion and is expected to increase worldwide by 2-4 % per annum. The continued success of the floriculture industry depends on the introduction of new species/cultivars with major alterations in key agronomic characteristics, such as resistance to pathogens. Fungal diseases are the cause of reduced yields and marketable quality of cultivated plants, including ornamental species. The fungal pathogen *Botrytis* causes extreme economic losses to a wide range of crop species, including ornamentals such as *Lilium*. *Agrobacterium*-mediated transformation was used to develop *Lilium* oriental cv. 'Star Gazer' plants that ectopically overexpress the *Rice Chitinase 10* gene (*RCH10)*, under control of the CaMV35S promoter. Levels of conferred resistance linked to chitinase expression were evaluated by infection with *Botrytis cinerea*; sporulation was reduced in an *in vitro* assay and the relative expression of the *RCH10* gene was determined by quantitative Reverse-Transcriptase PCR. The extent of resistance to *Botrytis,* compared to that of the wild type plants, showed a direct correlation with the level of chitinase gene expression. Transgenic plants grown to flowering showed no detrimental phenotypic effects associated with transgene expression. This is the first report of *Lilium* plants with resistance to *Botrytis cinerea* generated by a transgenic approach.

Keywords: *Lilium*, chitinase, *Agrobacterium*, *Botrytis*, *RCH10*, ornamental

Introduction

Fungal diseases are the cause of reduced yields and marketable quality of cultivated plants, including ornamental species. This results in significant loss of income and simultaneously increases the production costs as a direct result of disease prevention or control treatments (Inglis and Kawchuk 2002; Tanaka, et al. 2005; Zuker, et al. 1998). The ornamental industry has been gaining in strength and importance in recent years. The international trade in ornamentals is currently £60-75 billion (Chandler and Tanaka 2007; Tanaka, et al. 2005) and the worldwide demand for ornamentals is expected to increase by 2 to 4 % per year for the next 10 years (Grasotti and Gimelli 2011). The continued success of the floriculture industry depends on the introduction of new species/cultivars with major alterations in key agronomic characteristics, such as resistance to pathogens. *Lilium* is one of the six major bulb crops in the world (Le Nard and De Hertogh 1993) because of its attractive, large flowers, which for some cultivars these are highly scented. These attributes make the genus a perfect target for exploitation by the ornamental industry. The genus is composed of approximately 100 species distributed mainly throughout the cold and temperate regions of the Northern Hemisphere (Nishikawa, et al. 1999).

Genetic manipulation techniques have become very powerful tools to improve a range of crops. Such techniques have allowed the introduction of agronomically important traits, which would be difficult to achieve by conventional breeding programmes. Traditional methods to enhance pathogen resistance in crops are laborious and time consuming, taking 15 to 20 years to obtain a resistant cultivar. One approach has been to introgress genes present in wild relatives into commercial crops (Rommens and

74 Kishore 2000). However, this is time-consuming and the resultant resistance frequently
75 breaks down; an alternative approach is the use of transgenic technologies, although the
76 use of such approaches is subject to ongoing debate. Considering that ornamental crops
77 are not produced for human consumption and the fact that transgenic ornamentals have
78 been produced for several years, mainly in South America and commercialized in
79 Europe and Japan (for example the MOON® series of carnations from FLORIGEN®
80 (EFSA, 2006; Katsumoto *et al.,* 2007; Tanaka *et al.,* 2009)), there are opportunities for
81 new species and cultivars to be developed by transgenic approaches. This means that
82 such approaches are becoming important targets for plant breeding even under the
83 present regulatory conditions and ongoing GMO debate (Rommens and Kishore 2000).
84
85 One of the most promising approaches amongst the different strategies that are being
86 adopted is the introduction of genes encoding proteins that inhibit fungal growth.
87 Introducing genes encoding potential Anti-Fungal Proteins (AFPs) of diverse origin,
88 such as chitinases and/or β-1,3 glucanases, has been used for the control of several
89 fungal pathogens (Pierpoint, et al. 1996). The antifungal activity of plant chitinases
90 makes them an ideal target to induce enhanced resistance to fungal pathogens in
91 ornamental species (Wang, et al. 2003). Chitinases belong to family 3 of the tobacco
92 pathogenesis-related proteins and their substrate is chitin (Palli and Retnakaran 1999).
93 These enzymes break bonds between the C1 and C4 of two consecutive N-
94 acetylglucosamines of chitin, which is a main component of the cell wall in fungi. Plant
95 chitinases are classified into seven classes (I–VII) based on their primary structures and
96 have been found in many species of higher plants exhibiting complex developmental
97 and hormonal regulation (Shin, et al. 2008). Chitinases are involved in the plant

hypersensitive resistance response to microbial attack. Purified plant chitinases have been demonstrated to attack and partially digest isolated cell walls of several pathogenic fungi such as ascomycetes, basidiomycetes and deuteromycetes (Zhu and Lamb 1990). Successful transgene-induced pathogen resistance has been reported in plants such as tobacco (Zhu, et al. 1994), rose (Marchant, et al. 1998) chrysanthemum (Takatsu et al., 1999), strawberry (Vellicce, et al. 2006) and wheat (Shin, et al. 2008).

The fungal pathogen *Botrytis* causes huge economic losses to a very wide range of host crop species as the disease can infect several tissues, including flowers, leaves, fruits and stems (Staats *et al.,* 2005). *B. cinerea* causes grey mould, a common disease of flowers and one of the most important in *Lilium* (Hou and Chen, 2003). To avoid the use of chemicals, which would increase the cost of production and impact on the environment, current research on lily breeding has been directed towards the development of disease-resistant cultivars (Balode 2009: Conijn 2014). Some cultivars have been reported as being less susceptible to *Botrytis* infection, however this resistance have shown to be low and difficult to maintain in the progeny (Balode 2009; Daughtrey and Bridgen 2013). To date there are no reports of *Lilium* plants with enhanced pathogen resistance generated by transgenic approaches.

In this study, we investigated the ability of the anti-fungal *Rice Chitinase 10* gene (*RCH10*) to confer resistance by ectopic overexpression in *Lilium* oriental cv. 'Star Gazer'. Enhanced resistance was obtained in the transgenic plant lines, with resistance positively correlated with the level of expression of the *RCH10* chitinase gene. This

demonstrates the utility of this approach and presents significant opportunities for the development of transgenic *Lilium* with enhanced resistance to fungal infection.

Materials and Methods

Agrobacterium-mediated transformation

Plasmid pBI101 (provided by Dr G. Ribas Vargas; University of Nottingham), harbouring the rice chitinase gene *RCH10* (Zhu and Lamb 1990) driven by the CaMV35S promoter, and the *NEOMYCIN PHOSPHOTRANSFERASE* (*NPTII*) gene under the control of the *nos* promoter was transformed by electroporation into *Agrobacterium tumefaciens* strain AGL1. The latter was used to inoculate basal plate discs from *in vitro* derived bulblets of *Lilium* cv. 'Star Gazer'. *Agrobacterium*-mediated transformation was as previously described (Núñez de Cáceres, et al. 2011).

Putative transgenic plants regenerated from inoculated explants were grown *in vitro* until they produced bulbs of an average weight of ~600 mg. Bulbs were then transferred to individual containers containing peat, perlite and vermiculite (4:2:1 by vol), and kept for 10 weeks in the dark at 5°C to break dormancy. After this period, bulbs were transferred to 9cm pots filled with the same compost and maintained in a growth room (16/8h photoperiod; 22 °C ± 1°C day; 16 °C ± 1°C night). After 16 weeks, the aerial parts of plants with bulbs that weighed ~4 grams were cut back and kept at 5 °C in the dark for 10 weeks for floral induction. After this vernalization period, bulbs were returned to the growth room under the previously described conditions.

Molecular analysis of putative transgenic plants

DNA from putative transgenic plants was extracted using a rapid DNA isolation protocol (Edwards, et al. 1991). Leaf material was cleaned with 70% (v/v) ethanol and then ~100 mg was snap frozen in liquid nitrogen and ground to a fine powder using a micro-pestle; 600 µl of DNA extraction buffer (Tris-HCl 200mM, NaCl 250mM, EDTA pH 8.0 25mM, SDS 10% (w/v)) was added and vortexed until no tissue clumps were visible. Samples were incubated at 45°C for 15 min and centrifuged (9500 x *g;* 7 min). Supernatants were transferred into new tubes and ice-cold isopropanol added (1:1 (v/v)). Samples were incubated on ice for 5 min and centrifuged (9500 x *g;* 7 min). The resultant pellets were washed with 200µl of 70% (v/v) ethanol, centrifuged (9500 x *g*; 2 min) and resuspended in 30µl sterile water. To confirm transgene presence, PCR analysis for the *NPTII* gene was conducted using the primers nptII_F (5'-AGACAATCGGCTCTGAT-3') and nptII_R (5'- ATACTTTCTCGGCAGGAGCA-3') with an expected band size of 261 bp (35 cycles; 35 s at 94°C, 35 s 57°C; 35 s 72°C; and the primers 35S_F (5'- CACAATCCCACTATCCTTCGCAAGAC-3' and CHIT_R 5'- CAGCATCTGGTCGAAGAGC-3'), with an expected band size of 352 bp for the *RCH10* gene (33 cycles; 30 s 94°C; 45 s 58.2°C; 45 s 72°C). The absence of *Agrobacterium* in the transformed plants was confirmed by test cultures.

A modified CTAB (CetylTrimethylAmmonium Bromide) protocol (Li, et al. 2011) was used for RNA extraction. Leaf tissue (~100 mg) was excised and snap frozen in liquid nitrogen; 700 µl of pre-warmed extraction buffer (CTAB 2% (w/v); Tris-HCl 100mM; EDTA 20mM; PVP (Polyvinylpyrrolidone) 2% (w/v)) and 1.5% (v/v) β-mercaptoethanol were added, vortexed and incubated at 65°C for 30 min; vortexing every 10 min. After incubation, the RNA was purified using an equal volume of

chloroform-isoamyl alcohol (24:1 (v/v)) and centrifuged (9000 x *g*; 4°C 10 min); this step was repeated 3 times. The RNA was precipitated overnight (-20˚C; 1/3 vol 8 M lithium chloride; 9000 x *g*; 4°C; 20 min), dissolved in 400 µl DEPC (Diethyl pyrocarbonate) treated water, extracted with chloroform-isoamyl alcohol and precipitated with 1/10 vol. 3 M sodium acetate and 3 vol. 100% ethanol (-20°C; 45 min; 9000 x *g* at 4°C; 20 min). The pellet was washed with 200 µl of 70% (v/v) ethanol and resuspended in 25 µl of RNase free water.

cDNA was synthesized from total RNA using the Superscript III kit (Invitrogen) following the manufacturer's instructions. RT-PCR analysis was performed for the *NPTII* gene using the same primers and parameters for PCR; for the *RCH10* gene using the primers RCH10_F 5'- GCCTTCTGGTTCTGGATGAC-3' and RCH10_R 5'-AATCCAAGTTGGCGTCGTAG-3'; expected band size of 247 bp (33 cycles 30 s 94°C; 30 s 56°C; 30 s 72°C), and for a polyubiquitin housekeeping gene using the primers Polyb_F 5'-GAAGCAGCTGGAAGATGGAC-3' and Polyb_R 5'-GATCCCTTCCTTGTCGTGAA-3'; expected band size 196 bp (30 cycles 45 s 94°C; 45 s 58.5°C; 30 s 72°C).

Quantitative Reverse Transcriptase-PCR (qRT-PCR) analysis

qRT-PCR was performed on 22 transgenic plants carrying the *RCH10* gene; wild type plants and water were used as negative controls. SYBR Green qPCR Master Mix 2x (Fermentas) was used according to the manufacturer's instructions. Samples were analysed in triplicate using a Light Cycler 480 (Roche Diagnostics). Primers were as used for RT-PCR for the *RCH10* gene; normalisation of expression was conducted

193 using the actin-like gene *LP59* from *Lilium longiflorum* accession DQ019459 (Lactin_2
194 F 5'-TGGTGTGATGGTTGGTATGG-3' and Lactin_2 R 5'-
195 TTTGCCTTAGGGTTGAGTGG-3'). Parameters for the qRT-PCR were 5 min at 95°C,
196 then 45 cycles of 10 sec at 95°C, 30 sec at 56.5°C and 30 sec at 72°C.
197
198 ***Leaf segment inoculation with Botrytis cinerea***
199 Ten days before each inoculation experiment a 0.5 cm^2 plug of *Botrytis cinerea*
200 (BcVn2) (originally isolated from *Vicia narbonensis;* provided by L. Cheetham,
201 University of Nottingham) was subcultured onto Potato Dextrose Agar (PDA) medium
202 (23°C; dark). Spore solutions were prepared by a method modified from Fiddaman, et
203 al. (2000) by flooding the Petri dishes with 10 ml of spore nutrition solution (0.1 g/L of
204 glucose, 0.1 g/L of KH_2PO_4 and 0.5% (v/v) Tween 20). Plates were scraped to dislodge
205 the conidia and the suspensions filtered through four layers of sterile muslin. Spore
206 solutions were diluted with a sterile water/Phytagel solution to obtain a 0.35% (w/v)
207 final concentration of gelling agent. Spore number was counted with a haemocytometer
208 and adjusted to 5 x 10^5 spores/ml.
209
210 Mature leaves were excised from 16-month-old plants at the mature flowering stage and
211 cleaned briefly with paper tissue soaked with 70% (v/v) ethanol. 1cm^2 explants were
212 transferred abaxially to square Petri dishes each containing 50 ml of sterile Phytagel
213 (1.0% (w/v)). Three replicate samples were used for each transgenic line plus wild type,
214 with three different time points (7, 10 and 14 days post-inoculation); this was
215 subsequently replicated 3 times. Measurement of the extent of sporulation was used to
216 assess resistance to the fungal pathogen. Two inoculation sites were made on each leaf

explant by pipetting 10 µl of spore solution onto each site. Explants were kept in the light (180 µMol m^{-2} s^{-1} Phillips Daylight Tubes, TLD/ 58W 35V) at 23 °C ± 2°C. For sporulation counts, explants were transferred from Petri dishes into 7 ml Universal tubes each containing 1 ml of sterile water supplemented with 0.5 % (w/v) Tween 20. Spore counts were performed under the microscope with a haemocytometer and are based on the average of 3 technical replicates, with 3 biological replicates for each line at each time point.

Results

Generation of RCH10 transgenic lines of Lilium by Agrobacterium transformation

The *Rice Chitinase 10* gene *RCH10* was transformed into explants of *Lilium* cv. 'Star Gazer' by *Agrobacterium*-mediated transformation; 212 putative transgenic plants were recovered from 750 explants (Table 1), on average 12 weeks after inoculation, from four independent experiments. PCR analysis confirmed presence of the *RCH10* and *NPTII* genes into the putative transgenic plant lines (Fig 1). The transformation efficiency was on average 28.2% in the four experiments, which was greater than our previous report of 20.1% (Núñez de Cáceres, et al. 2011). Reverse Transcriptase (RT) - PCR analysis of the mature transgenic plants (40 weeks after explant inoculation) confirmed that both the *NPTII* and *RCH10* genes were expressed (Fig 2); 79% of the analysed transgenic plants expressed the *RCH10* gene, confirming stable transformation and integration of the transgene.

Conferred resistance to Botrytis cinerea varied among the transgenic lines and was positively correlated with RCH10 expression

An assessment of the effect of transgene expression on fungal resistance was conducted by determining the level of infection by *Botrytis cinerea* (BcVn2)*;* on *Lilium* explants. A *Botrytis* spore suspension was used to inoculate *Lilium* leaf explants and sporulation counts were conducted 7, 10 and 14 days post-inoculation. In all cases, *Botrytis* sporulation on explants from wild type plants was greater compared with that on explants from transgenic plants expressing the *RCH10* gene (Table 2). Dunnett's test was used to determine if there was any statistically significant difference between sporulation levels on the transgenic and wild type explants. A statistically significant difference in sporulation rates, with a confidence interval of 95%, was observed at all time points between explants from transgenic plants compared with those from the wild type plants. This indicates that expression of the *RCH10* transgene in lily plants conferred resistance to *Botrytis cinerea* infection (Fig 3).

The relative expression level of the *RCH10* transgene showed a direct correlation with the extent of *Botrytis* sporulation. Quantitative Reverse Transcriptase (qRT)-PCR results showed that the relative expression of the *RCH10* gene varied amongst the independent transgenic plants, ranging from 0.11 to 8.4 increased as compared to the wild type, which did not show any expression (Fig 4). The expression data was compared against the sporulation levels to determine if the extent of resistance to *Botrytis* linked directly to the level of chitinase expression in the plants. Spearman's correlation test was performed to analyse the correlation between the qRT-PCR chitinase expression and sporulation counts. A correlation coefficient (*rs*) of -0.997 was

obtained 7 days after inoculation, -0.842 at 10 days and -0.867 at 14 days after inoculation (Table 3). These results confirmed a strong correlation between the relative expression of the *RCH10* gene and sporulation levels at all time points after inoculation (Fig 5); as the expression of *RCH10* increased, sporulation decreased.

RCH10 transgenic plants showed normal development and flowering

Transgenic and wild type plants were grown alongside each other until flowering to determine if there were any phenotypic effects from the chitinase gene expression. Induction of flowering occurred approximately 75 weeks after inoculation of explants. No significant differences were observed between the transgenic and wild type plants, either during vegetative or reproductive growth (Fig 6). Height, width, stem colour, leaves and flower number and size were all comparable between the transgenic and wild type plants (Fig 6). Flowers showed similar size, colour and structure (Fig 6), with a similar time to flower in both cases. The only difference noted between lines was in bulb size; plants from larger bulbs flowered on average 2 weeks before those from smaller bulbs following a vernalisation period of 10 weeks. However, this characteristic did not appear to be linked to the presence, or expression, of the transgenes. Thus the enhanced pathogen resistance conferred by overexpression of the *RCH10* gene did not appear to affect any other plant functions.

Discussion

Stable transgenic *Lilium* plants exhibiting differences in *RCH10* chitinase expression showed enhanced resistance to the fungal pathogen *Botrytis cinerea.* The extent of *Botrytis* resistance correlated directly with the level of chitinase transgene expression,

288 with plants with higher transgene expression showing the most fungal resistance. This
289 was demonstrated by showing the presence of the transgene by PCR, quantification of
290 the transgene expression by qRT-PCR and subsequent comparison to the levels of
291 sporulation in leaf explants that had been inoculated with *B. cinerea* (BcVn2). This
292 work has demonstrated the application of using a chitinase gene to induce pathogen
293 resistance in *Lilium*. Expression of the chitinase gene was regulated using the
294 CaMV35S promoter, which gives high levels of expression throughout most plant
295 tissues. Importantly, no adverse phenotypic effects were observed from transgene
296 expression, with flowering time and flower development being unaffected.
297
298 These results are consistent with those reported in other species such as rose (Marchant,
299 et al. 1998), chrysanthemum (Takatsu, et al. 1999), peanut (Rohini and Rao 2001),
300 cucumber (Kishimoto, et al. 2002), strawberry (Vellicce, et al. 2006), lemon (Distefano,
301 et al. 2008) and wheat (Shin, et al. 2008) in which chitinase expression correlated with
302 the resistance shown by the transgenic plants. This enhanced resistance has important
303 implications in future breeding programmes and for the development of new elite
304 cultivars. It has the potential to impact significantly on the future control of fungal
305 infections in the field.
306
307 Combining resistance against different diseases is another possibility as multiple genes
308 can be introduced on a single construct into target plants. Introduction of glucanase
309 genes alone, or with chitinase genes, has been reported to enhance resistance to fungal
310 pathogens in other ornamental crops such as geranium (Mohandas, et al. 2009), African
311 violet (Ram and Mohandas 2003), petunia (Esposito, et al. 2000) and carnation (Zuker,

et al. 2001). The present work also provides the opportunity to stack resistance genes against viral, fungal, and bacterial diseases, to induce resistance to a wider spectrum of pathogens in *Lilium.*

This is the first report of *Lilium* plants with resistance to *Botrytis cinerea* using a transgenic approach, and the first description of stable *Agrobacterium*-mediated transformation with a transgene, other than *GUS* and *NPTII,* in any species or cultivar of *Lilium.* This analysis has been conducted using an *in-vitro* assay, however other researchers have found differences in the level of resistance in showed by transgenic plants tested *in vitro* and *in vivo* such as potato, wheat and barley (Wu *et al.,* 1995; Rommens and Kishore 2000; Anand *et al*., 2003; Balconi *et al.,* 2007; Shin *et al.,* 2008). The next step is therefore to test these transgenic lines under varying conditions in which wind, temperature and humidity fluctuations, nutrient levels in the plant and the interaction with other potential pathogens could have an impact on the level of resistance. Further research will focus also on determining the maintenance of resistance in subsequent generations by analysis of the progeny of these transgenic plants and whether resistance is also conferred against other fungal pathogens.

Author Contribution Statement

FFNCG carried out experiments and manuscript preparation; MRD was involved in devising the work and writing the manuscript; ECS provided technical assistance in the experiments; ZAW was involved in devising the study, data analysis and preparation of the manuscript. All authors read and approved the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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456

457 **Figure legends**

458 **Figure 1 PCR analysis of the *NPTII* and *RCH10* genes in transgenic plants *Lilium***
459 **cv. 'Star Gazer'**

460 Lanes: (M) DNA marker; (W) water: negative control; (P) plasmid DNA: positive
461 control; (WT) wild type DNA as negative control; (1) Line R169; (2) Line R660; (3)
462 Line R679; (4) Line R29; (5) Line R400; (6) Line R108; (7) Line R206; (8) Line R673;
463 (9) Line R504; (10) Line R688; (11) Line R666; (12) Line R165. All plant samples

showed presence of the *NPTII* selectable marker gene, although line R169 showed very low levels of amplification, and all except line R108 showed the presence of the *RCH10* transgene.

Figure 2 RT-PCR analysis of the *NPTII* and *RCH10* genes in transgenic lines of *Lilium* cv. 'Star Gazer'

Lanes: (M) DNA marker; (W) water- negative control; (P) plasmid DNA- positive control; (WT): wild type DNA as negative control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R165; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line 666; (11) Line R108; (12) Line R47. All plant samples showed expression of the *NPTII* selectable marker and the Polyubiquitin housekeeping gene, and all but four showed expression of the *RCH10* gene.

Figure 3 Sporulation on leaf explants from transgenic *Lilium* plants 14 days after *Botrytis cinerea* inoculation.

(a, d) Wild type explant; (b, e) Plant R679 with a low expression of the *RCH10* gene; (c, f) Plant R504 with very high expression of the *RCH10* gene. (a-c) bar=1cm. (d-f) bar= 0.4 mm.

Figure 4 qRT-PCR analysis of *RCH10* transgene expression in leaves of transgenic *Lilium* plants.

485 Relative expression of the *RCH10* gene from different transgenic plants (R688-R206)
486 compared to expression in a wild type (WT) plant. Expression was normalised using
487 expression of the *LP59* housekeeping gene. All plants analysed plants were at mature-
488 plant flowering stage.

489

490 **Figure 5 Sporulation versus relative expression of the *RCH10* gene** Sporulation
491 counts for leaf explants of transgenic plants are shown at 7, 10 and 14 days post-
492 inoculation and plotted against their corresponding relative level of expression of the
493 *RCH10* transgene. A correlation coefficient (rs) of -0.997 was obtained at 7 days post-
494 inoculation, -0.842 at 10 days post-inoculation and of -0.867 at 14 days post-
495 inoculation; indicating a very strong negative correlation between the relative
496 expression of the *RCH10* gene and sporulation at all time points.

497

498 **Figure 6 Phenotypic comparison of transgenic plants expressing the *RCH10* gene**
499 **compared with a wild type plant**

500 All plants showed equivalent size, colour, shape and number of flowers as in the wild
501 type plants. All flowers of transgenic plants had the same size and arrangement of floral
502 organs as in wild type. (a, f) Wild type; (b, g) Line R169 (low *RCH10* expression); (c,
503 h) Line R504 (very high *RCH10* expression); (d, i) Line R206 (medium *RCH10*
504 expression); (e, i) Line R673 (high *RCH10* expression); Bars: (a-d) = 9 cm. (e-i) = 5 cm.
505 *RCH10* expression based upon qRT-PCR data (Fig 4).

506

Agrobacterium-mediated transformation of *Lilium* cv. 'Star Gazer'					
Experiment No.	**No. of explants inoculated**	**No. of Kan resistant tissues**	**% of Kan resistant tissues**	**No. of transgenic plants**	**Transformation efficiency (%)***
1	100	49	49	26	26.0
2	200	94	47	48	24.0
3	300	184	61	98	32.6
4	150	67	44	40	26.6
Total	**750**	**394**	**52.5**	**212**	**28.2**

507

508

509 **Table 1** ***Agrobacterium*-mediated** ***RCH10*** **transformation of** ***Lilium*** Efficiency
510 obtained in four independent transformation experiments of *Lilium* cv. 'Star Gazer'.

511 (*) Transformation efficiency was calculated as the number of PCR-positive
512 regenerated transgenic plants compared to the number of explants inoculated.

513

Plant Line	Chitinase relative expression*	Sporulation (Spores/ml)		
		7 days	10 days	14 days
R504	Very high	2	11	60
R169	Low	30	49	142
R666	Medium	7	35	139
R673	High	6	14	73
R660	Low	9	23	99
R165	Low	15	74	194
R688	Very low	16	82	264
R206	Medium	7	28	105
R679	Very low	22	69	212
Wild Type	None	311	795	3568

Table 2 ***Botrytis cinerea*** **spore counts after inoculation of leaf explants from** ***RCH10*** **transgenic** ***Lilium*** **and wild type plants** *Expression based upon qRT-PCR data (Fig 4).

Spearman's Correlation Analysis (Expression, Spores)		
	r_s	p value
7 days	-0.997	<0.0001
10 days	-0.842	0.0011
14 days	-0.867	0.0006

Table 3. Spearman's Correlation Analysis. Correlation between the level of expression of the *RCH10* gene and the sporulation count was analysed at 7, 10 and 14 days after inoculation. Values closer to 1 represent a stronger correlation, the negative sign on the value denotes an inverse correlation.

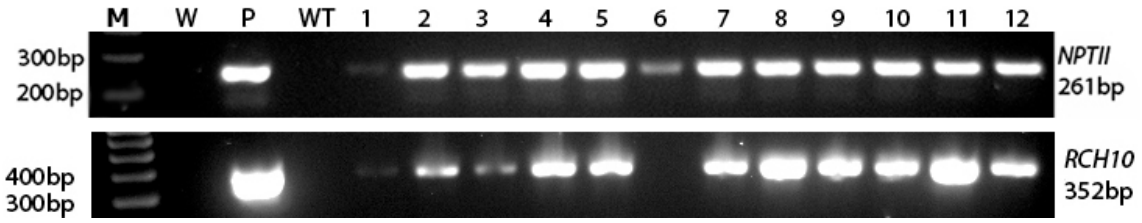


Figure 1 PCR analysis of the *NPTII* and *RCH10* genes in transgenic plants *Lilium* cv. 'Star Gazer'.

Lanes: (M) DNA marker; (W) water: negative control; (P) plasmid DNA: positive control; (WT) wild type DNA as negative control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R108; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line R688; (11) Line R666; (12) Line R165. All putative transformed plant samples showed positive insertion of the *NPTII* selectable marker gene, although line R169 showed very low levels of amplification, and all except line R108 showed the presence of the *RCH10* transgene.

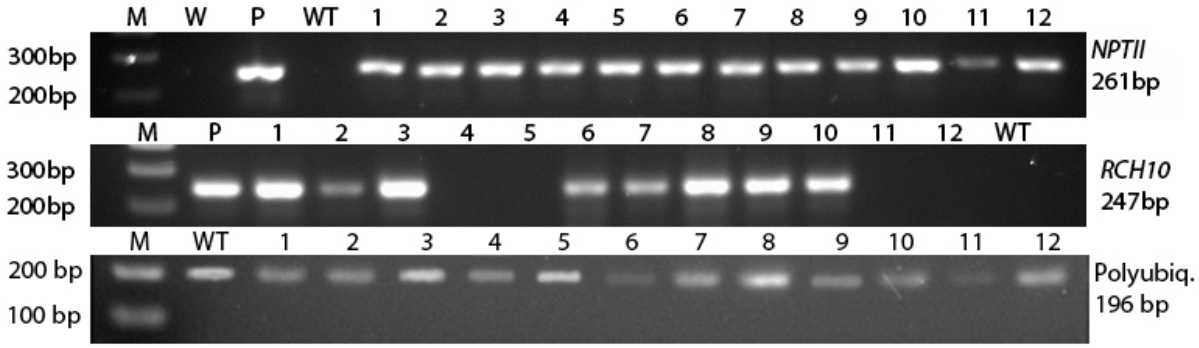


Figure 2 RT-PCR analysis of the *NPTII* and *RCH10* genes in transgenic lines of *Lilium* cv. 'Star Gazer'.

Lanes: (M) DNA marker; (W) water- negative control; (P) plasmid DNA- positive control; (WT): wild type DNA as negative control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R165; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line 666; (11) Line R108; (12) Line R47. All plant samples showed expression of the *NPTII* selectable marker and the Polyubiquitin housekeeping gene, and all but four showed expression of the *RCH10* gene.

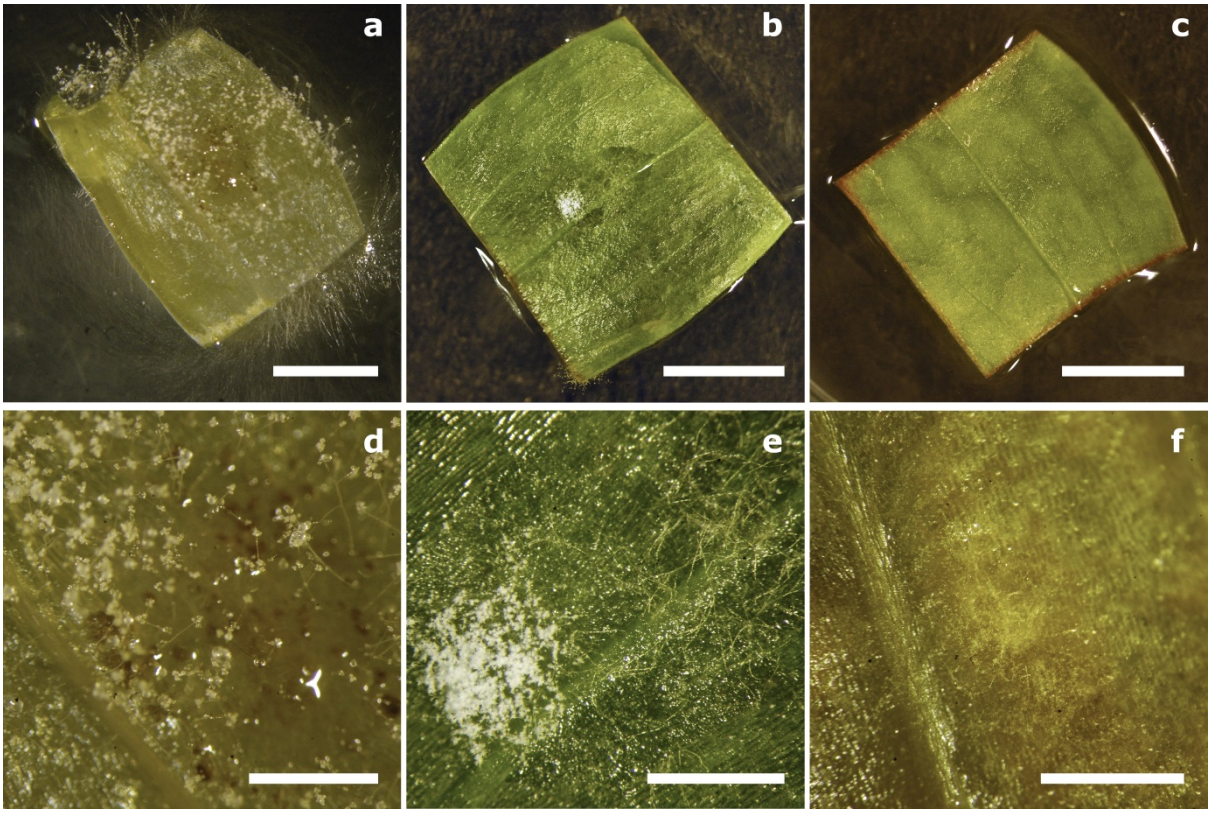


Figure 3 Sporulation on leaf explants from transgenic *Lilium* plants 14 days after *Botrytis cinerea* inoculation

(a, d) Wild type explant; (b, e) Plant R679 with a low expression of the *RCH10* gene; (c, f) Plant R504 with very high expression of the *RCH10* gene. (a-c) bar=1cm. (d-f) bar= 0.4 mm.

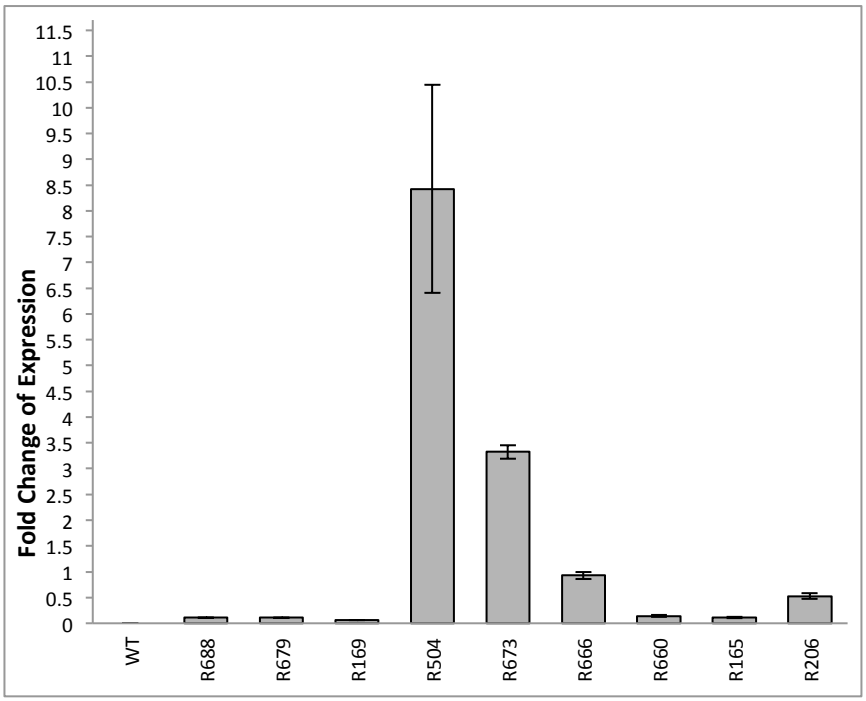
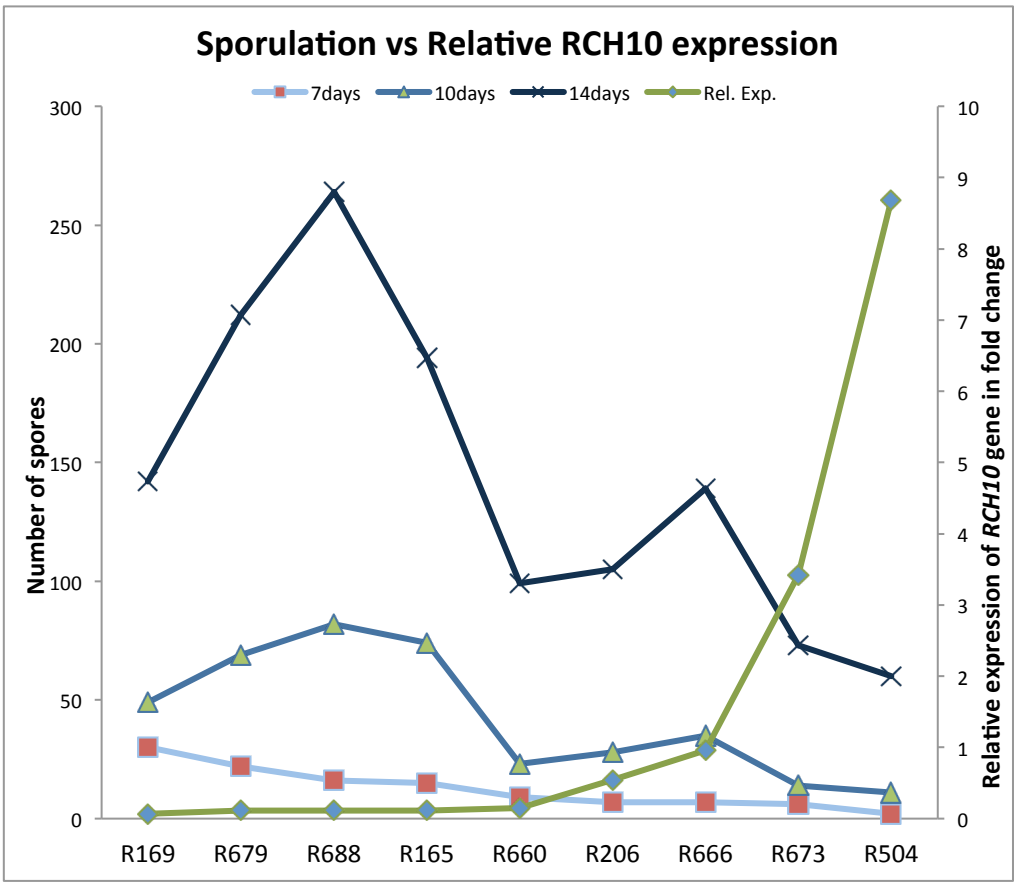


Figure 4 qRT-PCR analysis of *RCH10* transgene expression in leaves of transgenic *Lilium* plants

Relative expression of the *RCH10* gene from different transgenic plants (R688-R206) compared to expression in a wild type (WT) plant. Expression was normalised using expression of the *LP59* housekeeping gene. All plants analysed plants were at mature-plant flowering stage.



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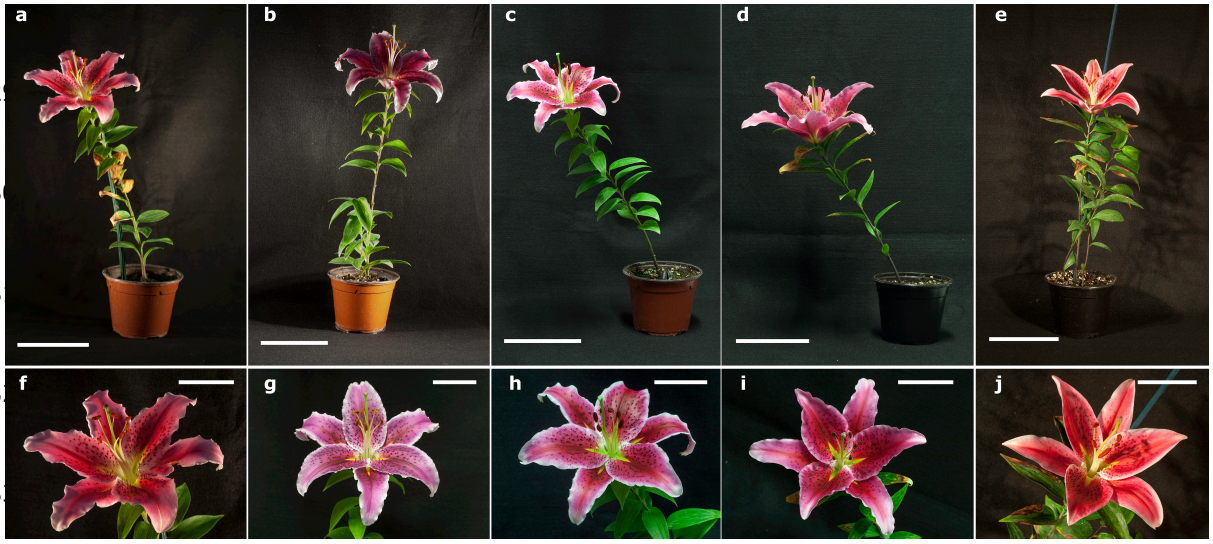

Figure 6 Phenotypic comparison of transgenic plants expressing the *RCH10* gene compared with a wild type plant

All plants showed equivalent size, colour, shape and number of flowers as in the wild type plants. All flowers of transgenic plants had the same size and arrangement of floral organs as in wild type. (a, f) Wild type; (b, g) Line R169 (low *RCH10* expression); (c, h) Line R504 (very high *RCH10* expression); (d, i) Line R206 (medium *RCH10* expression); (e, i) Line R673 (high *RCH10* expression); Bars: (a-d) = 9 cm. (e-i) = 5 cm. *RCH10* expression based upon qRT-PCR data (Fig 4).