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Meloydogine incognita (Nematoda) reproduction affected by plants overexpressing a steroid inducible gene-expression system

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ABSTRACT

This study reports the impact of an integral glucocorticoid expression system (GR-system) on plant and plant-nematode reproduction. Seedlings expressing a GR-system (transGR+) show affections of growth and development. It is well-known that plant-hormones such as gibberellins, auxins and steroids are essential for developmental and physiological processes, in biotic/abiotic stresses. Here is shown some phenotypic affections reported by tobacco expressing a whole mammalian GR-system, how those effects are rescued by external hormonal treatments and the influence on plant-nematode fecundity. Results demonstrate that: i) GR-system is constitutively expressed in plants and its functionality proved by the activation of the reporter-gene transcription (uidA) after induction with different steroid hormones, dexamethasone (DEX) and progesterone (PRO) as well; ii) transGR + growth affected at hypocotyls and roots development result mitigated by exogenous hormones applications; iii) no differential expression in the expansin-gene TExp4 assayed; iv) Significant reductions of J2-larvae assessed in the egg-masses and soil during the generations on transformed plants. Although the molecular mechanism behind these results is still unknown, all together these results suggest a role of GR as reflected by the impact on the plant development and reduction of *Meloydogine incognita* (Nematoda) second-stage juveniles in egg-masses and soil.

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1. Introduction

Phytohormones mediate important aspects of developmental processes in plants and participate in several aspects of plant nematode interactions [1,2]. Root-Knot Nematodes (RKN) are sedentary endoparasitic nematodes that interact with their hosts in a fascinating complex way. They infect their host and induce the re-differentiation of root-cells into the nematode feeding site called the giant-cells. Once inside the roots, nematodes extract important nutrients (water, ions and lipids) needed for their life from plants, thus causing severe damage to major crops [3]. The mechanisms guiding such alterations, however, are still poorly understood and, because *Meloidogyne* spp. affects thousands of plant host species it is presumed to interact with fundamental key steps of plant physiology. Among these key-steps, sterols surely play an important role and, the levels of i.e. stigmasterol, sitosterol, and campesterol seem to correlate with parasite infections. In fact, peculiar

endogenous levels may result in resistant cultivars compared to susceptible ones or to appear essential for own development and reproduction [2,4]. Although an endocrine system is still unknown in nematodes, there is evidence that many processes are regulated via hormonal pathways [5] and steroids may have endocrine functions in nematodes as well [6]. Reasonably, any endocrine disrupting means might influence nematode life cycle in view of plant protection planes. Many times, transgenic plant technology has been adopted to achieve details into parasitic strategy or for developing plant resistance based on gene-expression. Expression of specific genes such as proteinases, expansins, etc. are frequently reported in several studies aimed at protecting against phyto parasitic nematode.

In this study, efforts were aimed to demonstrate the efficacy of hormonal treatments to restore the phenotype in plants expressing the whole steroid-binding system and to prove the influence on plant-nematode reproduction.

Plants are known to produce a wide array of hormones (auxins, cytokinins, gibberellins and steroids) [7–9] ever those highly homologous to vertebrate counterparts and involved in proper root

elongation and hypocotyl growth [7,8,10]. The essential role of brassinosteroids in regulating plant growth and development was recognized decades ago, when mutants deficient in brassinosteroid biosynthesis or perception were discovered [11–13]. Since then, plant steroids such as brassinosteroids (BRs), stigmasterol and progesterone (PRO) have been demonstrated to be perceived by specific binding proteins as the membrane receptor BRI1, highly conserved leucine-rich repeat (LRR) kinase [14] and that resembles animal receptor kinases [14–18] or by a Membrane Steroid Binding Protein (MSBP1) which is a cytochrome b5-like acting as negative regulator of cell elongation and capable of influencing plant development by binding specifically to PRO or other steroids [19]. Moreover, plant defects were also described following the transformation with the GVG a chimeric-glucocorticoid system or by overexpression of the MSBP1 membrane-steroid binding protein [20–22]. The exact reason for aberrant phenotypes remained to be determined. Until now, the chimeric nature of the GVG system did not allow to recognize which domain/s could be responsible for such effects. Now, in this study a non-chimeric construct of the glucocorticoid expression system was employed sharing with the GVG only the hormone binding domain (HBD). The similitudes observed in our transgenic plants in term of dwarfism, associated to the response to steroids of these transgenic, leads to suggest for a role of the ligand binding domain itself. Indeed, these hormones are strategically important also for nematode parasitism, as testified by the loss of function mutations in either biosynthesis or signalling of genes for gibberellins, auxins and brassinosteroids that cause dwarfism or feeding-site alterations (i.e. the failure of the cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita* to induce normal syncytia or giant cells, respectively in the auxin-insensitive tomato mutants, dgt) [1]. In addition, differently from other plant hormones, transduction pathways of auxin and brassinosteroids are demonstrated to be strictly interconnected by an extensive complex mechanism [23,24] of intercellular transport of auxin that becomes essential for various aspects of plant growth and even for nematode infection [25].

Few are the basic biochemical differences between parasitic nematodes and higher plants or mammals. But, importantly the inability of nematodes to biosynthesize sterols obliges them to acquire it from diet or host, plants or animals [26]. For free-living nematodes growing in culture plates omitting sterols produce effects on development and growth [26]. Without sterols, worms can propagate for just a few generations and larvae fail to shed the old cuticles properly, gonad development becomes aberrant and their movement uncoordinated [27,28]. Generally, plant hatched juveniles have only a short free-living stage in the soil and host rhizosphere because J2 larvae do not feed during this period but use only the maternal lipid inherited and stored in the gut [29]. So, just the cholesterol passed on by mothers to the progeny can support larval development to adulthood. Notably, the amount of sterols transferred by the mothers is sufficient for the survival of the first generation and just partially for the second where development is arrested as dauer-like larvae. Starting from the second generation, animals without sterols are much more affected and many animals die or arrest their development as embryos or larvae [26].

In previous papers, transformants of *Nicotiana tabacum* bearing a glucocorticoid system (GR-system) were described [30–32]. Successively, other authors have hormonally characterized some transgenic *Nicotiana* spp. by gas chromatography and mass spectrometry [33,34]. Here in it is shown that the tobacco plants overexpressing a GR-system appear morphologically affected at hypocotyls and roots development, either *in vitro* or *in vivo*. It is also demonstrated biochemically that the hormone binding of GR-system can be activated by dexamethasone and progesterone. The morpho-physiological response of explants *in vitro* is shown, with and without hormones added to the medium. It is demonstrated that steroids added to the media can restore the phenotype to the transgenic seedlings. Finally, the repercussion of transGR + as host plants on nematode attractiveness and reproduction was determined. The nematode resistance was evaluated after a few and more consecutive generations.

2. Materials and methods

2.1. Molecular analysis on plant expressing GR-system

Nicotiana tabacum L. cv. white burley was co-transformed by Irdani et al. (1998) [30]. Plants expressing the GR-system were designated transGR+ and carried both an effector-vector: CaMV-GR, called pTI18 [31] and a reporter-vector: gre-*uidA* encoding β-glucuronidase (GUS-gene), called pTI20 [30]. Plants that expressed only the reporter vector were used as control and were designated transGR−. Regenerated plants were maintained in greenhouse until flowering and seeds-set. T2 seedlings, from three independent transGR + lines: (1), (2) and (6), were selected on LS medium [35] containing Kanamycin 100 μg/ml (LSK) plates and grown *in vitro* in growth chamber (16 h light/8 h darkness, at 24 ± 2 °C). Total RNA from tobacco leaves or roots, was extracted using the Tripure Isolation Reagent (Roche Applied Science). RNA extracts were quantified spectrophotometrically (260 nm) using Bio Photometer (Eppendorf) and 1 μg of RNA was subjected to reverse transcription (RT) reaction using ImPromII™ Reverse Transcriptase System (Promega). To detect transcripts in tobacco tissues, conventional end point amplification of cDNA were performed (RT-PCR). Primers for GR and new for tobacco-*Actin9*) and tobacco-*Expansin4* (TExp4) were designed from Genebank sequences (accession number X69885 and AF049353) [Table 1 of supplemental material] by using Primer3Plus software. Amplifications were carried out in a PTC-200™ thermocycler in a 25-μl mixture containing 1X PCR Key buffer (VWR International), 400 nM of each primer, 1.5 mM MgCl₂ and 0.2 mM dNTPs (Promega), 1 U *Taq* polymerase (VWR International) and 2 μl of cDNA following this cycling conditions: 95 °C, 5 min for initial denaturing step; 35 cycles of 94 °C, 1 min; 50 °C, 1 min; 72 °C, 2 min; 72 °C, for 10 min for final extension step. PCR products were visualized after electrophoresis separation in an ethidium-bromide stained agarose gel (1% w/v).

Real-time RT-PCR was performed to determine the level of GR transcripts in three tobacco-transgenic lines using the TaqMan technology and tobacco-*Actin9* mRNA as endogenous control. Primers and fluorescent probes were designed using Primer3Plus program and for multiplex real-time PCR two different labelling were used (6-FAM; JOE). The PCR was carried out in a MJ PTC-200 Chromo4 thermocycler (BioRad) using the following conditions: 95 °C for 10 min, followed by a total of 40 two-temperature cycles (94 °C for 1 min, 60 °C for 1 min).

Data output was released by Opticon Monitor software (version 2.03 MJ Research).

Quantification was done by using the $2^{-\Delta\Delta CT}$ method [36] because efficiencies of the amplification curves for GR and Tobacco-*Actin9* were equal [37]. The amount of GR transcripts, normalized to Tobacco-*Actin9* endogenous control and relative to the averaged level of expression of GR transcripts, is given by the equation: amount of GR = $2^{-\Delta\Delta CT}$. Hence, clones with a relative expression value below 1 have lower than average GR transcripts levels, while clones with a relative expression value above 1 have higher than average GR transcripts levels.

2.2. Hormones induction and GUS detection

The steroid hormones dexamethasone (DEX) and progesterone (PRO) purchased from Sigma Aldrich were dissolved in ethanol (EtOH) at 10 mM and diluted in the growth medium before use. The same volume of EtOH was added in the medium of control plants. Before hormone treatment, plants were grown *in vitro* for three weeks on agar medium then, the whole-plants were gently removed from agar and roots washed lightly in sterile-water before submerging it in a fresh liquid medium containing 30 μM of DEX, PRO or EtOH, respectively. After 72 h of hormone-induction, part of the root-apparatus were drawn and used for total protein extractions and GUS assays. Quantitative fluorimetric determinations of GUS activity were carried out as described by Jefferson et al. (1987) [38].

2.3. Hormone sensitivity assessment: DEX or PRO restores control-type phenotype in transGR+

Tobacco-transGR+ and control seeds were surface sterilized with 10% bleach solution for 10 min and washed three times with sterile water. The sterilized seeds were sown on Petri dishes containing LSK medium supplemented (with or without) DEX, PRO (0.1, 1, 10 μM) or EtOH. Fifteen days after germination, part of seedlings were aseptically transferred to magenta vessels (Lab Associates, NL) on the same medium as above and incubated in growth chamber under long-day growth conditions (16 h/8 h of light/darkness) at 24 ± 2 °C. Hypocotyl length was encountered 1 and 4 weeks after germination *in vitro*. Plants were also transferred to soil-pots for a further three months under controlled growth-conditions (see above). All experiments were performed in triplicate on an average of at least 25 seedlings.

2.4. NAA restores control-type root development in transGR+

A total of 80 leaf discs from either transGR+ and controls (≈1 cm Ø) were incubated on LS-LSK medium and on medium supplemented respectively with (1) 1 mg/L naphthalene acetic acid (NAA); (2) 0.8/0.2 mg/L NAA/kinetin (KIN); (3) 0.2/0.8 mg/L NAA/KIN; (4) 1 mg/L KIN; (5) 1 mg/L NAA/KIN or (6) devoid of hormones. Plates were grown *in vitro* in a growth chamber (16 h/8 h of light/darkness, at 24 ± 2 °C). Root formation data were recovered 8 weeks after plating.

2.5. Plant infections, nematode extraction and reproduction assessment

Root-knot nematode *Meloidogyne incognita* was maintained on tobacco (*Nicotiana tabacum* L.) plants in a greenhouse. Egg masses were extracted with 0.5% (v/v) NaOCl and second stage larvae (J2) hatched as described in Ref. [39].

Plant infections: *N. tabacum* w. t. transGR- and transGR+ [30] were grown in plastic pots (10 cm Ø) containing a sterilized mixture of sand (70%) and clay (30%) for 2 months and inoculated with approx. 500 s-stage juveniles (J2)/plant through three holes in the soil, just around the base of the plant stem. Plant infections were realized in successive steps, carried out in a growth chamber for 60 days with 18 h/6 h photoperiod at temperature of 24 ± 2 °C. These growth conditions allow to *M. incognita* to perform 1–2 generations on tobacco [40]. Firstly, all three genotypes of tobacco, w. t. transGR- and transGR+, were inoculated with fresh J2 extracted from tobacco w. t. thus indicated as "J2 from w. t.". Successively, J2 were kept developing repeatedly on same transgenic plants genotype. At cascade, J2 recovered from the transGR + plants were used for re-inoculation on other transGR+ and transGR-,

respectively. This explained the expression of "J2 grown a few-generations on transGR+" and, successively "J2 grown more-generations on transGR+".

Nematode staining and reproduction assessment: roots were removed from soil and washed gently in water for few minutes. Then, each root system was dipped in a 0,15 g/L phloxin-B colouring solution for 15 min to point out galls and egg masses. Stained roots were then observed under the microscope to assess nematode infectivity by estimating the egg masses [41]. All the egg masses (EM) were numbered and reported as average of EM/gr fresh roots (gr). The pool of juveniles was allowed to coming out from each egg-mass separately. Single EM was left disclose in water at 24 °C in multi-well plates. The precise J2 hatching were monitored day by day for as long as 13 days. Then, the recoveries were reported as the average of J2/egg-mass.

Nematodes extraction from soil: aliquots of 100 cc soil were achieved by each pot and processed by the method in Ref. [42]. Each soil aliquot was spread on two tissue paper filters (Ø 19 cm) mounted on a sieve of 1 mm mesh. The sieve with the soil was placed in a bowl (Ø 15 cm) and gently submerged with water up to cover the soil itself. The sieve with soil was incubated at room temperature (≈20 °C) for 48 h then removed and water collected passed through a 25 μm mesh. Nematode retained by the sieve were recovered carefully with a jet of water (wash-bottle) and determined by direct counting under stereomicroscope. Total juveniles number per Kg was reported (J2/kg).

2.6. Statistical analysis

Data were analysed for significant differences using analysis of variance (one-way ANOVA) followed by Tukey's test (P < 0.05) performed on PAST software (http://folk.uio.no/ohammer/past).

3. Results

3.1. Expression analysis of GR in N. tabacum

Both RT-PCR and quantitative real-time PCR were employed to study the expression of GR-system (Fig. 1a) in three independent tobacco lines. RT-PCR showed that GR was expressed either in leaves and roots of transGR+ (Fig. 1b). To further study the role of GR in hypocotyl suppression, a tobacco gene marker involved in cell elongation (TExp4) was included in our analysis. As shown in Fig. 1b, no obvious change in the tobacco-expansin4 was detected between transGR+ and transGR-. Tobacco expansins are a large family of proteins involved in cell wall loosening and, perhaps, other genes and/or different plant developmental stages should be further considered. The qRT-PCR analysis of transGR + revealed that expression level of GR enhanced 1.5–2 fold with respect to actin as internal control (Actin9). There were no significant changes among the GR expression in independent lines of transgenic and no expression signals in transGR-control plants (Fig. 1c). The functional analysis of GR-system was biochemically detected in transgenic plants as GUS activity, after inductions with DEX or PRO. In the absence of hormones, GUS activity in roots was barely detectable. GUS activity increased clearly after the 24 h of hormone treatment at 1 μM (data not reported) but, activities were more stable when measured after 3 days with 30 μM of hormones (Fig. 2). Both DEX and PRO induced high levels of GUS activity with respect to control plants which were treated with EtOH alone. Some preliminary data also suggest oestrogen (EST) as a possible inducer of GR-system (data not shown) but further investigation would be required.
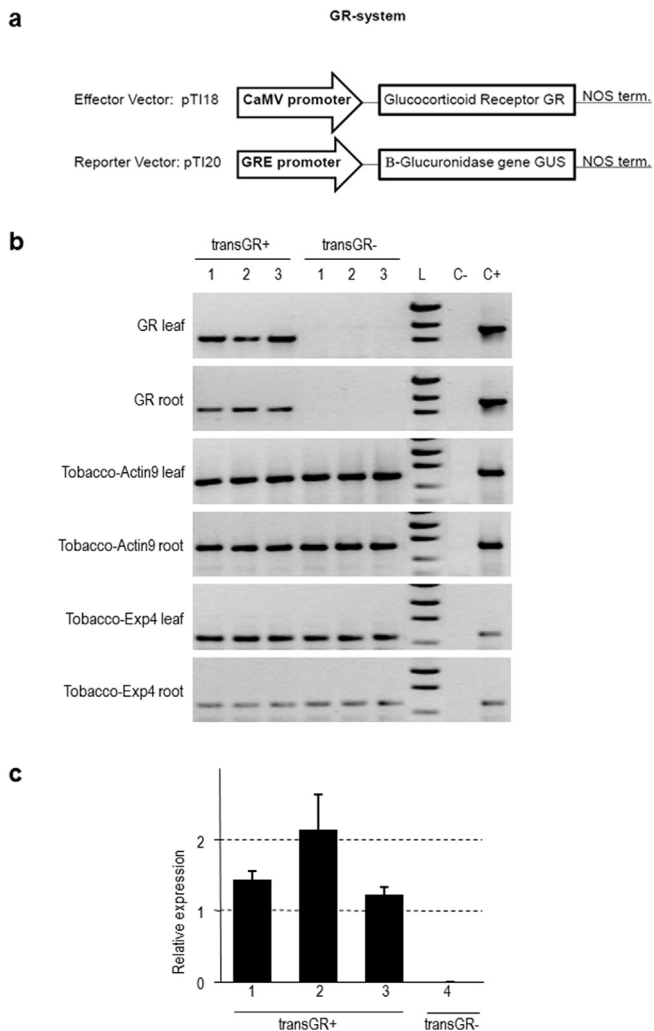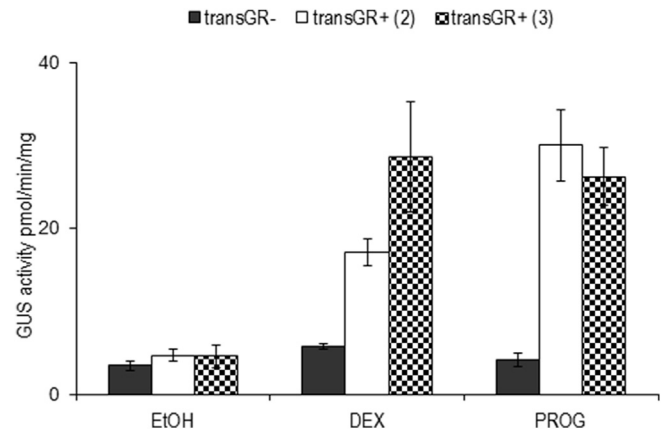
Fig. 1. Detection of GR expression in tobacco.
(a)Schematic representation of GR-system, the effector vector pTI18 bearing the glucocorticoid receptor and reporter-vector pTI20 [28,29]. (b) RT-PCR analysis of GR, actin and expansin transcripts from three independent transgenic lines (transGR+) respect to control lines (transGR−) bearing only the reporter-vector. (L) DNA molecular weight marker; (C-) no RNA; (C+) Plasmid-vector DNA (GR) or RNA from wild-type (actin, expansin4), used as positive control. (c) qRT-PCR analysis of GR transcripts of three independent transgenic lines (transGR+) and one control (transGR−). GR transcripts values were normalized respect to actin9 endogenous signal. The resulting mean values were relative to units mean ± SE of three independent amplifications.


Fig. 2. Inducible GUS activity in roots.
GUS assays after 72 h of induction with EtOH, DEX or PRO, in one control and two independent transgenic plants. TransGR + plants showed GUS expression in a hormone-dependent manner. Data reported as the mean ± SE of seven repetitions from transGR− (black) and transGR + lines (2) and (3) white and dotted, respectively.

3.2. Restored hypocotyl and root phenotypes after hormones treatments

Since transGR + appeared inhibited at hypocotyl development, progenies were repeatedly germinated on selective medium and grown *in vitro* up to 2 months, under long-day or dark conditions. In the light, transGR + expressing GR constitutively exhibited a dwarf phenotype with reduced root apparatus compared to control plants (Fig. 3a). TransGR + showed also a significant height reduction, approx. 55% when compared to control plants (Fig. 3b). The plant growth was measured again after another three months *in vivo*, still showing ≈55% of height and ≈66% of root systems compared to control plants (Fig. 3c). In the dark, the plant development of transgenic seedlings did not show any differences from controls, neither hypocotyl inhibition nor defect at apical hook were observed (data not shown).

The dwarf phenotype affecting transGR + resembled mostly those associated to the hormonal imbalance reported by others [19,34,43,44] or due to overexpression of certain regulatory domains [19,20,45]. The hypothesis of a sort of hormonal disturbance generated by transgene overexpression was tested by assaying the sensitivities of transGR + to exogenously applied phyto-hormones.

As GR demonstrated to bind DEX and PRO, the responses of these two hormones were checked. First transGR+ and transGR− lines were germinated on media containing increasing amounts of DEX or PRO (0–10 μM) for several days. PRO had already demonstrated to stimulate or inhibit the *Arabidopsis* hypocotyl growth depending on its concentration and light conditions [46], differently from DEX that had never been tested before. After 1 and 4 weeks of growth, tobacco hypocotyl length was scored in the light at low and high steroid concentrations. In Fig. 4 the hypocotyls elongation of transGR+ and transGR− on various hormones and concentrations are reported. Results show the plants growth promotion realized by DEX or PRO, especially at lower doses. Some clear distinctions between transGR+ and transGR− behaviour was registered. A strong growth promotion was observed soon after a week on DEX or PRO for transGR + seedlings at each concentration used (0.1–10 μM); a more modest stimulation was detected in transGR− either by DEX or PRO and only to the lower concentrations (0.1–1 μM). The effect of PRO becomes even inhibiting on transGR− when added at higher doses. Similar tendencies were observed in the seedlings kept longer (4 weeks) on the same plates where only more attenuated effects were noted. TransGR + plants demonstrated a fully restored hypocotyl length by exogenously application of DEX or PRO thus suggesting a role of transgene overexpression. Vice versa, no positive influence was noted at hypocotyl level in response to the exogenous auxin (0,1-1 μM NAA) (data not shown). Rather severe delays afflicted the seeds during germination on NAA, thus preventing any further analysis and comparison with the controls.

Furthermore, indirectly to investigate the hormonal state of transGR+, leaf explants were placed on different hormone concentrations/ratio and then, root growth was annotated. Roots were left to develop on six different media (1)−(6) then scored and reported in Fig. 5. In the absence of hormones, wild-type plants fully develop a normal root apparatus. On the contrary, transGR + showed very reduced root development by approx. 20%. Anyway, transGR + proved to restore a full root apparatus on the highest auxin supplemented media (1). Overall, transGR + significantly differentiated from controls. Severe root-
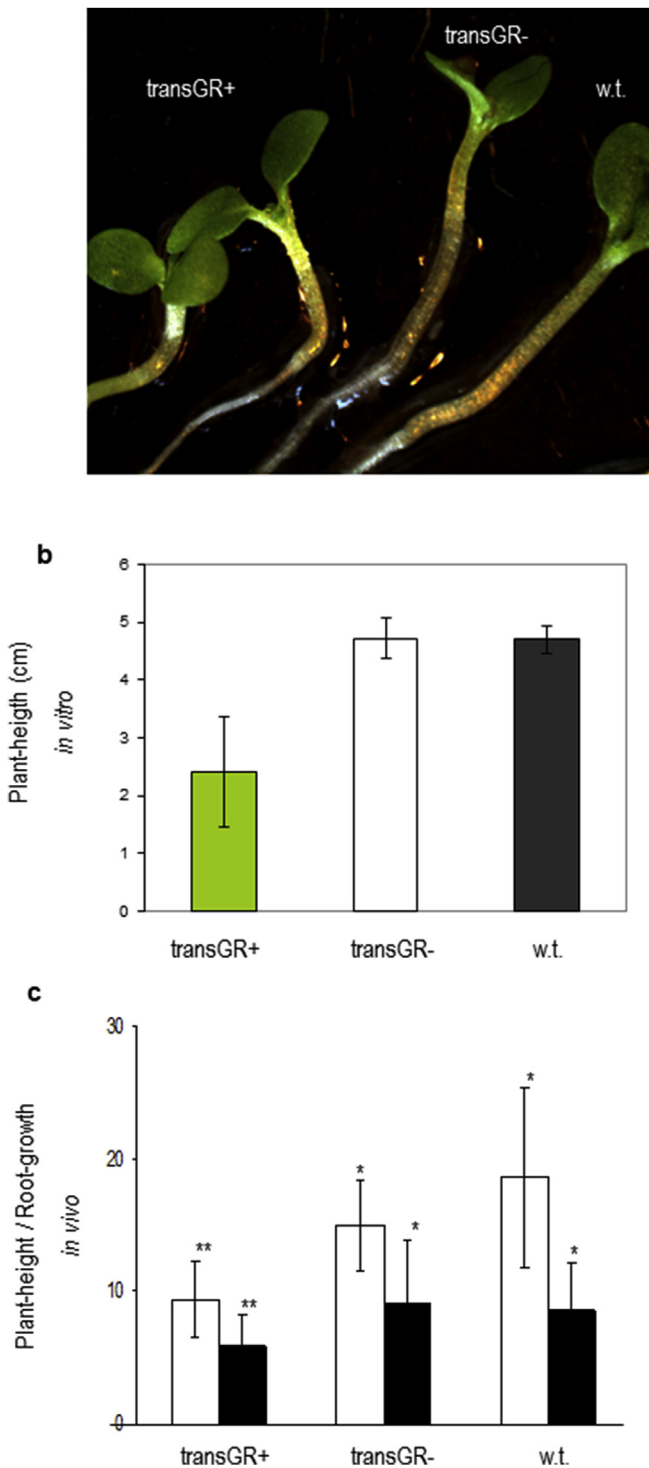
Fig. 3. Phenotype of GR overexpressing plants grown *in vitro* or *in vivo*.
(a)Small-phenotype of transGR + plants (left column) respect to controls: transGR− (middle); w. t. (right). (b) Effect of GR on seedlings-height, after 8 weeks of *in vitro* growth respect to controls. (c)Effect of GR expression on height and root development after 3 months of *in vivo* growth respect to control-plants. Plants-height (white-bar) and root-weight (black-bar).
Data reported are mean ± SE of 25, experiments repeated three times.
* Statistically significant differences (One-way ANOVA P < 0.05).

penury was observed on plates with low auxin contents (2) (3) while there was the absence of roots on cytokinin alone (4) or on 1:1 NAA/KIN ratio (5).

Only very high doses of exogenously applied NAA (1 μg/ml)

achieved the positive effect of root restoration in transGR + comparable to that of the control.

### 3.3. Nematode reproduction mitigated on transGR + plants

TransGR + plants were investigated through a series of plant infections with a RKN population known to require host essential hormones for grown and development. In that sense, sterols has been demonstrated to be necessary for nematode reproduction by the second generation. This study shows how J2 number released into the soil is significantly reduced to less than 50%, after a few generations on transGR+ (Fig. 6) with no significant difference between control plants, except for a slight decrease on transGR- (Fig. 6). Herein, the reduced recoveries of J2 from the soil of transGR + plants was better investigated in terms of both infectivity and reproduction rate of a plant nematode population of *M. incognita*. Infectivity was reported as a number of egg-masses per gram of roots (EM/gr). Although the absence of significant differences among the three genotypes, substantial decreases in egg-masses/gram were demonstrated after a few or more generations on transGR+ (28 and 20 EM/gr, respectively) respect to transGR- (34 and 26 EM/gr) or to wild type (39 EM/gr) (Fig. 7). Successively, the reproduction rate was evaluated as the average number of live larvae released from egg-masses (J2/EM), collected in the course of generations. The releases of J2/EM were estimated for all three genotypes: transGR+, w. t. and transGR- (Fig. 8). The values of J2/EM did not show significant difference in all genotypes when plants were inoculated with J2 coming from wild type plants (approx. 400 J2/EM). Instead, the average J2/EM developed on transGR+ and transGR-were significantly affected when J2 came after a few or more generations on transGR+. Interestingly, J2 decreased by 50% on both genotypes within a few generations, but more significant reductions were achieved after repeated generations on transGR+, until the reduction by 80% (Fig. 8).

4. Discussion

In this study, the overexpression of a steroid inducible system was quantitatively demonstrated in tobacco along with its functional activity by different steroids. Two main biological effects are investigated that are strictly dependent on the plant hormonal status: (i) the dwarfism exhibited by transGR+ with the proved hormonal rescue; and (ii) the increased plant resistance to the parasitic nematode reproduction.

Plants constitute a system in which the steroid binding properties of a mammalian glucocorticoid receptor appear mostly conserved. It is well-known that nuclear receptors upon activation by hormone binding require productive intracellular interactions with several other proteins and factors (i.e. heat-shock proteins, kinases, etc.). Thus, the functionality assessed here in tobacco confirms a high degree of conservation between plants and animals. The maintenance of these factors may reflect a similarity in signalling strategies among eukaryotes for important processes included homeostasis and development.

This study reports seedlings overexpressing GR-system that showed negative growth affections. Interestingly, the delayed growth resulted completely reverted by exogenous application of hormones (DEX, PRO or NAA). Hence, the correlation between the inhibitory growth effects by transgene joined to the capacity of binding with diverse steroids as well as the demonstrated reversibility of the phenotype by the same molecules suggest a critical role of GR-system at regulatory or signalling pathway in the plant development. No other hormones, potentially involved in growth (such as ethylene) was considered in this study, the reason being that the normal stature observed by transGR+ in the dark,
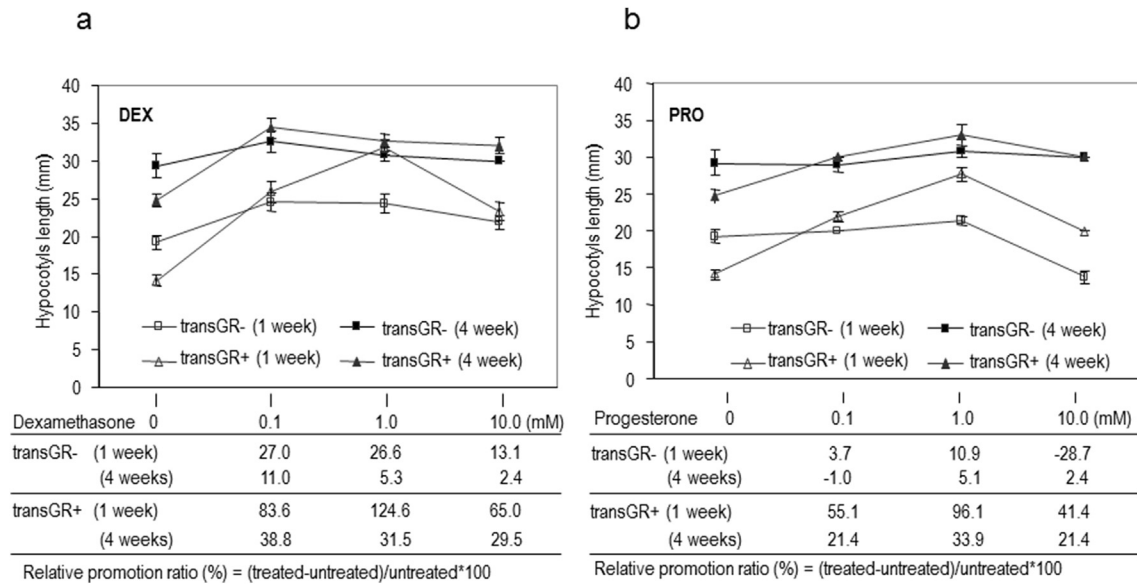
Fig. 4. Effect of dexamethasone (DEX) and progesterone (PRO) on GR overexpressing seedlings.
(a)Effect of DEX on hypocotyl elongation after 1 and 4 weeks of growth under the light.
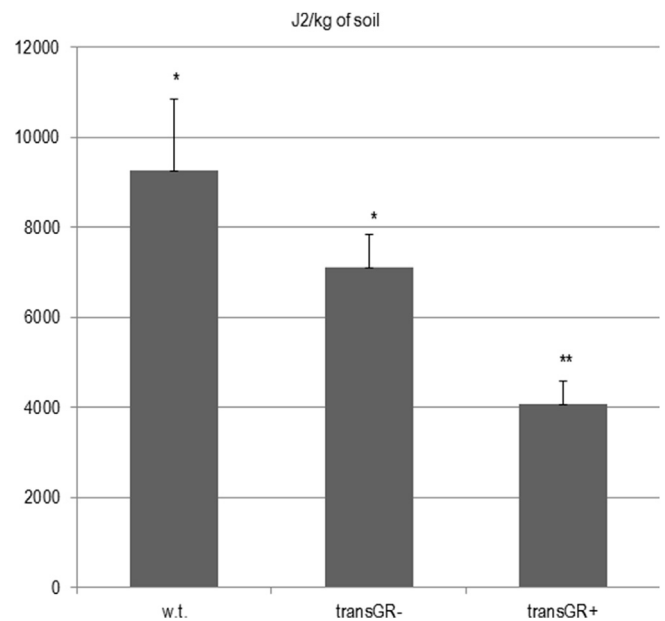(b)Effect of PRO on hypocotyl elongation after 1 and 4 weeks of growth under the light.
For each plant genotype was reported the mean ± SE of 25 plants. Experiments repeated three times. Seedlings were grown on media containing various concentrations of DEX or PRO for 7–14 days.
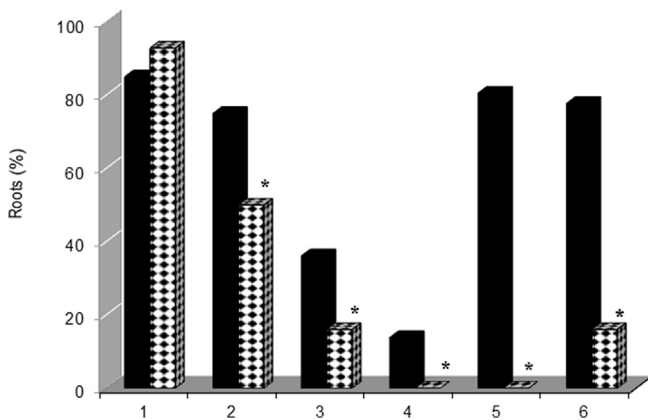
Fig. 5. Effect of differential hormones-ratio on roots development.
Results are the mean percentage of twenty leaf explants cut from a pool of leaves derived from transGR+ (squared-bar) or w. t. plants (black-bar) and left to grow on LS medium + (1)–(5) hormone composition: (1)1 mg/L naphthalene acetic acid (NAA); (2) 0.8/0.2 mg/L NAA/KIN; (3) 0.2/0.8 mg/L NAA/KIN; (4)1 mg/L kinetin (KIN); (5) 1 mg/L NAA/KIN or (6) devoid of hormones. Evaluation was made 60 days after start of culture. * Statistically significant differences (One-way ANOVA P < 0,05).

Fig. 6. Effect of GR overexpressing plants on nematodes recovered from soil.
Juveniles larvae (J2) extracted from samples soil and reported a. Each bar represents the mean ± SE of n = 6 plants, and bars with different (*) Statistical significant difference at P < 0.01.

weakened the idea of an ethylene-loss of function mutation. Instead, molecular analysis was performed to investigate the expression of a cell-elongation factor. For this reason, tobacco expansin 4 (TExp4) was included in the study since it results genetically closer to the AtExp1 gene, found silenced in MSBP1 overexpressing plants [19]. However, in this study TExp4 resulted equally expressed in transGR+ and control plants without any significant differences. Reasonably, this result could be expected by the awareness that a large number of expansins or extensins exist in tobacco and, perhaps the necessity that other sequences should be considered and examined. However, dwarfism is a common trait to plants altered in hormonal/steroid pathway (det1, msbp1, bri1) and, even if the mechanism by which the enhanced expression of GR-system influences on plant growth awaits further studies, this

study demonstrated that normal phenotype can be recovered by transGR + seedlings by exogenous steroids treatments. This data effectively demonstrated that ligands as DEX and PRO can re-establish hypocotyl length comparable to those of the controls treated at same doses, inducing growth promotion at low doses or inhibition at higher doses. Therefore, it is plausible that PRO as ubiquitous components in plants, or perhaps other cognate steroids detected in tobacco seeds and leaves [47,48] may be influenced by overexpression of this ligand-binding domain (HBD), thus

EM/gr
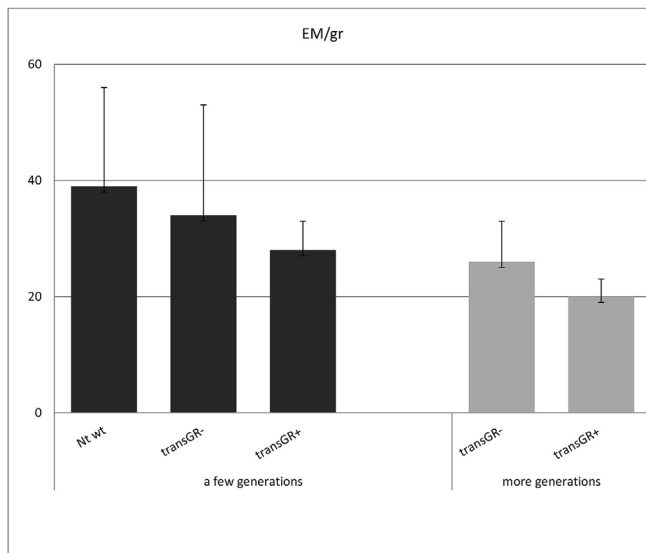
Fig. 7. Effect of GR overexpressing plants on egg-masses developed.
Number of egg masses per gram obtained on N.t. wt, transGR- and transGR + plants, infected with J2 left to grow a few or more generations on transGR + plants. Data are means of two independent experiments. Each bar represents the mean ± SE of n = 6 plants.
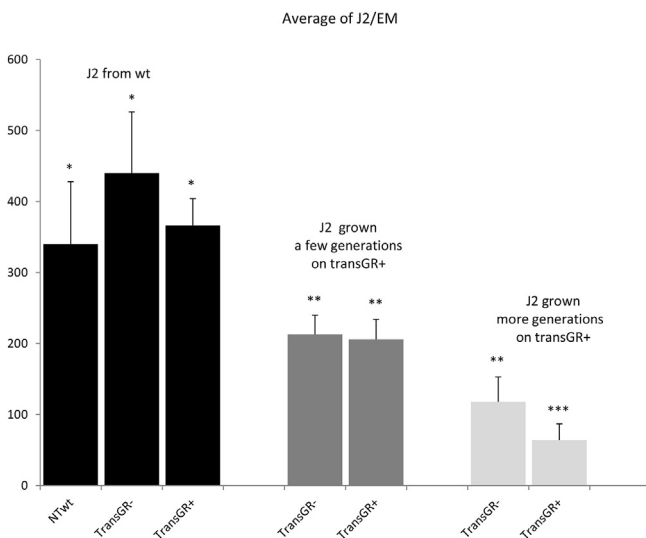
Average of J2/EM

Fig. 8. Nematode reproduction analysis through generations.
Average of juveniles (J2) per egg mass released from N.t. wt, transGR- and transGR + plants, infected with J2 coming from w. t. or left a few or more generations on transGR + plants. Data are means of three independent experiments. Each bar represents the mean ± SE of n = 36.
(*) Statistical significant difference at P < 0.05. *P*-values obtained by Duncan test analysis.

explaining the morphological changes reported. The no GUS activity observed without the exogenously addition of PRO or DEX is quite expected data considering the negligible traces of these hormonal components in plants. In fact, GUS fluorescence signals were gained only at higher doses of exogenous steroids. On the other hand, altered contents of several phyto-hormones have been already quantitatively reported by other authors in different *Nicotiana* species expressing exactly the same GR sequence [33,34]. Moreover, this data reported also a full restoration of the roots growth in transGR + by the addition of high doses of auxin (NAA),

thus also revealing a possible auxins and phytosteroids interconnection.

The demonstration that transGR + plants bind steroids suggests that other biological mechanisms may be affected. A significant achievement of this study was the consistent reduction in nematode reproduction observed on transGR + plants. Plant parasitic nematode reproduction is well-known and strictly dependent on the plant host tissues for composition or hormonal balance, especially at sterols. Here, the hormonal necessity of the nematode for investigating the impact of the transgene as an inhibitor factor for reproduction was exploited. In particular, it was noted that although transGR + plants appear equally attractive for RKN, showing an insignificant difference as egg-masses per gram, clearly they induced a relevant decrement in the absolute number of egg-masses. Since a significative difference of J2 per kg of soil was always recorded among the soil samples, the RKN reproduction was examined during the generations. J2 grown for a few or more generations on transGR + demonstrated to be strongly hit during the successive generations. The RKN reproduction was greatly impaired when larvae were left to grow repetitively on transGR+; this result seemingly in accordance with what has been reported on nematode growth effects on cholesterol-free medium [26]. Indeed, the progeny released by egg-masses resulted mitigated by 50% when larvae were grown for a few generations on transGR + but, it was reduced further to 85% when it was kept longer on transGR+. Overall, these data may reflect a physiological impairment into the transGR + roots and an obstacle to the reproduction. The mechanism by which the enhanced expression of GR system acts on plant growth awaits further studies. The overexpression of GR-system produced morpho-physiological changes in plants that may have diverse significances, among these changes the levels of regulatory compounds (i.e. hormones) or redundancy of receptors (i.e. ligand binding sites) equally important mechanisms in the development of organisms, including plants. Further studies combined with more powerful analytical tools could offer interesting details about the contents or the changes of these plant compounds bringing to light further knowledge in this important research field. In summary, a non-chimeric GR-system constitutively overexpressed in plants showed a role that afflicted plant growth and development but can be completely reverted by exogenous hormones addition. The capacity demonstrated by the GR-system to bind steroids as PRO suggests a possible influence at a physiological and/or cellular level in the plant. Interestingly, an important correlation between transGR + expressing plants and nematode progenies development was discovered in this study.

Competing interests

Authors' contributions

TI conceived and performed all the experiments, wrote the manuscript. RP conducted molecular analysis and biochemical assays. BP conducted transgenic-plant experiments. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2017.03.006.

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