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

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18

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

20

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

24

25

26 **Abstract**

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

46

47

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

49

## 50 **Introduction**

51 Fungal diseases are the cause of reduced yields and marketable quality of cultivated  
52 plants, including ornamental species. This results in significant loss of income and  
53 simultaneously increases the production costs as a direct result of disease prevention or  
54 control treatments (Inglis and Kawchuk 2002; Tanaka, et al. 2005; Zuker, et al. 1998).  
55 The ornamental industry has been gaining in strength and importance in recent years.  
56 The international trade in ornamentals is currently £60-75 billion (Chandler and Tanaka  
57 2007; Tanaka, et al. 2005) and the worldwide demand for ornamentals is expected to  
58 increase by 2 to 4 % per year for the next 10 years (Grasotti and Gimelli 2011). The  
59 continued success of the floriculture industry depends on the introduction of new  
60 species/cultivars with major alterations in key agronomic characteristics, such as  
61 resistance to pathogens. *Lilium* is one of the six major bulb crops in the world (Le Nard  
62 and De Hertogh 1993) because of its attractive, large flowers, which for some cultivars  
63 these are highly scented. These attributes make the genus a perfect target for  
64 exploitation by the ornamental industry. The genus is composed of approximately 100  
65 species distributed mainly throughout the cold and temperate regions of the Northern  
66 Hemisphere (Nishikawa, et al. 1999).

67

68 Genetic manipulation techniques have become very powerful tools to improve a range  
69 of crops. Such techniques have allowed the introduction of agronomically important  
70 traits, which would be difficult to achieve by conventional breeding programmes.  
71 Traditional methods to enhance pathogen resistance in crops are laborious and time  
72 consuming, taking 15 to 20 years to obtain a resistant cultivar. One approach has been  
73 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  $\beta$ -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

98 hypersensitive resistance response to microbial attack. Purified plant chitinases have  
99 been demonstrated to attack and partially digest isolated cell walls of several pathogenic  
100 fungi such as ascomycetes, basidiomycetes and deuteromycetes (Zhu and Lamb 1990).  
101 Successful transgene-induced pathogen resistance has been reported in plants such as  
102 tobacco (Zhu, et al. 1994), rose (Marchant, et al. 1998) chrysanthemum (Takatsu et al.,  
103 1999), strawberry (Vellicce, et al. 2006) and wheat (Shin, et al. 2008).

104

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

116

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



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

123

## 124 **Materials and Methods**

### 125 *Agrobacterium-mediated transformation*

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

133

134 Putative transgenic plants regenerated from inoculated explants were grown *in vitro*  
135 until they produced bulbs of an average weight of ~600 mg. Bulbs were then transferred  
136 to individual containers containing peat, perlite and vermiculite (4:2:1 by vol), and kept  
137 for 10 weeks in the dark at 5°C to break dormancy. After this period, bulbs were  
138 transferred to 9cm pots filled with the same compost and maintained in a growth room  
139 (16/8h photoperiod; 22 °C ± 1°C day; 16 °C ± 1°C night). After 16 weeks, the aerial  
140 parts of plants with bulbs that weighed ~4 grams were cut back and kept at 5 °C in the  
141 dark for 10 weeks for floral induction. After this vernalization period, bulbs were  
142 returned to the growth room under the previously described conditions.

143

### 144 *Molecular analysis of putative transgenic plants*

145 DNA from putative transgenic plants was extracted using a rapid DNA isolation  
146 protocol (Edwards, et al. 1991). Leaf material was cleaned with 70% (v/v) ethanol and  
147 then ~100 mg was snap frozen in liquid nitrogen and ground to a fine powder using a  
148 micro-pestle; 600 µl of DNA extraction buffer (Tris-HCl 200mM, NaCl 250mM, EDTA  
149 pH 8.0 25mM, SDS 10% (w/v)) was added and vortexed until no tissue clumps were  
150 visible. Samples were incubated at 45°C for 15 min and centrifuged (9500 x g; 7 min).  
151 Supernatants were transferred into new tubes and ice-cold isopropanol added (1:1  
152 (v/v)). Samples were incubated on ice for 5 min and centrifuged (9500 x g; 7 min). The  
153 resultant pellets were washed with 200µl of 70% (v/v) ethanol, centrifuged (9500 x g; 2  
154 min) and resuspended in 30µl sterile water. To confirm transgene presence, PCR  
155 analysis for the *NPTII* gene was conducted using the primers nptII\_F (5'-  
156 AGACAATCGGCTCTGAT-3') and nptII\_R (5'- ATACTTTCTCGGCAGGAGCA-3')  
157 with an expected band size of 261 bp (35 cycles; 35 s at 94°C, 35 s 57°C; 35 s 72°C;  
158 and the primers 35S\_F (5'- CACAATCCCACACTATCCTTCGCAAGAC-3' and CHIT\_R  
159 5'- CAGCATCTGGTCGAAGAGC-3'), with an expected band size of 352 bp for the  
160 *RCH10* gene (33 cycles; 30 s 94°C; 45 s 58.2°C; 45 s 72°C). The absence of  
161 *Agrobacterium* in the transformed plants was confirmed by test cultures.

162

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

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

176

177 cDNA was synthesized from total RNA using the Superscript III kit (Invitrogen)  
178 following the manufacturer's instructions. RT-PCR analysis was performed for the  
179 *NPTII* gene using the same primers and parameters for PCR; for the *RCH10* gene using  
180 the primers RCH10\_F 5'- GCCTTCTGGTTCTGGATGAC-3' and RCH10\_R 5'-  
181 AATCCAAGTTGGCGTCGTAG-3'; expected band size of 247 bp (33 cycles 30 s  
182 94°C; 30 s 56°C; 30 s 72°C), and for a polyubiquitin housekeeping gene using the  
183 primers Polyb\_F 5'-GAAGCAGCTGGAAGATGGAC-3' and Polyb\_R 5'-  
184 GATCCCTTCCTTGTCGTGAA-3'; expected band size 196 bp (30 cycles 45 s 94°C;  
185 45 s 58.5°C; 30 s 72°C).

186

### 187 ***Quantitative Reverse Transcriptase-PCR (qRT-PCR) analysis***

188 qRT-PCR was performed on 22 transgenic plants carrying the *RCH10* gene; wild type  
189 plants and water were used as negative controls. SYBR Green qPCR Master Mix 2x  
190 (Fermentas) was used according to the manufacturer's instructions. Samples were  
191 analysed in triplicate using a Light Cycler 480 (Roche Diagnostics). Primers were as  
192 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<sup>2</sup> 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<sub>2</sub>PO<sub>4</sub> 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/Phytigel 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<sup>5</sup> 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<sup>2</sup> explants were  
212 transferred abaxially to square Petri dishes each containing 50 ml of sterile Phytigel  
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

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

224

225

## 226 **Results**

### 227 ***Generation of RCH10 transgenic lines of Lilium by Agrobacterium transformation***

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

239

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

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

254

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

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

268

### 269 ***RCH10 transgenic plants showed normal development and flowering***

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

283

### 284 **Discussion**

285 Stable transgenic *Lilium* plants exhibiting differences in *RCH10* chitinase expression  
286 showed enhanced resistance to the fungal pathogen *Botrytis cinerea*. The extent of  
287 *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,



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

315

316 This is the first report of *Lilium* plants with resistance to *Botrytis cinerea* using a  
317 transgenic approach, and the first description of stable *Agrobacterium*-mediated  
318 transformation with a transgene, other than *GUS* and *NPTII*, in any species or cultivar  
319 of *Lilium*. This analysis has been conducted using an *in-vitro* assay, however other  
320 researchers have found differences in the level of resistance in showed by transgenic  
321 plants tested *in vitro* and *in vivo* such as potato, wheat and barley (Wu *et al.*, 1995;  
322 Rommens and Kishore 2000; Anand *et al.*, 2003; Balconi *et al.*, 2007; Shin *et al.*,  
323 2008). The next step is therefore to test these transgenic lines under varying conditions  
324 in which wind, temperature and humidity fluctuations, nutrient levels in the plant and  
325 the interaction with other potential pathogens could have an impact on the level of  
326 resistance. Further research will focus also on determining the maintenance of  
327 resistance in subsequent generations by analysis of the progeny of these transgenic  
328 plants and whether resistance is also conferred against other fungal pathogens.

329

### 330 **Author Contribution Statement**

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

335

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341

342 **Conflict of Interest**

343 The authors declare that they have no conflict of interest.

344

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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

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

467

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

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

476

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

479 (a, d) Wild type explant; (b, e) Plant R679 with a low expression of the *RCH10* gene; (c,  
480 f) Plant R504 with very high expression of the *RCH10* gene. (a-c) bar=1cm. (d-f) bar=  
481 0.4 mm.

482

483 **Figure 4 qRT-PCR analysis of *RCH10* transgene expression in leaves of transgenic**  
484 ***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 ( $r_s$ ) 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

<i>Agrobacterium</i> -mediated transformation of <i>Lilium</i> cv. ‘Star Gazer’					
Experiment No.	No. of explants inoculated	No. of		No. of transgenic plants	Transformation efficiency (%)*
		Kan resistant tissues	% of Kan resistant tissues		
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
<b>Total</b>	<b>750</b>	<b>394</b>	<b>52.5</b>	<b>212</b>	<b>28.2</b>

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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



514

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

515

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

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524			
<b>Spearman's Correlation Analysis (Expression, Spores)</b>			
525			
	$r_s$	p value	526
527			
7 days	-0.997	<0.0001	528
10 days	-0.842	0.0011	529
14 days	-0.867	0.0006	530
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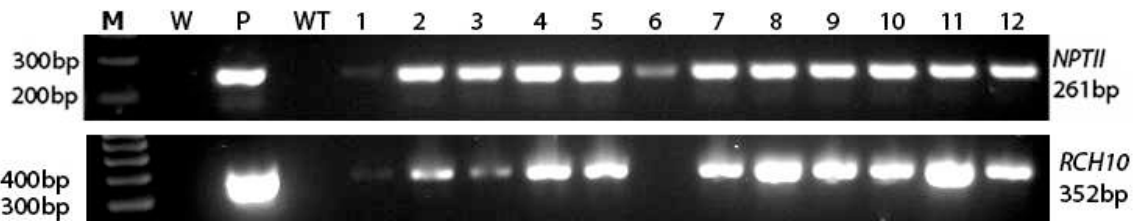
533 **Table 3. Spearman's Correlation Analysis.** Correlation between the level of  
534 expression of the *RCH10* gene and the sporulation count was analysed at 7, 10 and 14  
535 days after inoculation. Values closer to 1 represent a stronger correlation, the negative  
536 sign on the value denotes an inverse correlation.

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543 **Figure 1 PCR analysis of the *NPTII* and *RCH10* genes in transgenic plants *Lilium***  
544 **cv. ‘Star Gazer’.**

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

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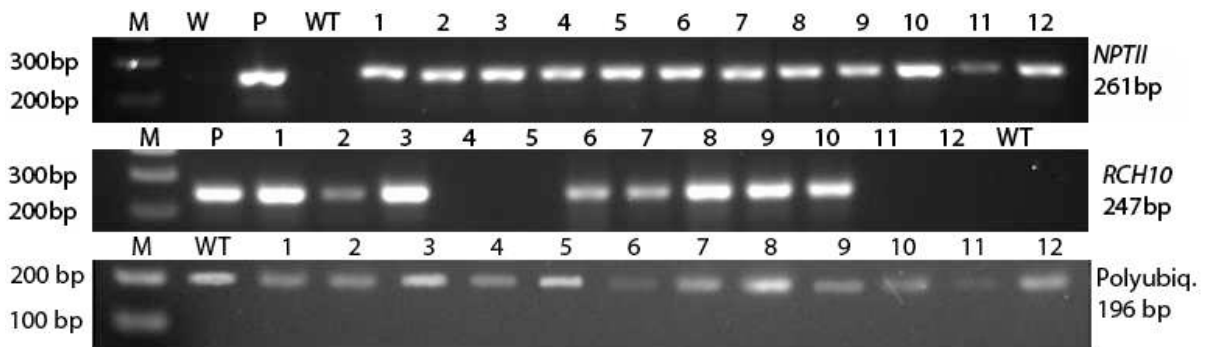
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561 **Figure 2 RT-PCR analysis of the *NPTII* and *RCH10* genes in transgenic lines of**

562 ***Lilium* cv. ‘Star Gazer’.**

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564 control; (WT): wild type DNA as negative control; (1) Line R169; (2) Line R660; (3)

565 Line R679; (4) Line R29; (5) Line R400; (6) Line R165; (7) Line R206; (8) Line R673;

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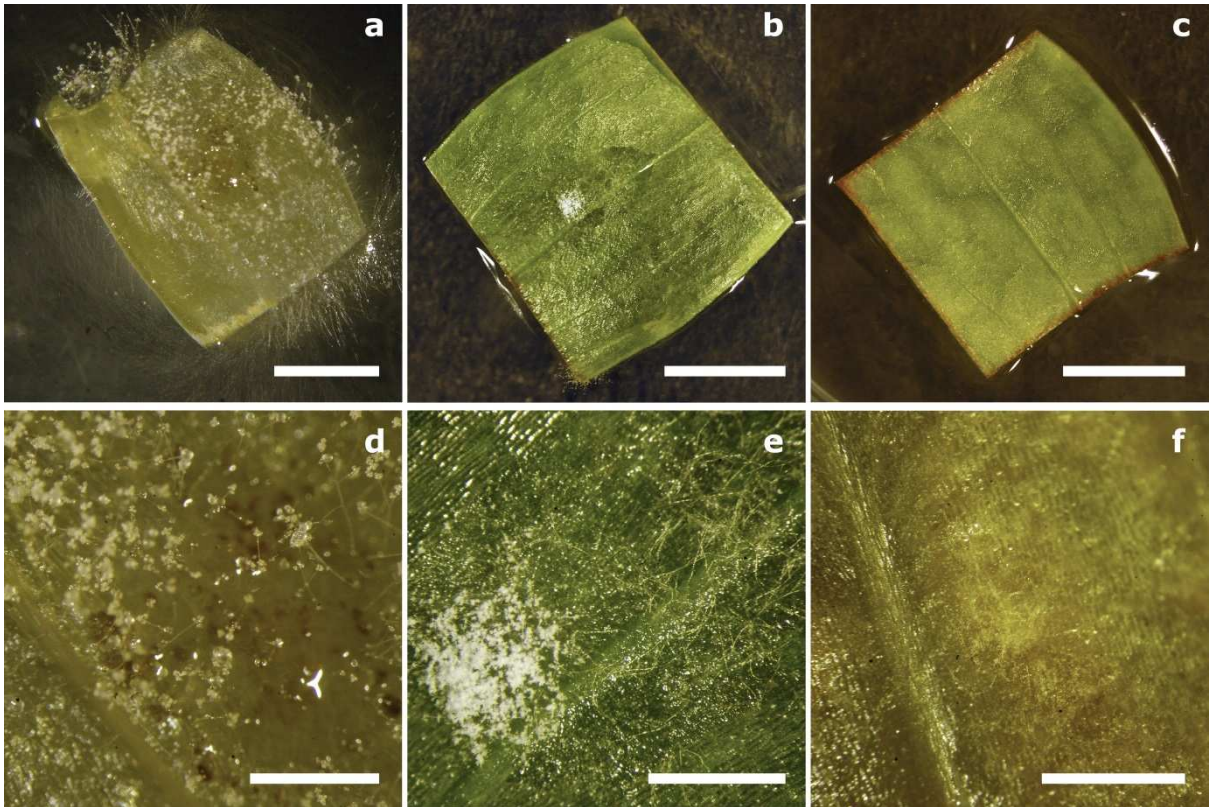
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584 **Figure 3 Sporulation on leaf explants from transgenic *Lilium* plants 14 days after**

585 ***Botrytis cinerea* inoculation**

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587 f) Plant R504 with very high expression of the *RCH10* gene. (a-c) bar=1cm. (d-f) bar=

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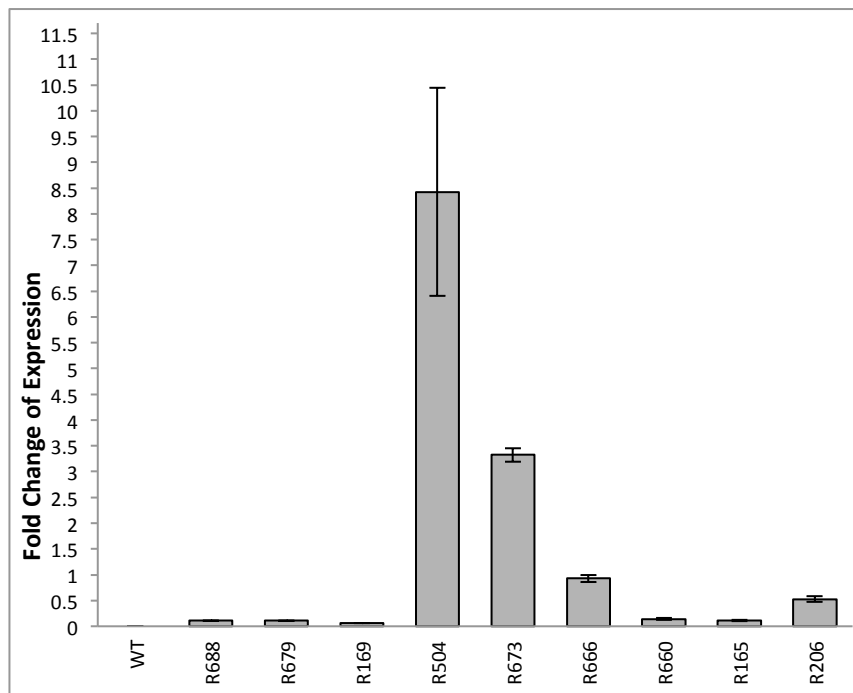
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604 **Figure 4 qRT-PCR analysis of *RCH10* transgene expression in leaves of transgenic**  
605 ***Lilium* plants**

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607 compared to expression in a wild type (WT) plant. Expression was normalised using  
608 expression of the *LP59* housekeeping gene. All plants analysed plants were at mature-  
609 plant flowering stage.

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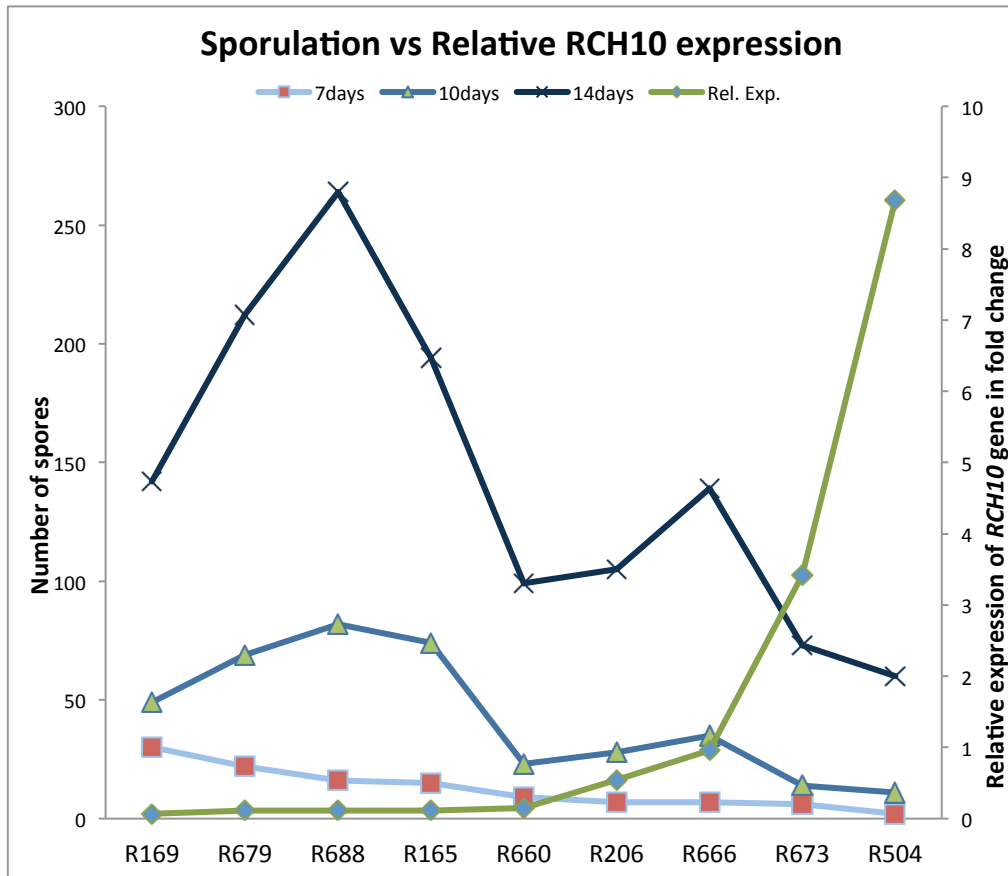
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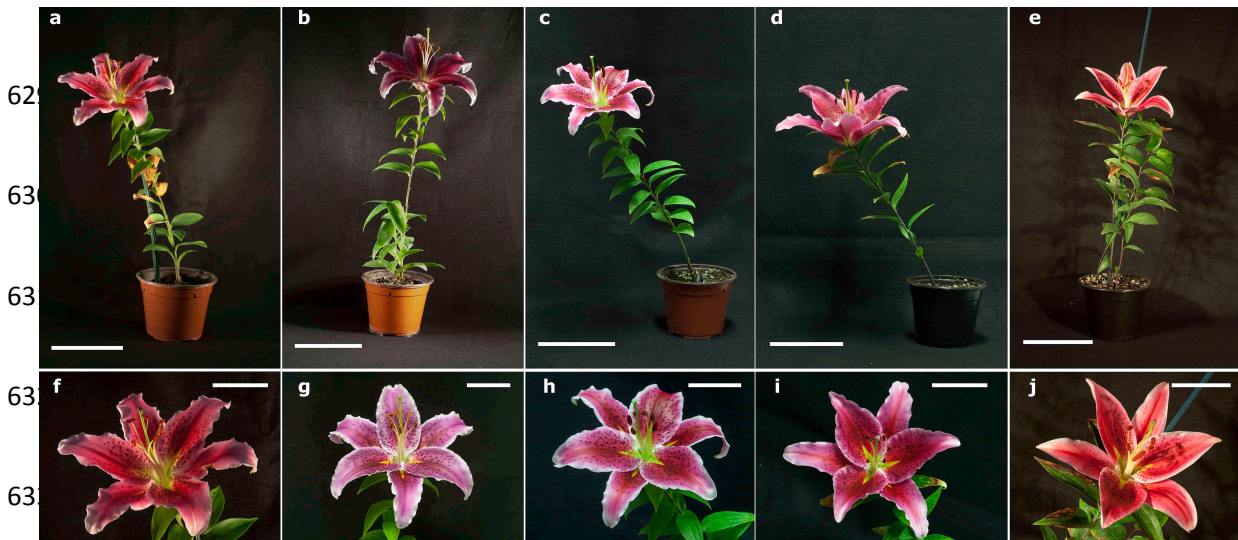
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622 **Figure 5 Sporulation versus relative expression of the *RCH10* gene** Sporulation  
623 counts for leaf explants of transgenic plants are shown at 7, 10 and 14 days post-  
624 inoculation and plotted against their corresponding relative level of expression of the  
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 642 expression); (e, i) Line R673 (high *RCH10* expression); Bars: (a-d) = 9 cm. (e-i) = 5 cm.  
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