

1 The rat glucocorticoid receptor integration in *Nicotiana*
2 *langsdorffii* genome affects plant responses to abiotic stresses and
3 to arbuscular mycorrhizal symbiosis

4 Patrizia Bogani¹, Elisa Calistri¹, Stefano Biricolti², Monica Ruffini Castiglione³,
5 Alessandra Turrini⁴, Manuela Giovannetti⁴, Marcello Buiatti¹, Patrizia Bogani¹

6 1.Dipartimento di Biologia Università di Firenze Florence Italy

7 2.Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente Università di Firenze
8 Florence Italy

9 3.Dipartimento di Biologia Università di Pisa Pisa Italy

10 4.Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali Università di Pisa, Pisa Italy

11 **Abstract**

12 The present study reports evidence of the pleiotropic effects caused by the insertion of the rat
13 glucocorticoid receptor (GR) into the genome of *Nicotiana langsdorffii*. Transgenic *N. langsdorffii*-
14 GR plants and the wild-type genotypes were analysed for their phenotypic and physiological
15 characteristics. The integration of the *GR* gene affected flowering, growth habit, leaf morphology
16 and stomatal pattern. Furthermore, GR plants showed an increased tolerance to heavy metal, drought
17 and heat stress as evidenced by electrolyte leakage and by cell dedifferentiation and differentiation
18 capability after recovery from stress treatments. We also monitored the establishment of the
19 beneficial symbiosis between transgenic plants and the mycorrhizal fungus *Funneliformis mosseae*
20 whose pre-symbiotic growth was significantly reduced by root exudates of *N. langsdorffii*-GR
21 plants. The observed pleiotropic responses of transgenic plants may be a consequence of the
22 hormonal imbalance, putatively due to the interaction of the GR receptor with the host genetic
23 background. Our findings suggest that *N. langsdorffii*-GR plants can be used as a functional model
24 system for the study of plant responses to a series of environmental stimuli.

25 **Keywords**

26 Abiotic stress Arbuscular mycorrhizal symbiosis *Nicotiana* Rat glucocorticoid receptor (GR)

27

28

29

30

31

32

33

34

36 **Introduction**

37 The similarity of steroid signalling pathways in plants and animals has been suggested by the
38 interactions of plant sterol derivatives with human systems. Rárová et al. (2012) showed that
39 brassinosteroids inhibit angiogenesis in human endothelial cells and interact with human steroid
40 receptors such as the oestrogen receptors α and β and the androgen receptor. Moreover, Steigerová
41 et al. (2012) found that brassinosteroids mediated the apoptosis of human cancer cells by inducing
42 cellular block and stopping cells at the G1 stage. The glucocorticoid receptor (GR) is a mammalian
43 nuclear hormone-receptor transcription factor (Giguere et al. 1986) that is activated by
44 glucocorticoids, a class of steroid hormones, synthesized in the adrenal cortex, and popular as anti-
45 inflammatory and immunosuppressive therapeutic agents (Mangelsdorf et al. 1995). Glucocorticoids
46 pass through the plasma membrane into the cytoplasm where they bind to GR receptor. In the
47 cytoplasm, the receptor in the unliganded form is complexed with heatshock proteins (HSPs),
48 namely Hsp90 and its cofactors (Cadepond et al. 1991; Kovacs et al. 2005). Upon hormone binding,
49 GR dissociates from HSPs and forms a homodimer that is translocated to the nucleus where it
50 activates the expression of targeted genes by binding to specific DNA sequences (glucocorticoid
51 response elements, GRE) present in their promoter regions (Yamamoto 1985; Cadepond et al. 1991;
52 Kovacs et al. 2005). Glucocorticoids may also bind to trans-membrane receptors for non-genomic
53 glucocorticoid-receptor-dependent modulation of signal transduction pathways (Sheppard 2003;
54 Patel et al. 2014).

55 Aoyama and Chua (1997) introduced a promoter containing the ligand-binding domain of the animal
56 glucocorticoid receptor into transgenic *Arabidopsis* plants based on the hypothesis that
57 “glucocorticoid itself does not cause any pleiotropic effect in plants”. These authors expected
58 transcriptional induction system to be activated by a synthetic molecule, dexamethasone (DEX).
59 Since then, many papers reported the use of ligand-binding domain of the animal glucocorticoid
60 receptor as an inducible system to activate downstream genes (see for instance Yu et al. 2004; Stamm
61 et al. 2012) without observing pleiotropic effects before the treatment with the ligand molecule
62 (DEX). However, the full length glucocorticoid receptor can induce a whole chain of events acting
63 as a transcription factor (Truss and Beato 1993).

64 In our laboratory, we transformed two different *Nicotiana* spp. with the constitutively expressed full-
65 length rat glucocorticoid receptor (GR) (accession number M14053) with the aim of testing the
66 hypothesis of direct interactions between animal GR and the plant’s physiological network. To this
67 aim, GR was not activated through the usage of DEX or other known steroid molecules. We observed
68 a number of “unintended effects” on the morphology and physiology of *Nicotiana* spp. transgenic
69 plants, suggesting that plant steroids might trigger, through the activation of GR, an “animal-plant”
70 signalling chain (Giannarelli et al. 2010; Fuoco et al. 2013). Actually, in our previous research an
71 imbalance of the hormonal pattern induced by the integration of the *GR* gene was demonstrated,
72 showing also an increased level of stress-related molecules. In particular, the modified hormonal
73 patterns of the transgenic lines strongly affected the response of the plants to metal stress (Fuoco et
74 al. 2013), conferring to GR plants a higher capability to survive heavy metal treatment.

75 In order to investigate whether the observed stress tolerance of GR lines might be extended to other
76 types of abiotic stresses and to the establishment of mycorrhizal symbiosis, a set of experiments was
77 carried out. In particular, in this work, (1) we examined the morphology and physiology of *N.*
78 *langsdorffii* transgenic plants as affected by GR integration; (2) we assessed the responses of the
79 transgenic plants to abiotic stresses induced by heavy metals, drought and heat, and to beneficial
80 interaction with the arbuscular mycorrhizal (AM) symbiont *Funneliformis mosseae*.

81

82 Materials and methods

83 Plant material

84 Transgenic *Nicotiana langsdorffii*-GR plants were obtained, as described in Giannarelli et al. (2010),
85 through the leaf disc transformation technique with *Agrobacterium tumefaciens* strain LBA4404
86 containing the binary vector pTI18 harbouring the rat glucocorticoid receptor (*GR*) gene (Irdani et
87 al. 2003) under the control of the CaMV35S promoter. Transformants were selected using 100 mg/L
88 kanamycin monosulfate (Sigma/Aldrich, USA) and *Agrobacterium* eliminated with 500 mg/L
89 carbenicillin (Sigma/Aldrich, USA). Individuals (T_1) randomly selected from all those obtained from
90 self-crossing independent transgenic lines (T_0) and individuals (T_2) from T_1 -selfed plants were
91 grown in a greenhouse under natural lighting with day length of 16 h and temperature ranging from
92 18 to 24 ± 1 °C. Harvested seeds were placed in a 1.5 mL centrifuge tube and surface sterilized with
93 70 % alcohol for 1 min, 25 % (v/v) Clorox bleach (6.0 % NaClO₃) for 20 min, followed by four
94 rinses with sterilized Milli-Q water (Millipore, USA). Seeds were then germinated in petri dishes
95 containing Linsmaier & Skoog (LS) medium (Sigma-Aldrich, USA), supplemented with 100 mg/L
96 of kanamycin. The selected T_1 seedlings were maintained under these conditions until further
97 transfers onto fresh medium at growth intervals of 30 days, or self-pollinated to produce T_2 -selfed
98 progeny after acclimatization of the plants. T_1 and T_2 plants as a pool of representative transgenic
99 plants were then screened for the presence and the expression of the *GR* transgene, as earlier
100 described (Giannarelli et al. 2010) and used for further analyses.

101 Plant morphology and stomatal features

102 A number of phenotypic features of *N. langsdorffii* wild-type and T_1 and T_2 -selfed transgenic plants
103 were studied and compared. To this aim, five T_1 and ten T_2 transgenic plants and relative isogenic
104 wild-type plants were acclimated for 1 month in a growth chamber at 24 ± 1 °C, and then transferred
105 to a greenhouse until flowering and seed production. All plants were screened for morphology, plant
106 height, internode number, flower number, flowering time and seed production. Plant height was
107 measured at maturity as the distance from the soil surface to the top of the inflorescence. The number
108 of internodes was determined starting from the base of the plant. Differences between and within
109 groups were analysed with PAST software version 3.0 (Hammer et al. 2001) by using the one-way
110 ANOVA package. Principal Component Analysis (PCA) was carried out by using the PCA package
111 found in the PAST software. As the plants to increase resistance to drought use stomata, analysis of
112 stomata was carried out through the examination of epidermal strips from fully expanded uniform
113 leaves belonging to five different wild-type and transgenic plants, which were mounted, on slides
114 with a drop of water. The number of stomata, guard cell length and width were determined on
115 randomly selected fields (50 stomata analysed for each sample) with the use of an optical microscope
116 Leitz DMRB equipped with a Leica DFC420 digital camera and ImageJ software (NIH) for image
117 analysis. Stomatal density, which refers to the number of stomata per unit area of the leaf, and
118 stomatal area were also estimated.

119 In vitro metal, water and heat treatments

120 Heavy metal stress induction

121 The concentrations of chromium (Cr) and cadmium (Cd) for the induction of metal stress in
122 *Nicotiana* plants were selected because of preliminary experiments where the effect of half-maximal

123 injury on survival of *N. langsdorffii* wild-type plants grown on media supplemented with different
124 concentrations of both metals was tested. In particular, treatments with 30 ppm Cd and 50 ppm Cr
125 reduced plant growth after 15 days, while higher concentrations caused plants death (Fuoco et al.
126 2013). These concentrations were, therefore selected for stress induction in this study. Ten
127 untransformed and ten transgenic plants were grown on the metal-supplemented media for 15 days.
128 Control treatment consisted of growing transgenic and non-transgenic plants on LS medium.

129 **Drought stress induction**

130 Polyethylene glycol (PEG 6000) was used to induce drought stress according to van der Weele et al.
131 (2000). PEG 6000 was added to the growth medium in order to lower its water potential. First, a
132 preliminary experiment was carried out to establish the concentration of PEG appropriate for
133 induction of drought stress (Supplementary Fig. S1). The concentration of 20 % of PEG was chosen
134 for mimicking severe drought condition. Subsequently, ten axenic wild-type and ten transgenic
135 plants were grown for 15 days in Wavin vessels in 50 mL LS medium conditioned (see
136 Supplementary materials) with 50 mL 20 % PEG solution. In addition to subjecting whole plants to
137 water stress, leaf discs were also used to assess the different responses of GR plants to water stress.
138 Survival was evaluated for 90 days on regeneration medium (RM) with 20 % PEG.

139 **Heat stress induction**

140 Experiments were carried out to examine the effect of different temperature on the level of
141 electrolyte leakage from leaf discs of *N. langsdorffii* wild-type plants (Supplementary Fig. S2). Ten
142 in vitro grown whole plants of the different genotypes of *Nicotiana*, both transformed and
143 untransformed, were subjected to heat stress after 4 weeks of incubation on LS medium. The plants
144 were maintained in a SANYO incubator (MIR-153; Richmond Scientific Ltd) at 50 °C for 2 h prior
145 to further analyses.

146 After stress treatments, leaf discs were cut out from plants under a sterile hood. Electrolyte leakage,
147 the percentage of survival and recovery of plants in terms of in vitro shoot regeneration capability
148 were used to evaluate plant responses.

149 For heat stress, bud break and re-growth of the plants recovered from stress condition and maintained
150 in a growth chamber at 24 ± 1 °C have also been estimated.

151 **In vitro callus induction and shoot differentiation**

152 Leaf discs from wild-type and transgenic plants were placed on callus inducing medium [CIM, LS
153 containing 0.4 mg/L dichlorophenoxyacetic acid (2,4-D)] and on the regeneration medium [RM, LS
154 containing 1 mg/L 6-benzyl aminopurine (BAP) and 0.1 mg/L naphthaleneacetic acid (NAA)]. The
155 number of explants producing callus, or at least one shoot, was recorded. All the hormones were
156 purchased from Sigma/Aldrich, USA. Percentage values were compared by Chi-squared test.

157 **Electrolyte leakage assay**

158 Thirty leaf discs were collected from randomly picked plants, and placed in test tubes (10 per tube)
159 containing 5 mL of 1 M sucrose solution. Tubes were capped and allowed to equilibrate for 30 min
160 at 25 °C in a growth chamber. Then the electrical conductivity of each sample was measured by
161 using a conductivity metre (PABISCHTOP μ S 5650). Leaf discs were then frozen at -80 °C and
162 equilibrated at room temperature before measuring the total conductivity. Electrolyte leakage was

163 expressed as percentage according to Arora et al. (1998). Data were subjected to pair-wise
164 comparisons by Student's *t* test.

165

166 Bioassay with the symbiotic fungus *F. mosseae*

167 The experiments were setup using the arbuscular mycorrhizal fungus *F. mosseae* (Nicol. and Gerd.)
168 Gerdemann and Trappe (isolate IMA1) maintained in the collection of the Department of
169 Agriculture, Food and Environment, University of Pisa, Italy. *F. mosseae* sporocarps were extracted
170 from soil pot culture by wet sieving and decanting through a 100- μ m-pore-size sieve (Gerdemann
171 and Nicolson 1963) then flushed into petri dishes and stored at 4 °C until used. Sporocarps were
172 grown in a “double sandwich system”, as described in Turrini et al. (2004), with the aim of studying
173 the early stages of the AM fungal life cycle in the presence of GR-transformed roots. Briefly,
174 sporocarps were manually collected with forceps under a dissecting microscope (Wild, Leica,
175 Milano, Italy) and placed on 47-mm-diameter cellulose ester Millipore™ membranes (0.45- μ m-
176 diameter pores). Transformed and control plant roots were sandwiched between two membranes,
177 with one of them containing 10 sporocarps and a third membrane containing another 10 sporocarps,
178 was superposed to complete the double sandwich. In this way, sporocarps growing on internal
179 membranes, in contact with the roots, could differentiate appressoria and establish mycorrhizal
180 symbiosis, whereas those growing on the external membrane, exposed to root exudates, could show
181 host recognition responses. The bioassay was carried out on T₁ and T₂ kanamycin-resistant GR plants
182 and untransformed isogenic controls. Six individuals (T₁) randomly selected from all those obtained
183 from selfed-independent transgenic lines (T₀) and ten individuals (T₂) from T₁-selfed plants were
184 used as replicates together with an equivalent number of wild-type plants. Each “sandwiched”
185 experimental plant was placed into a 7-cm diameter pot containing sterile quartz grit and maintained
186 under controlled conditions (18–24 °C, 16–8 h photoperiod). Five membranes containing 10
187 sporocarps were covered with an empty membrane, buried in sterile quartz grit, and used to monitor
188 sporocarp germination, which started about 10 days post-inoculation. Plants were watered daily and
189 were not fertilized during the growth period. Twenty-five days after inoculation, plants were
190 removed from pots, “the sandwiches” were opened and both internal and external membranes were
191 stained with 0.05 % Trypan blue in lactic acid, in order to assess pre-symbiotic hyphal growth and
192 hyphal differential morphogenesis induced by host root exudates. Plant root systems were cleared in
193 10 % KOH, stained with 0.05 % Trypan blue (Phillips and Hayman 1970), and assessed for the
194 establishment of mycorrhizal symbiosis. Hyphal length and colonized root length were evaluated
195 using the gridline intersect method (Giovannetti and Mosse 1980). In order to quantify the entry
196 points developing arbuscules (infection units), colonized roots were mounted in lactic acid on
197 microscope slides and observed under a Reichert-Jung Polyvar light microscope (Vienna, Austria).
198 A second harvest was performed 50 days post-inoculation only for T₂ plants. The data were
199 subjected to Student's *t* test.

200 Results

201 Effect of the integration of the *GR* receptor gene on growth and phenotype of 202 transgenic plants

203 T₁ and T₂-selfed transgenic plants, selected for the kanamycin-resistant phenotype and the
204 expression of the integrated transgene (data not shown), were analysed for their phenotypic
205 characteristics. In general, transgenic plants exhibited a modified phenotype showing significant
206 differences in the leaf morphology (Fig. 1), number of internodes and plant height, a delay in

207 flowering time and a reduced number of flowers and seeds as compared to the wild type. Moreover,
208 a very relevant modification of transgenic plant development was the striking change in the
209 phyllotactic pattern from 3/4 in the wild type to 2/5 in transgenic lines (Table 1). Principal
210 Component analysis (PCA) was also used to record the effects of the GR receptor integration on
211 phenotype and morphology. As evidenced in the results of PCA, transgenic genotypes were
212 distinguished from wild type ones (Fig. 2). In addition, differences in stomatal characteristics were
213 observed between transformed and untransformed genotypes (Fig. 3a, b). These differences included
214 the size, area and density of stomata. Stomatal size, reported as length and width of guard cells, was
215 more than double in wild-type plants compared to the transgenic lines (Fig. 3c, d); stomatal area,
216 measured as both total area and stomatal aperture area, was almost double in wild type compared to
217 transgenic lines. This significant reduction in the size and area of stomata in the transgenic lines was
218 accompanied by a strong increase in stomatal density (Fig. 3e).
219

220 Recovery of transformed and untransformed *Nicotiana* plants from stress: in 221 vitro morphogenetic response and ion leakage analysis

222 **Heavy metal stress**

223 Prior to screening callus and shoot regeneration capability of untransformed and transgenic plants
224 grown on the toxic media for 15 days, we used the electrolyte leakage analysis as an indicator of
225 stress injury. Results reported in Fig. 4a showed lower values in transgenic plants compared to wild
226 type. In particular, the treatment of plants with 50 ppm Cr induced a significant increase in the
227 leakage of electrolytes in wild-type plants, but not in the transgenic ones. On the other hand, Cd
228 treatment did not show significant differences between transgenic and control plants.

229 Dedifferentiation and differentiation capability in leaf discs from wild-type and transgenic plants
230 were heavily affected by the metal stress. While no differences could be found in the capability of
231 wild-type and transgenic plants to both dedifferentiate and differentiate in the absence of treatments
232 with heavy metals (data not shown), the callus forming capability was heavily reduced after metal
233 treatments in both transformed and non-transformed leaf discs. A statistically significant difference
234 was detected only in Cd treatment. On the contrary, in transgenic explants shoot differentiation was
235 not affected by the metal treatment in comparison to the non-transformed explants, which showed
236 very low shoot regeneration (Fig. 4b).

237 **Drought stress**

238 Stress caused by PEG 6000 induced an increase in the leakage of electrolytes both in wild-type and
239 GR plants but, as observed for heavy metal-stressed plants, electrolyte leakage was significantly
240 lower in transgenic plants than in wild type (Fig. 5a). The capability of leaf discs from stressed plants
241 to dedifferentiate and/or differentiate after recovery in standard conditions of growth was confirmed
242 both for untransformed and transformed plants. Therefore, drought stress did not induce significant
243 differences in these parameters (data not shown). On the other hand, the morphogenetic response of
244 transgenic leaf discs grown for 90 days on a regeneration medium conditioned with PEG was quite
245 different when compared to that of untransformed *N. langsdorffii* explants. The survival of
246 transgenic leaf discs with shoot-forming calli was 76 % in the presence of 20 % PEG and only 26 %
247 survival in wild type (Fig. 5b).
248

249 **Heat stress**

250 Exposure of leaf discs to the range of temperatures up to 48 °C did not cause any injury to leaf tissue
251 (supplementary Fig. S2). However, leaf discs from transgenic plants exposed to 48 °C showed a
252 significantly higher dedifferentiation/differentiation capability than in wild-type explants (Fig. 6a,

253 b) and once again, electrolyte leakage in transgenic plants was lower than in wild type (Fig. 6c).
254 Finally, leaf discs from both wild-type and GR plants did not survive on a regeneration medium after
255 heat stress treatment at 50 °C. Only whole in vitro transgenic plants recovered from heat treatments,
256 showing bud break and re-growth after transferring the plants to standard growth temperature
257 (Fig. 6d).

258 Bioassay with the symbiotic fungus *F. mosseae*

259 Two different stages of the life cycle of the AM fungus *F. mosseae* were analysed, the pre-symbiotic
260 mycelia growth together with host recognition responses and the establishment of the mycorrhizal
261 symbiosis. Twenty-five days after inoculation, the growth of pre-symbiotic mycelium was affected
262 by GR plant root exudates. Both T₁ and T₂ GR-transformed plants caused a significant reduction of
263 hyphal growth compared with non-transformed ones (Table 2). Moreover, even if transgenic plants
264 were able to elicit hyphal differential morphogenesis (branching), the area of the membranes covered
265 by differentiated hyphae was significantly lower in the presence of T₂ GR plants, compared with
266 controls (Table 2). These data were confirmed by the second harvest, carried out 50 days post-
267 inoculation on T₂ GR and control plants (Table 2). As to the establishment of mycorrhizal symbiosis,
268 appressoria, coils and arbuscules were produced both on GR and on control plants (Fig. 7). It is
269 interesting to note that mycorrhizal colonization was of the Paris type, both in transformed and in
270 control plants (Fig. 7). Neither the percentage of colonized root length nor the number of infection
271 units was significantly affected in T₁ and T₂ GR-transformed plant roots, 25 days after inoculation.
272 The same trend was observed for the establishment of mycorrhizal symbiosis after 50 days in T₂ GR
273 plants (Table 2).

274

275 Discussion

276 In this study, a number of pleiotropic effects as a result of the integration of the rat glucocorticoid
277 receptor *GR* gene in *N. langsdorffii* genome are described. Diverse morphological modifications in
278 transgenic plants compared with the wild-type genotype were revealed, such as leaf morphology,
279 plant height, number of internodes, flowers and seeds, stomatal size, area and density. We observed
280 different responses of GR transgenic plants to abiotic stress-heavy metal, drought and heat stress
281 and to the beneficial symbiont *F. mosseae*, suggesting an interaction of the GR receptor,
282 constitutively expressed in transgenic plants, with the plant steroid signalling system. It is important
283 to note that in our experiments animal or synthetic steroids did not artificially induce the activity of
284 the GR receptor. In a previous study, we demonstrated that the integration of the *GR* gene into
285 genomes of *N. langsdorffii* and *N. glauca* plants drastically changed the whole plant hormonal
286 system and consequently the in vitro morphogenesis of transgenic plants (Giannarelli et al. 2010).
287 In particular, the auxin/cytokinin ratio was reduced in *N. glauca* but increased in the case of *N.*
288 *langsdorffii*, and ABA increased in *N. langsdorffii*. Such changes led to modifications of the ratios
289 of callus/root/shoot in in vitro cultures.

290 In the present study, phenotypic changes in transgenic plants compared to wild type were observed.
291 Such findings might be ascribed to the hormonal modification observed in previous studies
292 (Giannarelli et al. 2010). Moreover, transgenic *N. langsdorffii*-GR plants showed lower values of
293 electrolyte leakage than wild genotypes after heavy metal, drought and thermal stress. These findings
294 confirm our previous data demonstrating that the presence of the GR-steroid receptor in *N.*
295 *langsdorffii* reduced the absorption levels of Cd and Cr, compared to levels of absorbed Cd and Cr
296 in wild-type genotypes, by inducing a complex modification of the whole hormonal network (Fuoco
297 et al. 2013). Fuoco et al. (2013) also showed that transgenic plants exhibited higher values of the

298 components of the known complex of plant defence to stress, such as S-abscisic acid (S-ABA), 3-
299 indoleacetic acid (IAA), salicylic acid, total polyphenols, chlorogenic acid and antiradical activity.

300 One of the main features of the plant defence system from drought is the closure of stomata. Our
301 GR-transformed plant showed modifications in the stomatal characteristics. Such modifications may
302 be linked to indirect interference with epidermal patterning factors in the transgenic lines, leading to
303 a constitutively modified stomatal development in comparison with the control plants; this new
304 pattern enhances the adaptation of plants to drought. Transgenic plants showed a higher stomatal
305 density and size, and leaf morphological traits which control the effects of water scarcity by
306 increasing (Zhang et al. 2006) and decreasing, respectively (Spence et al. 1986), in periods of
307 drought (Doheny-Adams et al. 2012). To this regard, it is worth noting that the stomatal development
308 is regulated by the brassinosteroids, the naturally occurring plant steroids, by triggering mitogen-
309 activated protein kinases (MAPKK) signalling system (Kim et al. 2012).

310 All the data so far discussed suggest that the insertion of the *GR* gene into the genome is capable of
311 inducing in *Nicotiana* plants a constitutive series of physiological changes leading to stress
312 resistance, similar to those induced by genetic engineering of plants with microbial genes (Bettini et
313 al. 2003). As dynamic changes in metabolism and signalling hormonal network may also affect the
314 performance of mycorrhizal symbionts (Hause et al. 2007; Giovannetti et al. 2012), we analysed the
315 response of transgenic *N. langsdorffii* plants to the AM fungus *F. mosseae*. In our experiments,
316 plants expressing GR receptors showed a reduced pre-symbiotic hyphal growth and branching, while
317 maintaining the same levels of mycorrhizal colonization, compared with controls. Moreover, during
318 the establishment of the symbiosis, the development of intra-radical fungal structures, i.e. hyphal
319 coils and arbuscules, was normal both in GR and in control plants. Our data demonstrate that GR *N.*
320 *langsdorffii* mainly affected the pre-symbiotic events, interfering with the molecular dialogue
321 between the two partners, and are similar to those obtained by other authors (Foo 2013), who
322 observed a reduced colonization rate, while internal hyphae and arbuscules appeared to be normal,
323 in an IAA-deficient pea bushy (*bsh*) mutant. Similarly, the auxin-resistant *dgt* and the auxin hyper-
324 transporting *pct* tomato mutants, both producing low levels of IAA, failed to stimulate hyphal
325 branching, while maintaining a normal mycorrhizal colonization, even if reduced (Hanlon and
326 Coenen 2011). We can thus hypothesize that the changes in metabolic and hormonal levels in GR
327 plants induced either exudation of molecules hindering hyphal growth (i.e. specific/non-specific
328 inhibitors) or reduction of the production of strigolactones-rhizosphere signalling molecules. These
329 have been recently classified as a new hormone class (Gomez-Roldan et al. 2008; Umehara et al.
330 2008), which elicit mycelia differential morphogenesis (Giovannetti et al. 1993; Giovannetti et al.
331 1996; Akiyama et al. 2005). Recent studies showed a link between low-root auxin content and
332 strigolactone exudation (Foo 2013), providing new insights into the role of such hormones in
333 mycorrhizal symbiosis, as auxin regulates the expression of *PsCCD7* and *PsCCD8* genes for
334 strigolactone biosynthesis (Foo et al. 2005; 2013; Johnson et al. 2006). GR plants, producing high
335 levels of IAA (Giannarelli et al. 2010; Fuoco et al. 2013), showed negative effects on the early events
336 in the mycorrhizal symbiosis, suggesting that also high auxin contents may negatively affect
337 strigolactone production. As GR plants showed elevated levels of ABA whose biosynthesis shares
338 common pathways with that of strigolactones (both deriving from carotenoids), an interesting link
339 with the data on mycorrhizal symbiosis may be suggested. In a recent work on ABA-deficient
340 mutants, a correlation was demonstrated between ABA and strigolactones, indicating that ABA can
341 be a regulator of strigolactones biosynthesis through a yet unknown mechanism (López-Ráez et al.
342 2010). Further investigations on hormone networks regulating strigolactone production could be
343 carried out utilizing also *N. langsdorffii* plants expressing the GR receptor, which, showing high
344 levels of IAA and ABA, can represent a useful tool for exploring such an interesting issue.

345 All the present data suggest that the insertion of the *GR* gene into the *N. langsdorffii* genome induced
346 the activation of a signalling pathway leading to an efficient response to abiotic and biotic stimuli,
347 in the absence of animal steroids. The identification of putative plant steroids liable to interact with
348 the animal glucocorticoid receptor expressed by the GR transgenic plants remains a goal to be
349 achieved. On the other hand, a growing body of data suggests that brassinosteroids might be the
350 putative inducers of the activity of the glucocorticoid receptor. These considerations are based on
351 the effects of brassinosteroids in human systems (Rárová et al. 2012; Steigerová et al. 2012), and the
352 resemblance of plant steroid signalling processes to those of ecdysteroids in *Drosophila* (Thummel
353 and Chory 2002), both sharing a range of common developmental and physiological responses to
354 stress. To support this idea, there is a large body of evidence for the anti-stress behaviour of
355 brassinosteroids (Dhaubhadel et al. 2002; Choudhary et al. 2012; Kanwar et al. 2012; Li et al. 2012).

356 The use of the animal GR system in plants, as reported by Brockmann et al. (2001), raises several
357 important questions: (1) it is not clear how plant heat shock proteins function in the proper folding
358 and binding of the animal GR receptor protein, which may affect upon the leakiness of the
359 glucocorticoid receptor protein. To this purpose, it should be highlighted that the constitutive
360 expression of GR receptor, being a complexant of the Hsp90 protein (Cadepond et al. 1991; Kovacs
361 et al. 2005), may lead to a modification of the homeostasis of such an important protein. (2) The
362 developmental aspects of intracellular targeting of the GR protein in different plant tissue are still
363 poorly understood, making it difficult to recognize between the effects of the GR protein activated
364 by steroids and the unknown targeting of the GR protein within the cell.

365 Despite these uncertainties that call for further investigations, the GR-transformed plants represent
366 an interesting model system to study the response of plants to abiotic and biotic stimuli.

367 Acknowledgments

368 This work was supported by PRIN-2005-2007-2009 (co-ordinator Prof. Roger Fuoco) grants from
369 the Italian Ministry of Education, University and Research (MIUR). We thank Dr. Mary Lokken for
370 the editing of the English language.

371

372 References

- 373 1. Akiyama K, Ken-ichi M, Hideo H (2005) Plant sesquiterpenes induce hyphal branching in
374 arbuscular mycorrhizal fungi. *Nature* 435:824–827. doi: 10.1038/nature03608
- 375 2. Aoyama T, Chua NH (1997) A glucocorticoid mediated transcriptional system in transgenic
376 plants. *Plant J* 11(3):605–612. doi: 10.1046/j.1365-313X.1997.11030605.x
- 377 3. Arora R, Pitchay DS, Bearce BC (1998) Water-stress-induced heat tolerance in geranium leaf
378 tissues: a possible linkage through stress proteins? *Physiol Plant* 103:24–34.
379 doi: 10.1034/j.1399-3054.1998.1030104.x
- 380 4. Bettini P, Michelotti S, Bindi D, Giannini R, Capuana M, Buiatti M (2003) Pleiotropic effect of
381 the insertion of the *Agrobacterium rhizogenes* rol D gene in tomato (*Lycopersicon esculentum*
382 Mill.). *Theor Appl Genet* 107(5):831–836
- 383 5. Brockmann B, Smith MW, Zaraisky AG, Harrison K, Okada K, Kamiya Y (2001) Subcellular
384 localization and targeting of glucocorticoid receptor protein fusions expressed in transgenic
385 *Arabidopsis thaliana*. *Plant Cell Physiol* 42(9):942–951. doi: 10.1093/pcp/pce120
- 386 6. Cadepond F, Schweizer-Groyer G, Segard-Maurel I, Jibard N, Hollenberg SM, Giguere V,
387 Evans RM, Baulieu EE (1991) Heatshock protein 90 as a critical factor in maintaining
388 glucocorticosteroid receptor in a not functional state. *J Biol Chem* 266:5834–5841

- 389 7. Choudhary SP, Volkan H, Bhardwaj R, Yu J, Phan Tran LS (2012) Interaction of
390 brassinosteroids and polyamines enhances copper stress tolerance in *Raphanus sativus*. J Exp
391 Bot 63(15):5659–5675. doi: 10.1093/jxb/ers219
- 392 8. Dhaubhadel S, Browning KS, Gallie DR, Krishna P (2002) Brassinosteroids function to protect
393 the translational machinery and heatshock protein synthesis following thermal stress. Plant J
394 29:681–691. doi: 10.1046/j.1365-313X.2002.01257.x
- 395 9. Doheny-Adams T, Hunt L, Franks PJ, Beerling DJ, Gray JE (2012) Genetic manipulation of
396 stomatal density influences stomatal size, plant growth and tolerance to restricted water supply
397 across a growth carbon dioxide gradient. Phil Trans R Soc B 367:547–555
- 398 10. Foo E (2013) Auxin influences strigolactones in pea mycorrhizal symbiosis. J Plant Physiol
399 170:523–528. doi: 10.1016/j.jplph.2012.11.002
- 400 11. Foo E, Bullier E, Goussot M, Foucher F, Rameau C, Beveridge CA (2005) The branching gene
401 *RAMOSUS1* mediates interactions among two novel signals and auxin in pea. Plant Cell 17:464–
402 474. doi: 10.1105/tpc.104
- 403 12. Foo E, Yoneyama K, Hugill C, Quittenden L, Reid JB (2013) Strigolactones: internal and
404 external signals in plant symbioses? Plant Signal Behav 8:e23168.1–e23168.4.
405 doi: 10.4161/psb.23168
- 406 13. Fuoco R, Bogani P, Capodaglio G, Del Bubba M, Abollino O, Giannarelli S, Spiriti MM,
407 Muscatello B, Doumett S, Turetta C, Zangrando R, Zelano V, Buiatti M (2013) Response to
408 metal stress of *Nicotiana langsdorffii* plants wild-type and transgenic for the rat glucocorticoid
409 receptor gene. J Plant Physiol 170:668–675. doi: 10.1016/j.jplph.2012.12.009
- 410 14. Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal *Endogone* species extracted from
411 soil by wet sieving and decanting. T Brit Mycol Soc 46:235–246
- 412 15. Giannarelli S, Muscatello B, Bogani P, Spiriti MM, Buiatti M, Fuoco R (2010) Comparative
413 determination of some phytohormones in wild-type and genetically modified plants by gas
414 chromatography-mass spectrometry and high-performance liquid chromatography-tandem mass
415 spectrometry. Anal Biochem 398:60–68. doi: 10.1016/j.ab.2009.10.038
- 416 16. Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM (1986) Functional domains of the human
417 glucocorticoid receptor. Cell 46:645–652
- 418 17. Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular
419 mycorrhizal infection in roots. New Phytol 84:489–500. doi: 10.1111/j.1469-
420 8137.1980.tb04556.x
- 421 18. Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993) Differential hyphal
422 morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytol
423 125:587–594. doi: 10.1111/j.1469-8137.1993.tb03907.x
- 424 19. Giovannetti M, Sbrana C, Citernesi AS, Avio L (1996) Analysis of factors involved in fungal
425 recognition responses to host-derived signals by arbuscular mycorrhizal fungi. New Phytol
426 133(1):65–71. doi: 10.1111/j.1469-8137.1996.tb04342.x
- 427 20. Giovannetti M, Avio L, Barale R, Ceccarelli N, Cristofani R, Iezzi A, Mignolli F, Picciarelli P,
428 Pinto B, Reali D, Sbrana C, Scarpato R (2012) Nutraceutical value and safety of tomato fruits
429 produced by mycorrhizal plants. Br J Nutr 107:242–251. doi: 10.1017/S000711451100290X
- 430 21. Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun EA, Pillot J-P, Letisse F,
431 Matusova R, Danoun S, Portais J-C, Bouwmeester H, Bécard G, Beveridge CA, Rameau C,
432 Rochange SF (2008) Strigolactone inhibition of shoot branching. Nature 455:189–194.
433 doi: 10.1038/nature07271
- 434 22. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package
435 for education and data analysis. Palaeontol Electron 4.
436 http://palaeoelectronica.org/2001_1/past/issue1_01.htm
- 437 23. Hanlon MT, Coenen C (2011) Genetic evidence for auxin involvement in arbuscular
438 mycorrhizal initiation. New Phytol 189:701–709. doi: 10.1111/j.1469-8137.2010.03567.x

- 439 24. Hause B, Mrosk C, Isayenkov S, Strack D (2007) Jasmonates in arbuscular mycorrhizal
440 interactions. *Phytochem* 68:101–110. doi: 10.1016/j.phytochem.2006.09.025
- 441 25. Irdani T, Caroppo S, Ambrogioni L (2003) Response of *Nicotiana tabacum* plants
442 overexpressing a glucocorticoid receptor to *Meloidogyne incognita* (*Nematoda Tylenchida*)
443 infestation. *Redia* LXXXVI:35–38r
- 444 26. Johnson X, Breich T, Dun EA, Goussot M, Haurigné K, Beveridge CA, Rameau C (2006)
445 Branching genes are conserved across species. Genes controlling a novel signal in pea are
446 coregulated by other long-distance signals. *Plant Physiol* 42:1014–1026.
447 doi: 10.1104/pp.106.087676
- 448 27. Kanwar MK, Bhardwaj R, Arora P, Chowdhary SP, Sharma P, Kumar S (2012) Plant steroid
449 hormones produced under Ni stress are involved in the regulation of metal uptake and oxidative
450 stress in *Brassica juncea* L. *Chemosphere* 86(1):41–49.
451 doi: 10.1016/j.chemosphere.2011.08.048
- 452 28. Kim TW, Michniewicz M, Bergmann DC, Wang ZY (2012) Brassinosteroids regulates stomatal
453 development by GSK3-mediated inhibition of a MAPK pathway. *Nature* 482:419–424.
454 doi: 10.1038/nature10794
- 455 29. Kovacs JJ, Murphy PJM, Gaillard S, Zhao X, Wu JT, Nicchitta CV, Yoshida M, Toff DO, Pratt
456 WB, Yao TP (2005) HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation
457 of glucocorticoid receptor. *Mol Cell* 18:601–607. doi: 10.1016/j.molcel.2005.04.021
- 458 30. Li Z, Xu Z, He G, Yang G, Chen M, Li L, Ma Y (2012) A mutation in *Arabidopsis* BSK5
459 encoding a brassinosteroid-signaling kinase protein affects responses to salinity and abscisic
460 acid. *Biochem Biophys Res Comm* 426:522–527. doi: 10.1016/j.bbrc.2012.08.118
- 461 31. López-Ráez JA, Verhage A, Fernández I, Garcíá JM, Azcón-Aguilar C, Flors V, Pozo MJ (2010)
462 Hormonal and transcriptional profiles highlight common and differential host responses to
463 arbuscular mycorrhiza fungi and the regulation of the oxylipin pathway. *J Exp Bot* 61:2589–
464 2601. doi: 10.1093/jxb/erq089
- 465 32. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schützs G, Umesono K, Blumberg B,
466 Kastner P, Mark M, Chambon P, Evans RM (1995) The nuclear receptor superfamily: the
467 second decade. *Cell* 83:835–839. doi: 10.1016/0092-8674(95)90199-X
- 468 33. Patel R, Williams-Dautovich J, Cummins CL (2014) Minireview: new molecular mediators of
469 glucocorticoid receptor activity in metabolic tissues. *Mol Endocrinol* 28:999–1011.
470 doi: 10.1210/me.2014-1062
- 471 34. Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic
472 and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans Br Mycol*
473 *Soc* 55(1):158–161, IN16–IN18
- 474 35. Rárová L, Zahler S, Liebl J, Kryštof V, Sedlák D, Bartůněk P, Kohout L, Strnad M (2012)
475 Brassinosteroids inhibit in vitro angiogenesis in human endothelial cells. *Steroids* 77:1502–
476 1509. doi: 10.1016/j.steroids.2012.08.011
- 477 36. Sheppard KE (2003) Corticosteroid receptors, 11 β hydroxysteroid dehydrogenase and the heart.
478 *Vitam Horm* 66:77–112
- 479 37. Spence RD, Wu H, Sharpe PJH, Clark KG (1986) Water stress effects on guard cell anatomy
480 and the mechanical advantage of the epidermal cells. *Plant Cell Environ* 9:197–202.
481 doi: 10.1111/1365-3040.ep11611639
- 482 38. Stamm P, Ravindran P, Mohanty B, Tan EL, Yu H, Kumar PP (2012) Insights into the molecular
483 mechanism of RGL2-mediated inhibition of seed germination in *Arabidopsis thaliana*. *BMC*
484 *Plant Biol* 12(1):179. doi: 10.1186/1471-2229-12-179
- 485 39. Steigerová J, Rárová L, Oklešť'ková J, Křížová K, Levková M, Šváchová M, Kolář Z, Strnad
486 M (2012) Mechanisms of natural brassinosteroid-induced apoptosis of prostate cancer cells.
487 *Food Chem Toxicol* 50:4068–4076. doi: 10.1016/j.fct.2012.08.031
- 488 40. Thummel CS, Chory J (2002) Steroid signalling in plants and insects-common themes, different
489 pathways. *Genes Dev* 16:3113–3129. doi: 10.1101/gad.1042102

- 490 41. Truss M, Beato M (1993) Steroid hormone receptors: interaction with deoxyribonucleic acid
491 and transcription factors. *Endocr Rev* 14(4):459–479. doi: 10.1210/edrv-14-4-459
- 492 42. Turrini A, Sbrana C, Pitto L, Ruffini Castiglione M, Giorgetti L, Briganti R, Bracci T,
493 Evangelista M, Nuti MP, Giovannetti M (2004) The antifungal Dm-AMP1 protein from *Dahli*
494 *amerckii* expressed in *Solanum melongena* is released in root exudates and differentially affects
495 pathogenic fungi and mycorrhizal symbiosis. *New Phytol* 163:393–403. doi: 10.1111/j.1469-
496 8137.2004.01107.x
- 497 43. Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H,
498 Kamiya Y, Shirasu K, Yoneyama K, Kyojuka J, Yamaguchi S (2008) Inhibition of shoot
499 branching by new terpenoid plant hormones. *Nature* 455:195–200. doi: 10.1038/nature07272
- 500 44. r Weele CM, Spollen WG, Sharp RE, Baskin TI (2000) Growth of *Arabidopsis thaliana*
501 seedlings under water deficit studied by control of water potential in nutrient-agar media. *J Exp*
502 *Bot* 51:1555–1562. doi: 10.1093/jexbot/51.350.1555
- 503 45. Yamamoto KR (1985) Steroid receptor regulated transcription of specific genes and gene
504 networks. *Annu Rev Genet* 19:209–252. doi: 10.1146/annurev.ge.19.120185.001233
- 505 46. Yu H, Ito T, Zhao Y, Peng J, Kumar P, Meyerowitz EM (2004) Floral homeotic genes are targets
506 of gibberellin signaling in flower development. *Proc Nat Acad Sci USA* 101(20):7827–7832.
507 doi: 10.1073/pnas.0402377101
- 508 47. Zhang YP, Wang ZM, Wu YC, Zhang X (2006) Stomatal characteristics of different green
509 organs in wheat under different irrigation regimes. *Acta Agron Sin* 32:70–75

510

511 **Table 1**
 512 Effect on plant phenotype and growth of the integration of the *GR* receptor gene into the genome of *Nicotiana*
 513 *langsdorffii*. Phyllotaxis was measured as a fraction of angle full rotation ($135^\circ = 2/5$ of angle full rotation;
 514 $144^\circ = 3/8$ of angle full rotation)

Genotypes	<i>H</i> (cm)	Int. No.	<i>F</i> (d)	Flower No.	Phyllotaxis	Seed No.
NLWT	131.81 ± 3.15	33.36 ± 2.25	109.54 ± 3.46	28.72 ± 3.16	135°	2993.09 ± 696.69
NLGRT1	99.80 ± 4.44	31.60 ± 3.78	174.00 ± 10.60	14.00 ± 6.09	144°	nd
NLGRT2	99.00 ± 11.2	43.50 ± 4.91	163.40 ± 17.80	20.30 ± 5.89	144°	886.90 ± 528.83

515 *nd* not determined

516

517

518

519 **Table 2**
 520 Analysis of *Funneliformis mosseae* hyphal growth and development in the presence of root exudates (pre-
 521 symbiotic stage) and in the roots (symbiotic stage) of transgenic GR (NLGR) and wild type (NLWT) *Nicotiana*
 522 *langsdorffii* (generations T₁ and T₂)

523

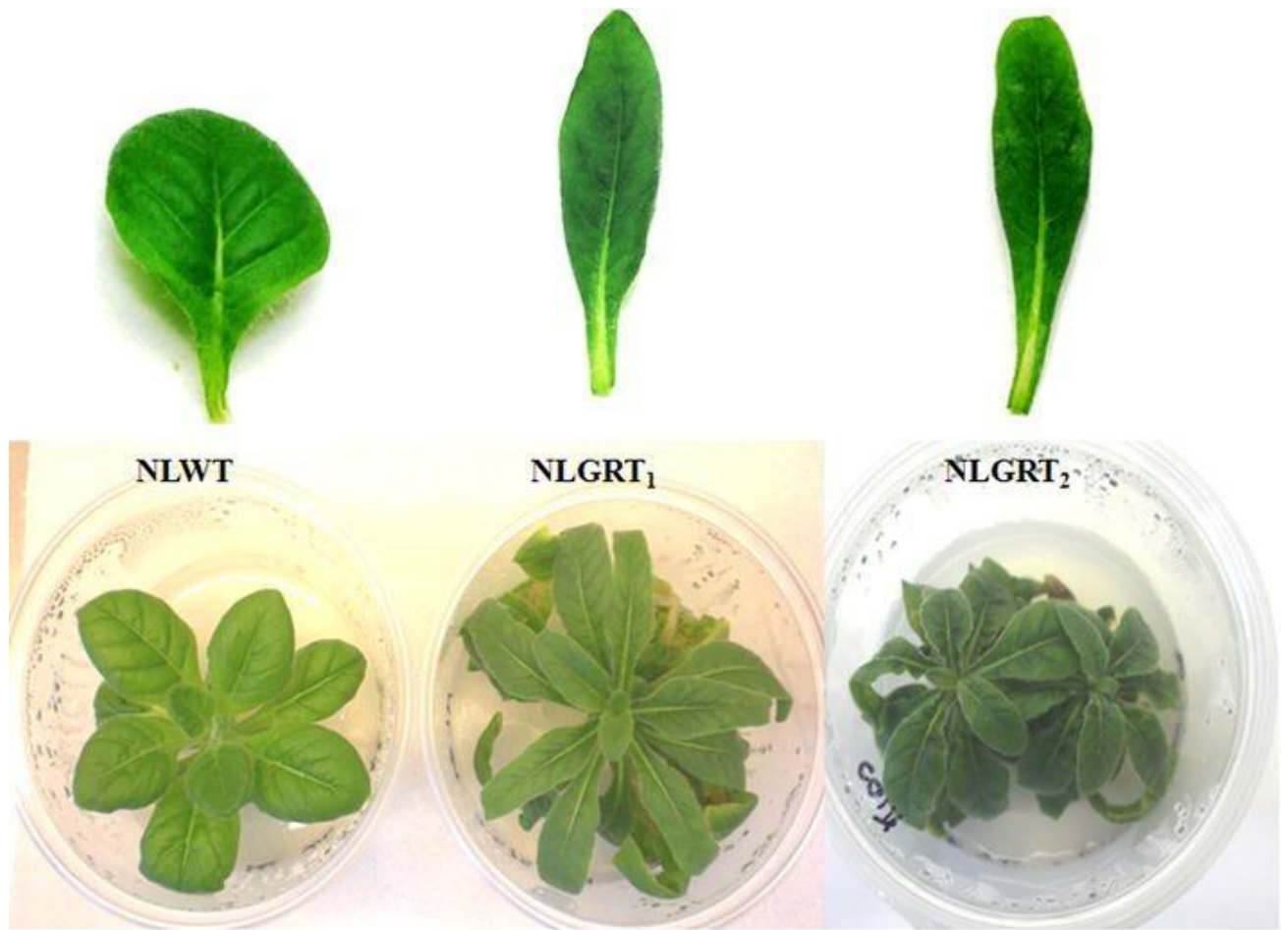
Plant generation	Harvest time			NLWT plants	NLGR plants	P
T ₁	25 days	post- inoculation	Pre-symbiotic mycelial length (mm)	700.00 ± 20.00	155.00 ± 32.80	0.000004
			No of infection units/cm root length	0.27 ± 0.06	0.09 ± 0.06	0.09
T ₂	25 days	post- inoculation	Pre-symbiotic mycelial length (mm)	3239.50 ± 133.72	2228.48 ± 168.37	0.0002
			Pre-symbiotic mycelial length per germ tube (mm)	55.44 ± 3.74	36.26 ± 3.14	0.001
			Area covered by differential hyphal morphogenesis (mm ²)	97.00 ± 12.96	57.67 ± 12.41	0.04
			Percentage of colonized root length (%)	3.31 ± 0.83	5.11 ± 1.95	0.21
			No of infection units/cm root length	0.24 ± 0.07	0.46 ± 0.20	0.26
	50 days	post- inoculation	Pre-symbiotic mycelial length (mm)	4025.80 ± 429.97	3272.66 ± 204.68	0.037
Pre-symbiotic mycelial length per germ tube (mm)			71.29 ± 6.18	57.04 ± 4.14	0.023	

Plant generation	Harvest time		NLWT plants	NLGR plants	P
		Area covered by differential morphogenesis (mm ²)	92.00 ± 20.77	41.12 ± 9.21	0.045
		Percentage of colonized root length (%)	7.63 ± 1.23	13.10 ± 3.34	0.382

524

525

526



527

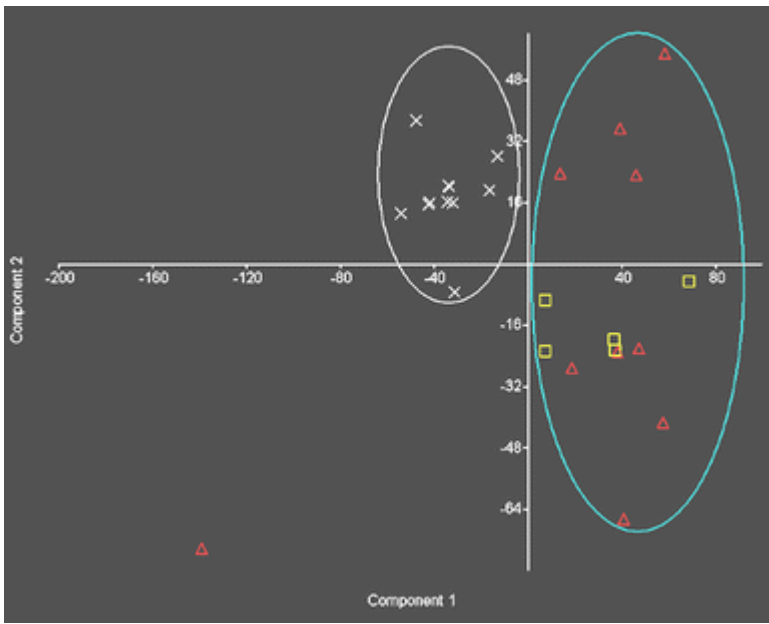
528

529

Fig. 1 Morphological phenotypes of *Nicotiana langsdorffii* transgenic leaves and in vitro plants (NLGRT1, NLGRT2), and wild-type plants (NLWT)

530

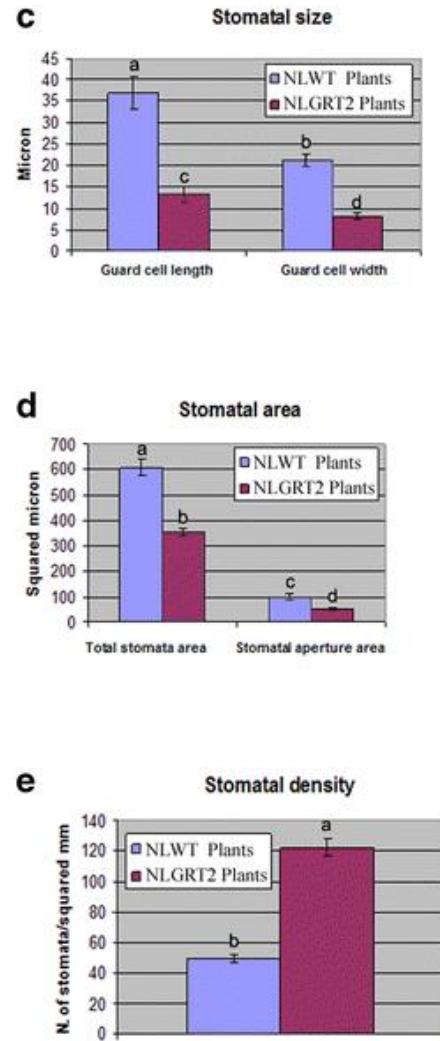
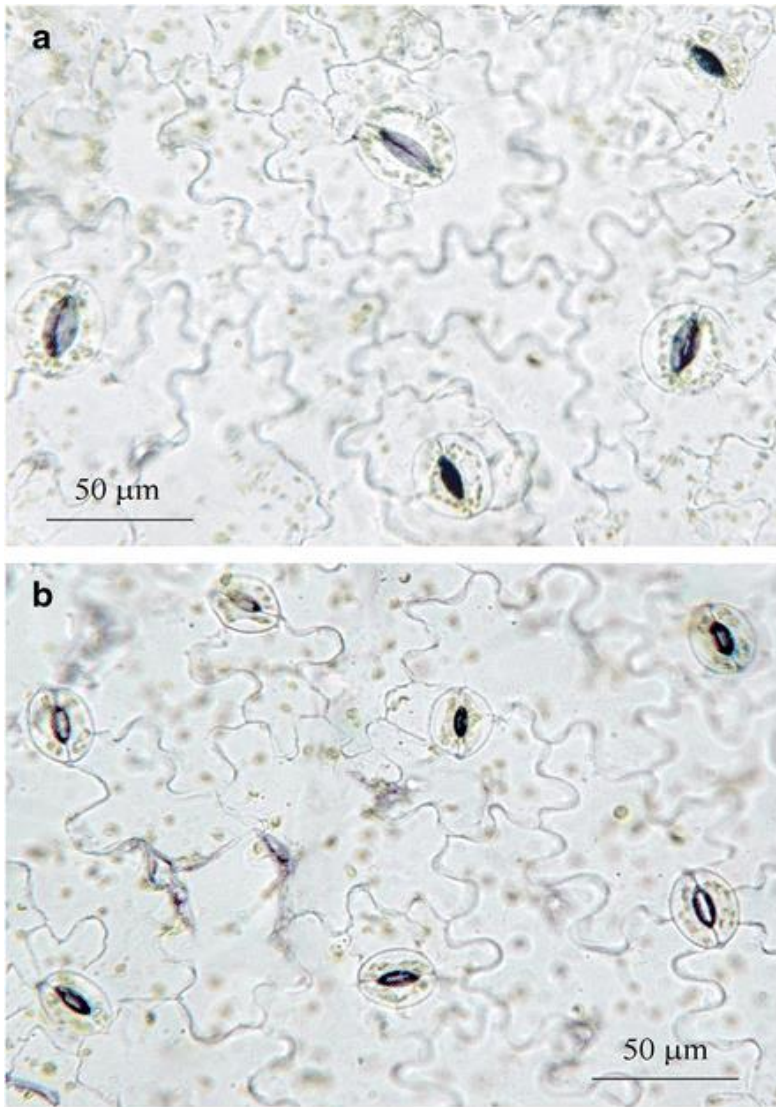
531



532
533

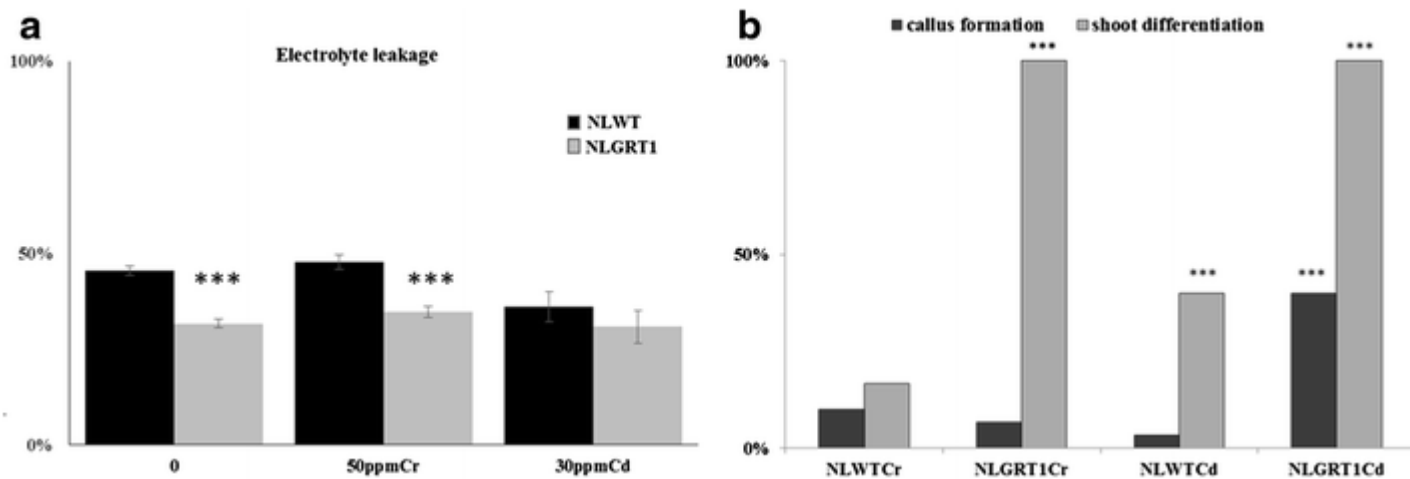
534 **Fig. 2** Principal Component Analysis of phenotypic differences observed in transgenic and wild-type
535 *Nicotiana langsdorffii* plants. NLWT, *N. langsdorffii* wild type (*multiple sign*); NLGRT1, T₀ selfed
536 *N. langsdorffii* plant transgenic for the *GR* gene (*square*); NLGRT2, T₁-selfed transgenic plants
537 (*triangle*)
538

539



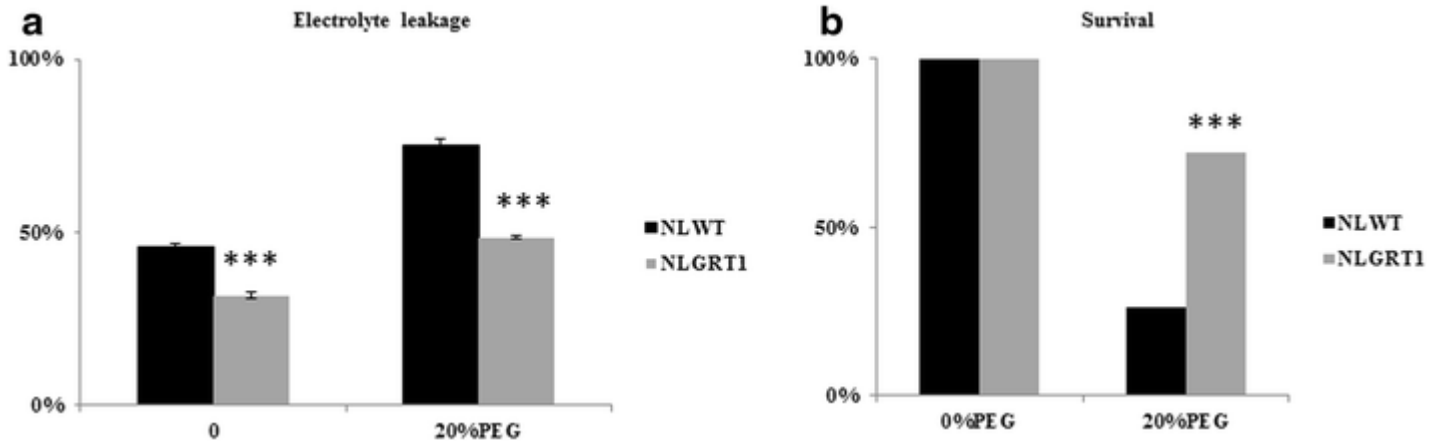
540
541
542
543
544
545
546
547

Fig. 3 Effect of the integration of the GR receptor gene into the genome of *Nicotiana langsdorffii* on stomatal characteristics. **a, b** Micrographs of leaf epidermal strips from wild-type (NLWT) and transgenic plants (NLGRT2), respectively. **c–e** Histograms representing the mean values of stomatal size, stomatal area and stomatal density, respectively (\pm standard error), referring to NLWT and NLGRT2 plants. *Different letters* denote significant differences at $P < 0.01$

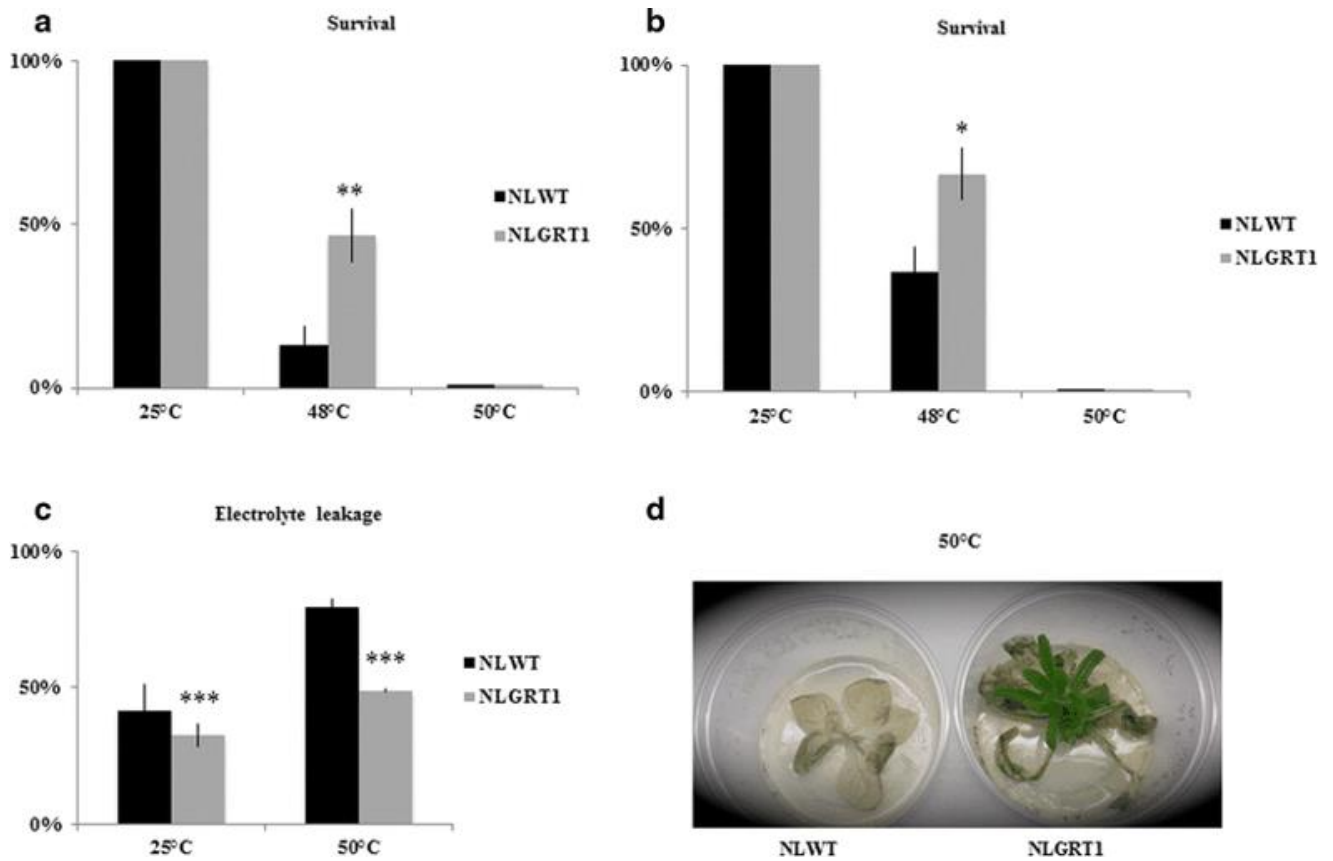


548
549
550
551
552
553
554
555

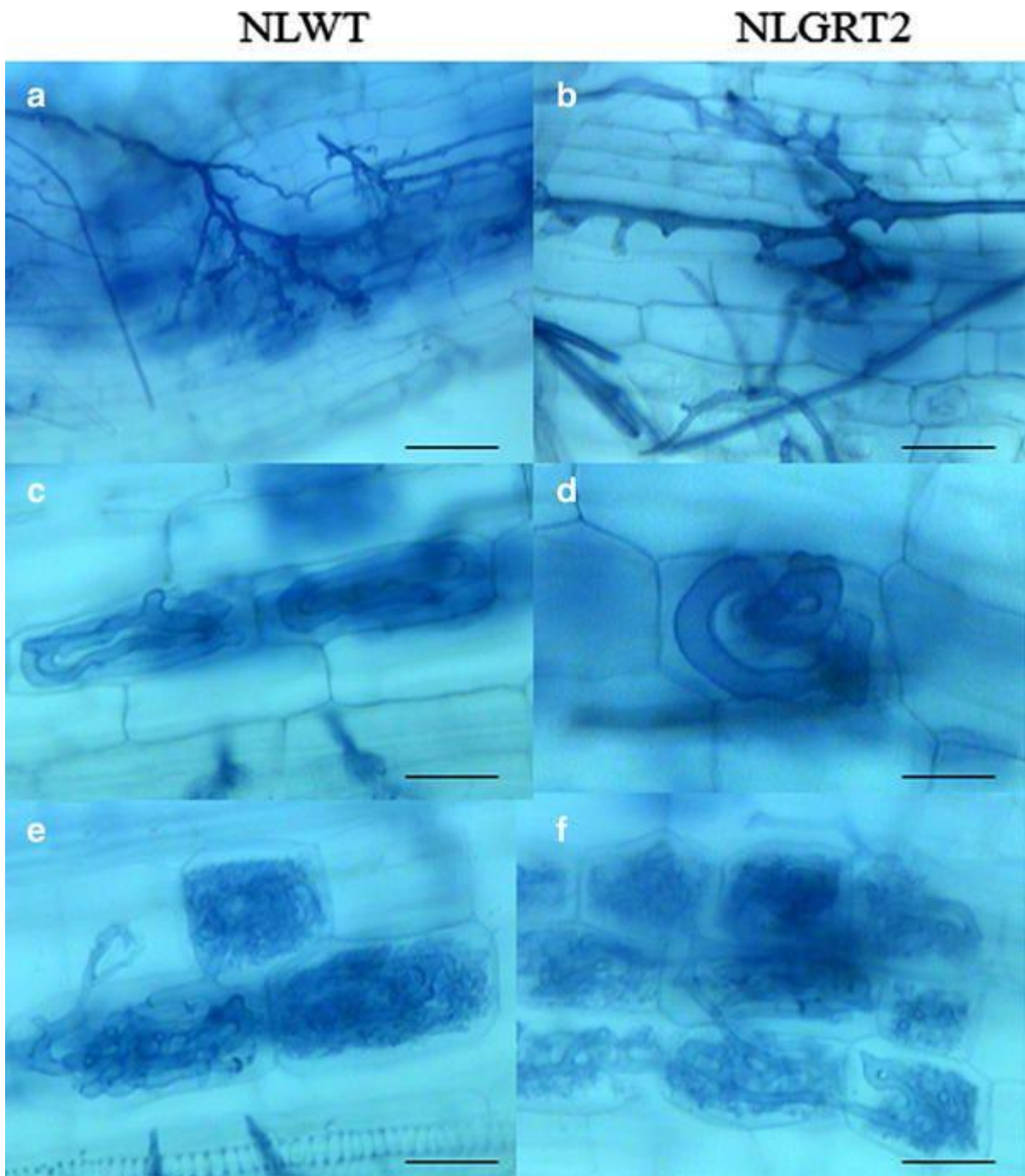
Fig. 4 Response of *Nicotiana langsdorffii* wild-type plants (NLWT) and transgenic plants (NLGRT1) plants to metal stress induction. **a** Electrolyte leakage from leaf discs of NLWT and NLGRT1 plants recovered following 15 days of 50 ppm chromium (Cr) and 30 ppm cadmium (Cd) treatments ($n = 10$; standard deviations bars refer to three replicates); **b** dedifferentiation and differentiation capability of leaf discs of NLWT and NLGRT1 recovered plants. ($n = 30$). *** $P < 0.001$; pair-wise comparisons were determined between NLWT vs NLGRT1 plants



556
 557 **Fig. 5** Response of *Nicotiana langsdorffii* wild-type (NLWT) and transgenic plants (NLGRT1) to
 558 water stress induction. **a** Electrolyte leakage assay ($n = 10$; *standard deviations bars* refer data from
 559 three replicates). **b** % survival of leaf discs from NLWT and NLGRT1 grown for 90 days on RM
 560 medium conditioned with 20 % PEG 6000 ($n = 90$). $P < 0.0001$
 561



562
 563 **Fig. 6** Response of NLWT and transgenic plants (NLGRT1) recovered following heat stress:
 564 dedifferentiation (a) and differentiation (b) capability of leaf discs of NLWT and NLGRT1 plants
 565 recovered following 2 h of treatment at different temperatures; electrolyte leakage assay ($*P < 0.05$;
 566 $**P < 0.01$; $***P < 0.001$) (c); re-growing capability of NLWT and NLGRT1 plants recovered
 567 following a treatment at 50 °C for 2 h (d)
 568



569
570
571
572
573
574
575
576

Fig. 7 Light photomicrographs of fungal structures formed by *Funneliformis mosseae* on the roots of *Nicotiana langsdorffii* wild type plants (NLWT) (**a, c, e**) and on *N. langsdorffii*-GR transgenic plants (**b, d, f**) (NLGR). **a, b** Fungal extraradical hyphae and appressoria formed on the root surface. *Scale bars a*, 76 μm ; *b*, 38 μm . **c, d** Coils formed in cortical root cells. *Scale bars c*, 24 μm ; *d*, 19 μm . **e, f** Arbuscules produced within cortical root cells plant roots (**e, f**). *Scale bars e*, 22 μm ; *f*, 24 μm