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

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

1.Dipartimento di Biologia Università di Firenze Florence Italy

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

3.Dipartimento di Biologia Università di Pisa Pisa Italy

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

Abstract

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

Keywords

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

Introduction

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

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

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

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

Materials and methods

Plant material

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

Plant morphology and stomatal features

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

In vitro metal, water and heat treatments

Heavy metal stress induction

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

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

Drought stress induction

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

Heat stress induction

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

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

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

In vitro callus induction and shoot differentiation

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

Electrolyte leakage assay

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

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

Bioassay with the symbiotic fungus *F. mosseae*

The experiments were setup using the arbuscular mycorrhizal fungus *F. mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (isolate IMA1) maintained in the collection of the Department of Agriculture, Food and Environment, University of Pisa, Italy. *F. mosseae* sporocarps were extracted from soil pot culture by wet sieving and decanting through a 100-µm-pore-size sieve (Gerdemann and Nicolson 1963) then flushed into petri dishes and stored at 4 °C until used. Sporocarps were grown in a "double sandwich system", as described in Turrini et al. (2004), with the aim of studying the early stages of the AM fungal life cycle in the presence of GR-transformed roots. Briefly, sporocarps were manually collected with forceps under a dissecting microscope (Wild, Leica, Milano, Italy) and placed on 47-mm-diameter cellulose ester Millipore™ membranes (0.45-µm-diameter pores). Transformed and control plant roots were sandwiched between two membranes, with one of them containing 10 sporocarps and a third membrane containing another 10 sporocarps, was superposed to complete the double sandwich. In this way, sporocarps growing on internal membranes, in contact with the roots, could differentiate appressoria and establish mycorrhizal symbiosis, whereas those growing on the external membrane, exposed to root exudates, could show host recognition responses. The bioassay was carried out on T_1 and T_2 kanamycin-resistant GR plants and untransformed isogenic controls. Six individuals (T_1) randomly selected from all those obtained from selfed-independent transgenic lines (T_0) and ten individuals (T_2) from T_1-selfed plants were used as replicates together with an equivalent number of wild-type plants. Each "sandwiched" experimental plant was placed into a 7-cm diameter pot containing sterile quartz grit and maintained under controlled conditions (18–24 °C, 16–8 h photoperiod). Five membranes containing 10 sporocarps were covered with an empty membrane, buried in sterile quartz grit, and used to monitor sporocarp germination, which started about 10 days post-inoculation. Plants were watered daily and were not fertilized during the growth period. Twenty-five days after inoculation, plants were removed from pots, "the sandwiches" were opened and both internal and external membranes were stained with 0.05 % Trypan blue in lactic acid, in order to assess pre-symbiotic hyphal growth and hyphal differential morphogenesis induced by host root exudates. Plant root systems were cleared in 10 % KOH, stained with 0.05 % Trypan blue (Phillips and Hayman 1970), and assessed for the establishment of mycorrhizal symbiosis. Hyphal length and colonized root length were evaluated using the gridline intersect method (Giovannetti and Mosse 1980). In order to quantify the entry points developing arbuscules (infection units), colonized roots were mounted in lactic acid on microscope slides and observed under a Reichert-Jung Polyvar light microscope (Vienna, Austria). A second harvest was performed 50 days post-inoculation only for T_2 plants. The data were subjected to Student's *t* test.

Results

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

T_1 and T_2-selfed transgenic plants, selected for the kanamycin-resistant phenotype and the expression of the integrated transgene (data not shown), were analysed for their phenotypic characteristics. In general, transgenic plants exhibited a modified phenotype showing significant differences in the leaf morphology (Fig. 1), number of internodes and plant height, a delay in

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

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

Heavy metal stress

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

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

Drought stress

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

Heat stress

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

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

Bioassay with the symbiotic fungus *F. mosseae*

Two different stages of the life cycle of the AM fungus *F. mosseae* were analysed, the pre-symbiotic mycelia growth together with host recognition responses and the establishment of the mycorrhizal symbiosis. Twenty-five days after inoculation, the growth of pre-symbiotic mycelium was affected by GR plant root exudates. Both T_1 and T_2 GR-transformed plants caused a significant reduction of hyphal growth compared with non-transformed ones (Table 2). Moreover, even if transgenic plants were able to elicit hyphal differential morphogenesis (branching), the area of the membranes covered by differentiated hyphae was significantly lower in the presence of T_2 GR plants, compared with controls (Table 2). These data were confirmed by the second harvest, carried out 50 days post-inoculation on T_2 GR and control plants (Table 2). As to the establishment of mycorrhizal symbiosis, appressoria, coils and arbuscules were produced both on GR and on control plants (Fig. 7). It is interesting to note that mycorrhizal colonization was of the Paris type, both in transformed and in control plants (Fig. 7). Neither the percentage of colonized root length nor the number of infection units was significantly affected in T1 and T2 GR-transformed plant roots, 25 days after inoculation. The same trend was observed for the establishment of mycorrhizal symbiosis after 50 days in T2 GR plants (Table 2).

Discussion

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

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

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

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

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

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

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

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

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Table 1

Effect on plant phenotype and growth of the integration of the *GR* receptor gene into the genome of *Nicotiana langsdorffii*. Phyllotaxis was measured as a fraction of angle full rotation (135° = 2/5 of angle full rotation; 144° = 3/8 of angle full rotation)

Genotypes	*H* (cm)	Int. No.	*F* (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

nd not determined

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 T_1 and T_2)

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

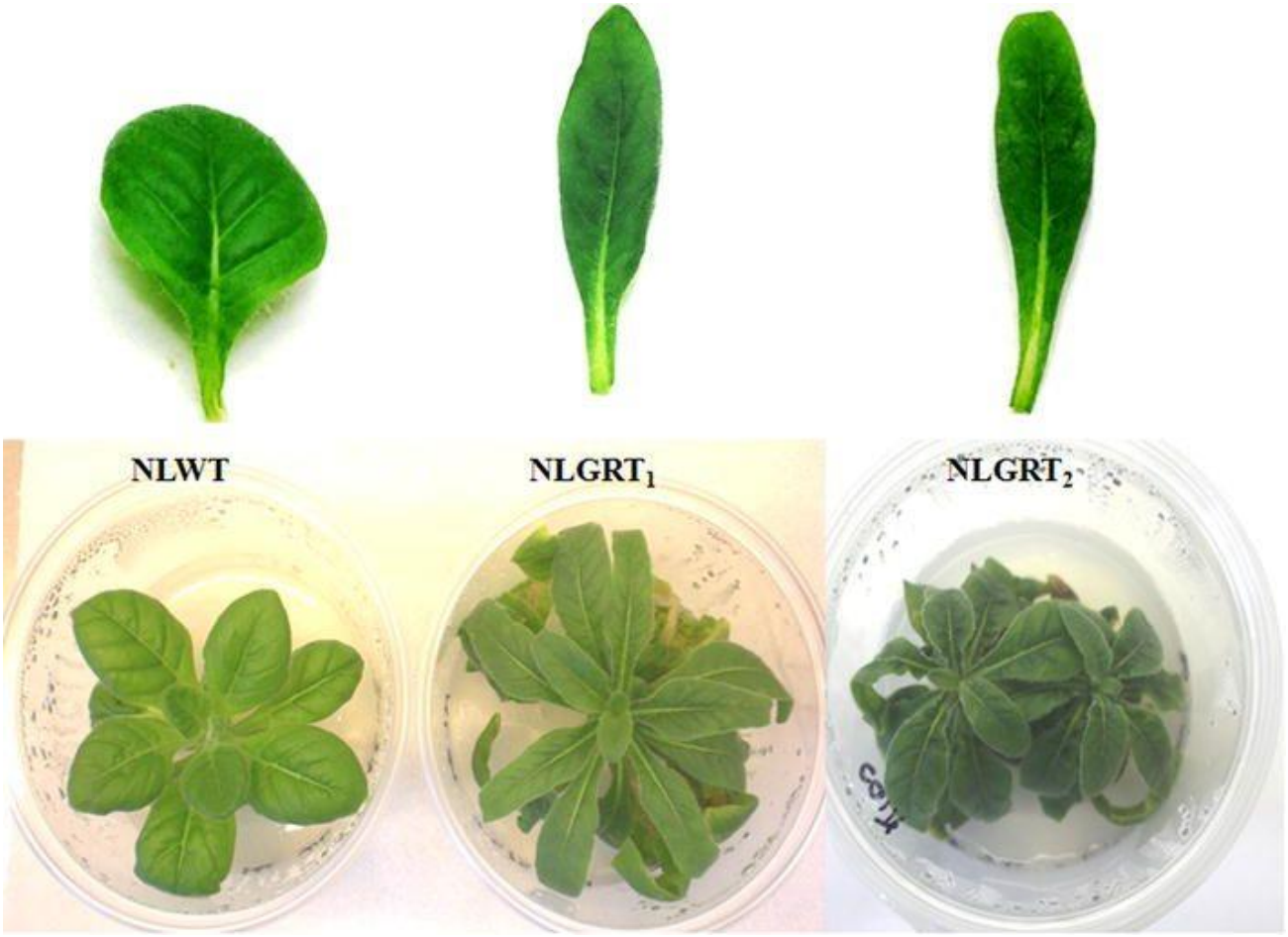

Fig. 1 Morphological phenotypes of *Nicotiana langsdorffii* transgenic leaves and in vitro plants (NLGRT1, NLGRT2), and wild-type plants (NLWT)

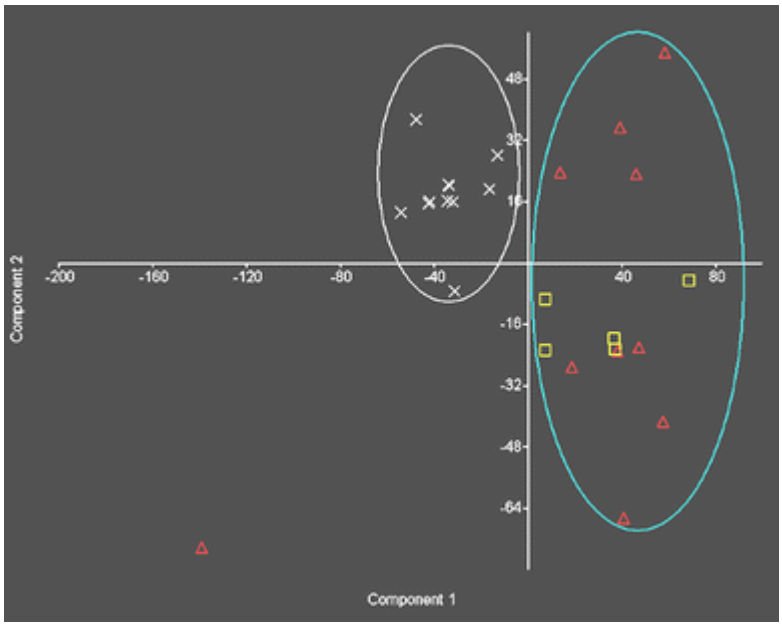

Fig. 2 Principal Component Analysis of phenotypic differences observed in transgenic and wild-type *Nicotiana langsdorffii* plants. NLWT, *N. langsdorffii* wild type (*multiple sign*); NLGRT1, T_0 selfed *N. langsdorffii* plant transgenic for the *GR* gene (*square*); NLGRT2, T_1-selfed transgenic plants (*triangle*)

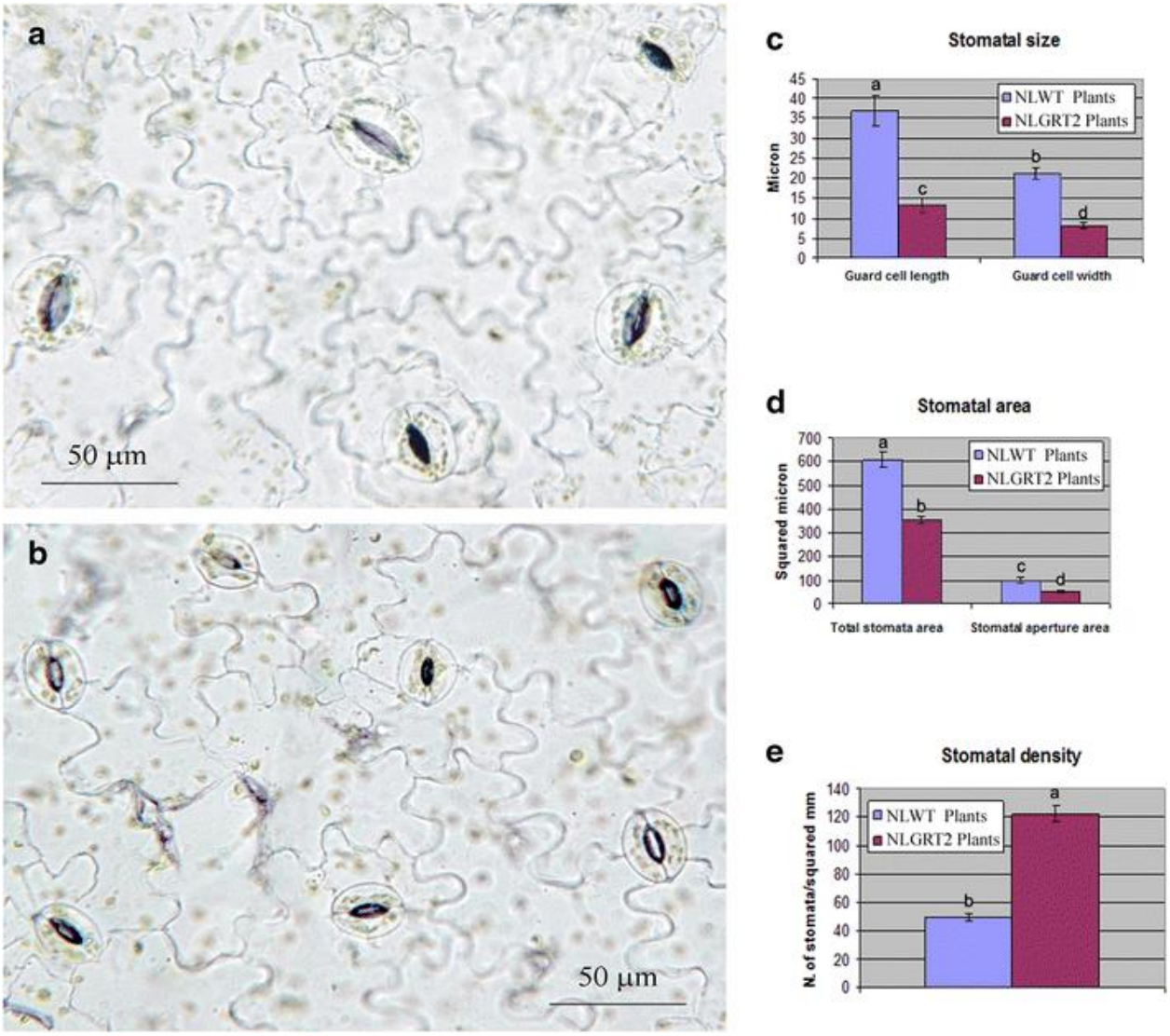

Fig. 3 Effect of the integration of the GR receptor gene into the genome of *Nicotiana langsdorffii* on stomatal characteristics. **a**, **b** Micrographs of leaf epidermal strips from wild-type (NLWT) and transgenic plants (NLGRT2), respectively. **c**–**e** Histograms representing the mean values of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

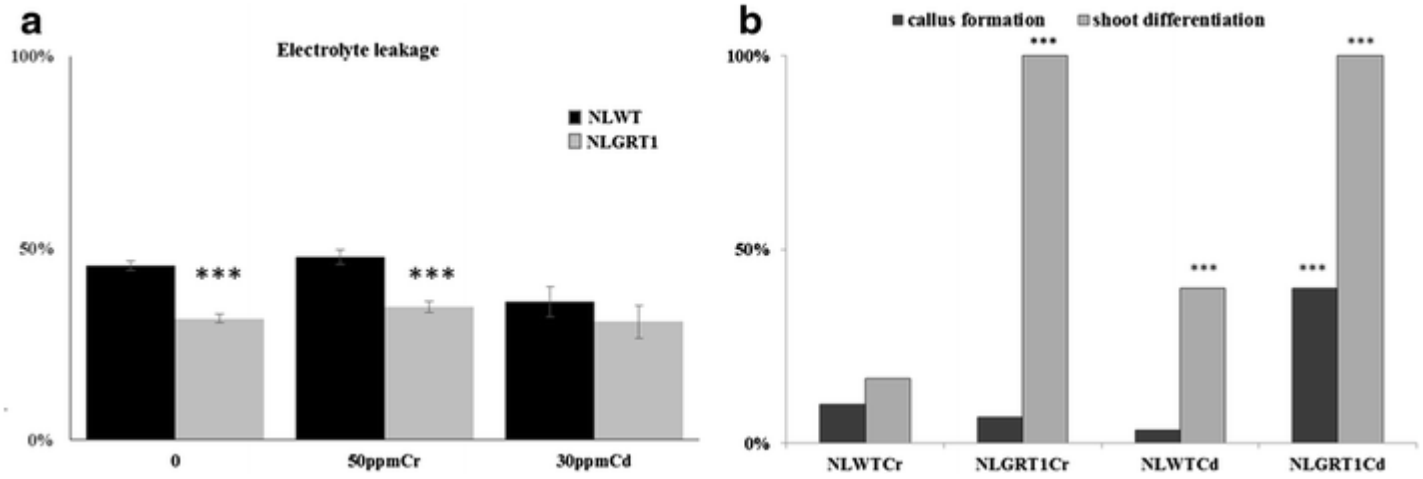

Fig. 4 Response of *Nicotiana langsdorffii* wild-type plants (NLWT) and transgenic plants (NLGRT1) plants to metal stress induction. **a** Electrolyte leakage from leaf discs of NLWT and NLGRT1 plants recovered following 15 days of 50 ppm chromium (Cr) and 30 ppm cadmium (Cd) treatments (n = 10; *standard 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

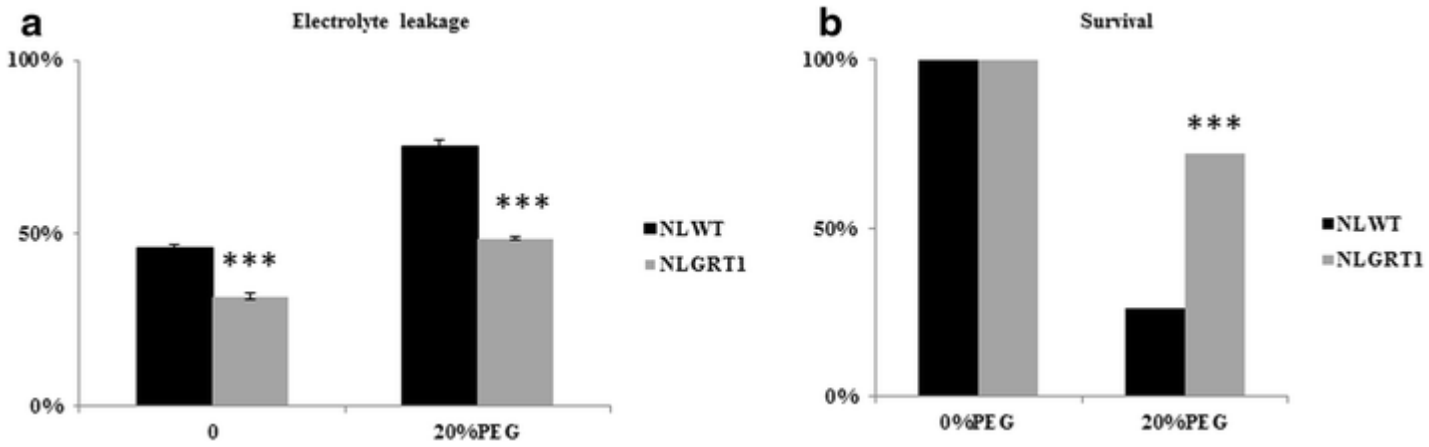

Fig. 5 Response of *Nicotiana langsdorffii* wild-type (NLWT) and transgenic plants (NLGRT1) to water stress induction. **a** Electrolyte leakage assay (n = 10; *standard deviations bars* refer data from three replicates). **b** % survival of leaf discs from NLWT and NLGRT1 grown for 90 days on RM medium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

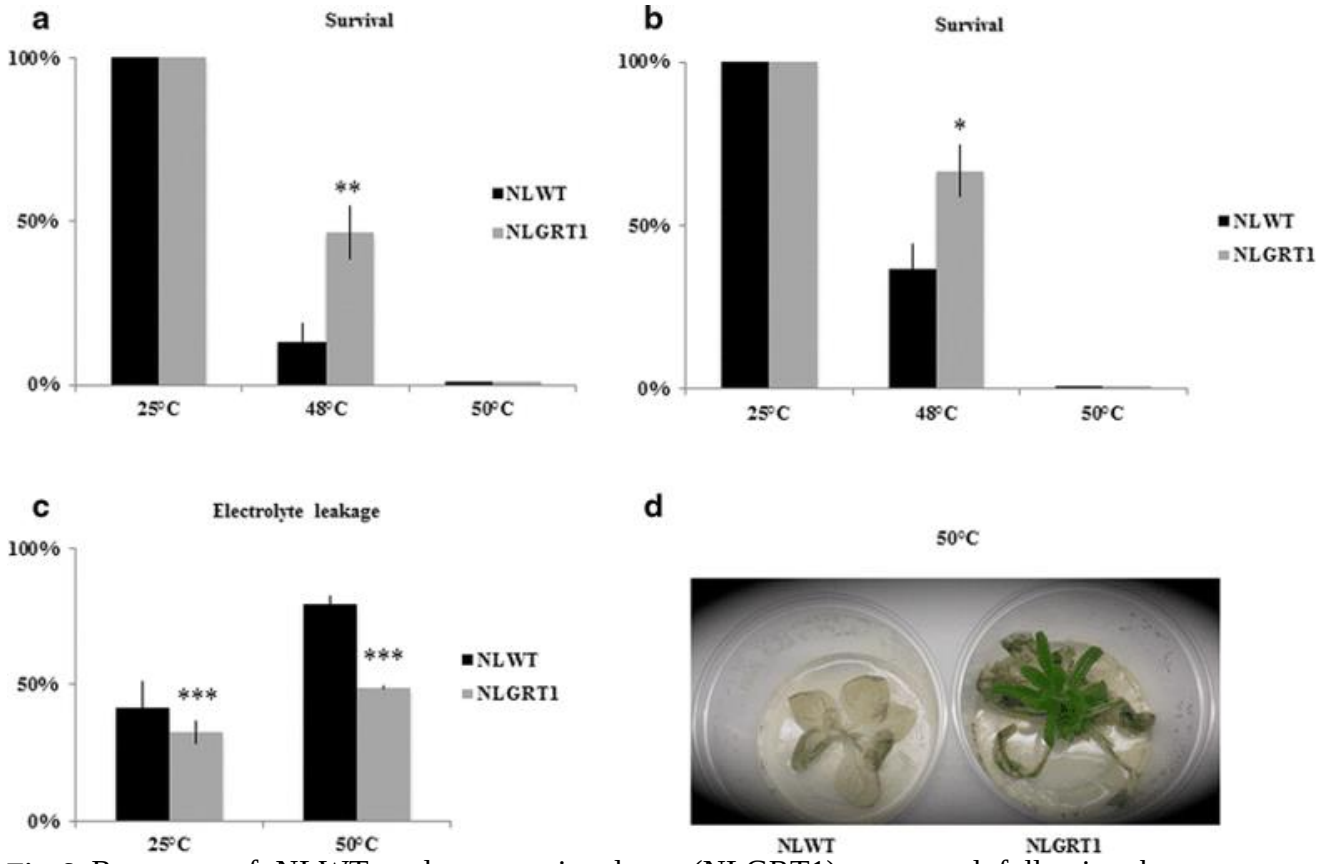

Fig. 6 Response of NLWT and transgenic plants (NLGRT1) recovered following heat stress: dedifferentiation (**a**) and differentiation (**b**) capability of leaf discs of NLWT and NLGRT1 plants recovered following 2 h of treatment at different temperatures; electrolyte leakage assay (*$P < 0.05$; **$P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

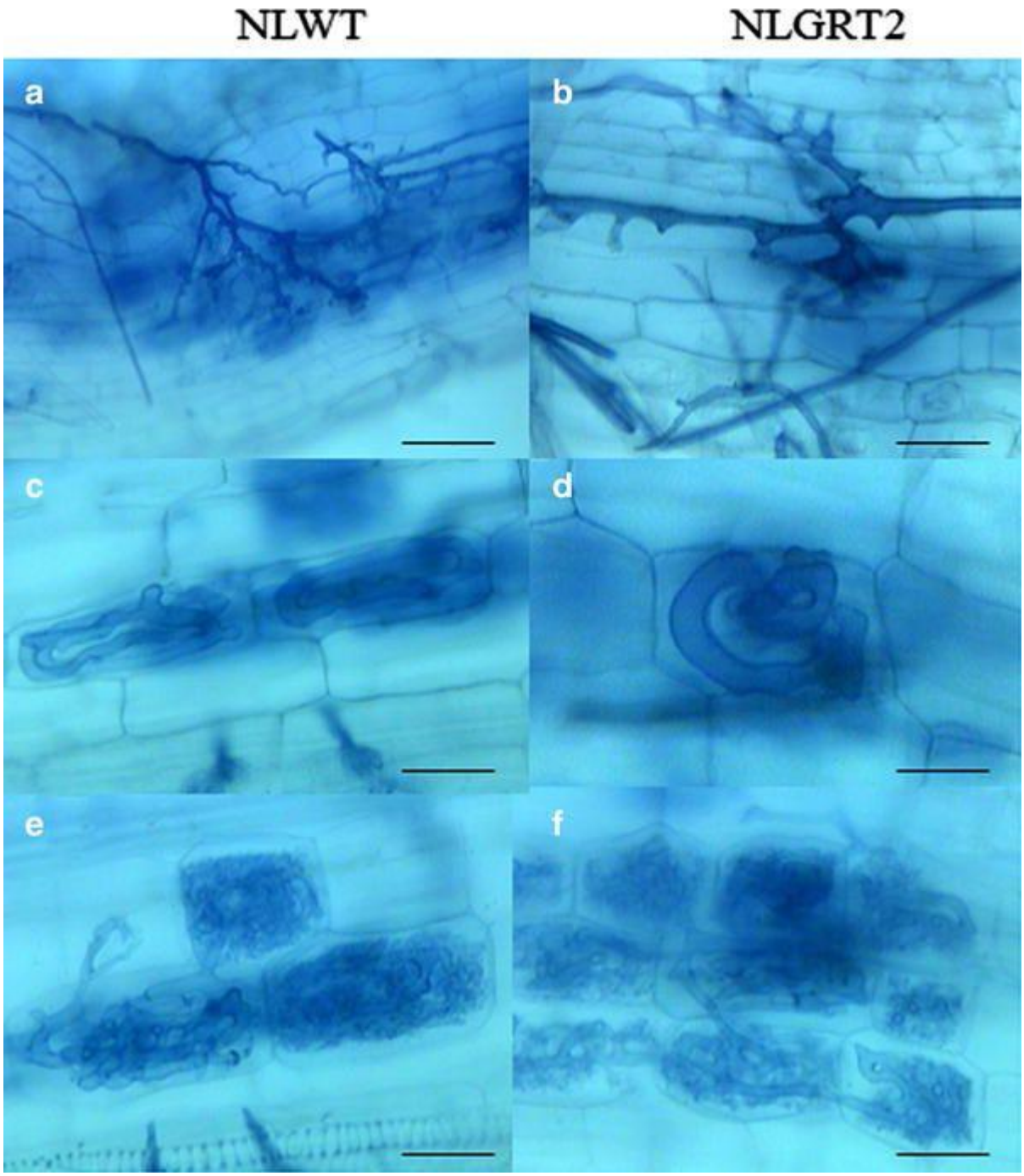

Fig. 7 Light photomicrographs of fungal structures formed by *Funneliformis mosseae* on the roots of *Nicotiana langsdorffii* wild type plants (NLWT) (**a**, **c**, **e**) and on *N. langsdorffii*-GR transgenic plants (**b**, **d**, **f**) (NLGR). **a**, **b** Fungal extraradical hyphae and appressoria 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