



(51) International Patent Classification:

*A01N 43/08* (2006.01)      *A01N 65/00* (2009.01)  
*A01N 43/12* (2006.01)      *A01P 21/00* (2006.01)

(21) International Application Number:

PCT/EP2010/056198

(22) International Filing Date:

6 May 2010 (06.05.2010)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

09159625.4      7 May 2009 (07.05.2009)      EP

(71) Applicant (for all designated States except US): **GMI - GREGOR-MENDEL-INSTITUT FÜR MOLEKULARE PFLANZENBIOLOGIE GMBH** [AT/AT]; Dr. Bohr-Gasse 3, A-1030 Vienna (AT).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **GREB, Thomas** [DE/AT]; Anzbachgasse 55, A-1140 Vienna (AT). **AGUSTI, Javier** [ES/AT]; Sandwirtgasse 17/Top 2, A-1060 Vienna (AT).

(74) Agent: **SONN & PARTNER Patentanwälte**; Riemergasse 14, A-1010 Vienna (AT).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**

— of inventorship (Rule 4.17(iv))

**Published:**

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: USE OF STRIGOLACTONES

(57) Abstract: The invention describes the use of strigolactones for promoting secondary growth of plants and, thereby, for accumulating biomass, for increasing shoot stability and transport capacity along growth axes.



WO 2010/128112 A2

## Use of Strigolactones

The present invention relates to methods and compositions for promoting plant growth.

In addition to longitudinal growth, lateral expansion of growth axes is essential for land plants for creating extended shoot and root systems. Lateral or secondary growth is mediated by the activity of the cambium, a two-dimensional meristematic tissue, which is organised as a cylinder enclosing the centre of growth axes. Tissue formation by the cambium is mostly limited to vascular tissue which is essential to increase transport capacities and mechanical support of shoot and root systems. In contrast to the current understanding of molecular mechanisms controlling apical meristems and in spite of its essential role for many aspects of plant growth, knowledge of the molecular regulation of the cambium is very limited.

The vascular system in plants is the main long-distance transport system responsible for the distribution of assimilates, nutrients, water and signalling molecules, but also provides mechanical stability to the plant body. Therefore, beyond scientific aspects, the understanding of the molecular control of vascular development and, especially, the control of cambium activity, which is the origin of secondary vascular tissue, has implications for various aspects of crop production. This holds true especially considering anthropogenic changes in the global environment. For example, as vascular tissue provides mechanical support to plants, increased secondary growth has the potential to increase the resistance of annual crops to mechanical stresses such as wind and heavy rainfall. Furthermore, second generation biofuels are gained from whole plants and represent sustainable alternatives to burning fossil fuels. Lateral growth contributes significantly to the accumulation of biomass in perennial plants and an increase of cambial activity could make the usage of promising species even more efficient or could cause species on which there is currently no focus to become interesting for such applications.

Most importantly, wood formation in trees represents the most prominent result of secondary growth. Wood is not only a major source of energy but also provides material for building

and many other products for which the mechanical properties and the aesthetics of this material are essential. In addition, developing wood cells represent one of the most important sinks for excess atmospheric CO<sub>2</sub>, thereby reducing one of the major contributors to global warming.

Another aspect which is relevant in this connection is shoot architecture of crop species. During domestication, there has been a strong selection for plants with strong apical dominance, a trait which facilitates the harvesting process due to more synchronised fruit ripening and decreased assimilate transfer into side shoots.

Strigolactones are natural compounds extracted from root exudates of various plant species and were originally described as agents inducing the germination of seeds of parasitic plants of the groups *Striga* and *Orobanche*. These parasitic weeds are serious pests in many countries and are particularly harmful to some crops of major economic interest, such as crops of sorghum, maize, sugar cane, beans, etc.. Strigol was first identified in the false host cotton and later also in the *Striga* hosts maize, sorghum and millet. The structurally related alethrol was identified in cowpea, a host of *S. gesneroides*. Alethrol, orobanchol and a third unidentified germination stimulant have been isolated from root exudate of red clover. Strigolactones also enhance the seed germination of certain nonparasitic plants. Moreover, upon phosphate depletion, they function as root-derived signals causing elevated hyphal branching and spore germination of symbiotic mycorrhizal fungi, to enhance the uptake of soil nutrients. They are present in the root exudates of mono- and dicotyledonous plants (for example, rice and pea) and have additionally been detected in their shoots.

With strigolactones, it was possible to envisage suicide germination, a new approach in the struggle against parasitic plants (suicide germination consists in treating agricultural soils, which are likely to be infested with parasitic plants). Synthetic strigolactones, such as GR7 and GR24, are easier to obtain than the natural compounds and have therefore been regarded as being more suitable for application on an industrial scale. With synthetic strigolactones, the suicide germination treatment is carried out at a time when the plant to be culti-

vated is not yet growing in said soils. By disseminating said synthetic strigolactones, germination of the dormant seeds of the parasitic plants is induced and the parasitic plants, without a host plant to infest, ultimately die through lack of nutrition (US 2008/0318773 A1).

In US 2008/0318773 A1 strigolactones are used to stimulate the growth and/or development of arbuscular mycorrhizal fungi (AM fungi). This method is described as an advanced mycorrhization technique which is aimed at optimizing the production of fungic inoculum, and an improved use of AM fungi in soils or cultivation substrates. Administration of strigolactones is made for intensifying the symbiotic interaction between AM fungi and cultivated plants. In doing so, strigolactones (selected from GR24, GR7, Nijmegen-1, demethylsorgolactone, strigol, alectrol, sorgolactone, orobanchol and their structural analogs) are e.g. added to the cultivation medium (cultivation soil). An effect of strigolactones on the plants has not been described in US 2008/0318773 A1.

The role of strigolactones in the induction of *Orobancha cumana* seed germination is described in Galindo et al. (J. Agric. Food Chem., 50 (2002), 1911-1917), Ayman et al. (Plant Growth Regulation 48 (2006), 221-227) and De Luque et al. (Phytochem. 53 (2000), 45-50).

In DE 10 2005 048 904 A1 strigolactones from hops are applied for bioremediation of abandoned landfills and contaminated sites in soil for stimulating hyphae forming organisms, such as Mycorrhiza, which organisms then carry out the bioremediation.

In WO 2006/098626 A2, it is suggested to create crop species that do not induce germination of parasitic plant seeds anymore and therefore are resistant to parasitic plants by transforming plant cells with gene silencing vectors e.g. against enzymes involved in carotenoid catabolism to strigolactone germination stimulants. Also recombinant trap and catch crops overproducing strigolactone are suggested.

More recently, it was reported that strigolactones inhibit shoot branching (Gomez-Roldan et al., Nature 455 (2008), 189-194; Umehara et al., Nature 455 (2008), 195-200). This effect was very specific and lead to the speculation that strigolactones may be used to specifically regulate shoot branching with

minimal side effects and without the need for transgenic technology. In parallel, this also suggested that strigolactones may have a principal role in mediating the detection of nutrient availability by roots. It was even speculated that strigolactone levels are increased by plants in response to inorganic phosphate deficiency.

It is an object of the present invention to provide methods and compositions which are useful in agriculture, especially which are useful in promoting secondary (or lateral) growth of plant growth axes or in producing biomass.

Therefore, the present invention provides the use of strigolactones for promoting secondary growth of plants.

Quite surprisingly, it is shown with the present invention that strigolactones, for which up to now only a narrow spectrum of activity within plants was reported (among them the inhibition of shoot branching!), have a major role in triggering secondary growth in plants. By using this effect, the invention can specifically be used to enhance secondary growth in plants, preferably in cultured plants, especially in crop plants and to provide enhanced shoot stability, transport capacity and, generally, an accumulation of biomass. Moreover, it could also be shown that with the present invention an enhanced transport capacity in the plants along plant growth axes is safeguarded. All these effects may be obtained by direct application of strigolactones to the plants of interest. Accordingly, the present invention also relates to the use of strigolactones for enhancing shoot stability in plants and the use of strigolactones for increasing transport capacity in plants. The present invention therefore does not need the assistance of further organisms, such as (arbuscular) mycorrhizal fungi (as in US 2008/0318773 A). Specifically in view of the prior art, wherein strigolactones are only used indirectly with such fungi (as growth stimulants for these fungi), it was indeed surprising that administration of strigolactones directly to the plant of interest has the beneficial effects described herein, (specifically in view of the known effect of inhibition of shoot branching being connected to such direct application!).

Although the present invention may also be realised by enhancing the strigolactone production in plants by enhancing the

internal production pathways in plants (for example by classical breeding programmes (extremely time consuming in crop plants !) or recombinant manipulation as in WO 2006/098626 A2 (with respect to strigolactone production)(various problems connected)), the present invention is preferably put into practice by the "pharmacological" approach, i.e. by administering strigolactones directly to the plants.

In the examples, the feasibility of the present invention is shown in an Arabidopsis model, which serves as a suitable model for other plants in general for which this invention is usable. Arabidopsis is also most suitable for addressing detailed scientific questions concerning the present invention (e.g. by using mutants with influence on the strigolactone pathway).

However, the present invention, of course, preferably provides its beneficial output in economically important plants, in which the enhancement of secondary growth by administration of strigolactones is used for agricultural improvement.

The use of strigolactones in field applications is also promising, because strigolactones are only involved in a small repertoire of (other) responses; their activity is generally regarded as being specific (in contrast to e.g. auxins). In addition, strigolactones are not toxic per se.

Since in monocots, stems rarely show secondary growth, dicots (wherein stems frequently have secondary growth) are preferred plants which are treated according to the present invention. Due to the economic importance of the wood-forming properties of the present invention, trees represent obvious targets for strigolactone application. Accordingly, trees, especially trees used for industrial wood production are preferred plants to be treated with strigolactones according to the present invention. Preferred tree types are: oak, chestnut, black locust, mulberry, osage-orange, sassafras, maple, ash, hickory, hackberry, beech, pine, buckeye, poplar, willow, catalpa, elm, walnut, mahogany, balsa, black ironwood, birch, eucalyptus, fir or alder.

In addition, a lack of shoot stability requires often attaching crop plants to mechanical support (e.g. tomato, grape, peas, ornamental flowers) or leads to a loss in yield due to me-

chanical influences (e.g. in rape seed by wind, heavy rain fall). The enhancement of secondary growth by strigolactone application according to the present invention leads to an increase in shoot stability making the manual attachment dispensable and reducing crop failures.

Another preferred group of plants according to the present invention are those which are used for fiber production from sprout axes, such as flax (*Linum usitatissimum*), jute (*Corchorus capsularis* and *Corchorus olitorius*), hemp (*Canabis sativa*) or ramie (*Boehmeria nivea*). In these or related plants, the present invention is used for enhancing the fiber production. Collected fibers usually originate from bast which is, in addition to wood, the second tissue produced by the vascular cambium.

Other preferred plants are those industrially used plants (vegetables) which are based on a (secondary) growth of shoot and root axes, such as kohlrabi (*Brassica oleracea gongylodes*), potato, carrot, sugar beet or garden beet.

On the other hand, the effect of the present invention can also be used for monocotyledonous plants e.g. for accumulation of biomass and to increase shoot stability. Secondary growth does not exist in most of monocotyledonous plants. However, some monocotyledonous species perform secondary growth which is based on the activity of a cambium-like tissue (e.g. *Cordyline*, *Draena*, *Yucca*). As strigolactone signal transduction is also present in monocots, strigolactone administration according to the present invention is also applicable in this case and induces secondary growth in species which do not perform this process under standard conditions. For example those, which are often subjected to damage by wind and rain, such as wheat and barley, *Miscanthus* or switchgrass, or which are harvested as whole plants to serve as animal feed or as sources of biofuels, such as maize and sugarcane, can be regarded as further preferred plants to be treated according to the present invention. Accordingly, a preferred embodiment of the present invention is drawn to the use of strigolactones for the accumulation of biomass in monocotyledonous plants, especially those with a cambium-like tissue.

The plants to be treated according to the present invention may preferably be plants without (arbuscular) mycorrhizal fungi

in (symbiotic) connection therewith. In view of US 2008/0318773 A) the direct effects of strigolactones according to the present invention were not expectable from these teachings. On the other hand, the present invention can also be used in combination with the plants which use the symbiotic action of mycorrhizal fungi, e.g. by stimulating both, the fungi and the plants, the latter e.g. by specific administration to specific parts like stems, roots or leaves or to whole plants.

With the present invention, an accumulation of biomass can be achieved in non-parasitic plants (in industrial plant production), preferably in plants used for agricultural mass production, especially in trees used for industrial wood production and crop plants. Industrial plant production includes agriculture with High-yielding varieties (HYVs) and intensive farming using monocultures (e.g. from 1 Are upwards for small plants, like vegetables or 5 Are or more for crops or trees and the like).

According to the present invention any strigolactone is applicable which has a proper biological activity of germination stimulation annotated to this class of compounds. Many naturally occurring strigolactones have a decreased stability, especially under basic conditions. For such strigolactones, proper stabilizers should be added when applied according to the present invention. On the other hand, many stable artificial strigolactones are known, which are preferably used according to the present invention. Specific examples have been termed "GR" (germination releaser) (Johnson et al., J. Chem. Soc. Perkin I 6 (1981), 1734). Proper bioassays are e.g. described in Mangnus et al. (J. Plant Growth Regul. 11 (1992), 91) or in Bergmann et al. (J. Plant Physiol. 142 (1993), 338, wherein a bioassay based on the concentration of half maximal stimulation of germination ("C<sub>1/2</sub>") is described. Examples of preferred strigolactones to be used according to the present invention are strigol, strigyl acetate, orobanchol, orobanchyl acetate, 5-deoxystrigol, sorgolactone, alectrol, 3-[[ (2,5-Dihydro-3-methyl-2-oxo-5-furanyl)oxy]methylene]-3,3a,6,6a-tetrahydrocyclopenta[b]furan-2-one (GR7), 3-[[ (2,5-Dihydro-3-methyl-2-oxo-5-furanyl)oxy]methylene]-3,3a,4,8b-tetrahydroindeno[1,2-b]furan-2-one (GR24), Methyl 2-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-3-[4-



methyl-5-oxo-2,5-dihydrofuran-2(R)-yloxy]acrylate (Nijmwegen 1), demethylsorgolactone, or mixtures thereof. Specifically mixtures of various stereoisomers are preferred, because in these mixtures, especially at higher concentrations, differences in activity between such stereoisomers are hidden, because all of them are active (Welzel et al., Chem. Commun. 1999 (1999), 2017).

Due to stability aspects, GR24, GR7 or Nijmwegen 1 (or other strigolactones with comparable stability and  $c_{1/2}$  in the same order of magnitude (10-1000%, 20-500%, 50-200%)) are specifically preferred strigolactones according to the present invention. These preferred embodiments are also characterised by being producible in an economically feasible manner.

Of course, also other strigolactones are applicable which share the particular structure and configuration of the rings denoted B, C and D of the strigol molecule (see US 2008/0318773). Accordingly, any compound having a structure analogous to GR7, GR24 and other strigolactones explicitly mentioned herein, that is to say any compound having the structure of the chain of rings denoted B, C and D of strigolactones and an appropriate biological strigolactone activity can be used according to the present invention. Preferably, the strigolactones to be used according to the present invention have at least a biological activity of 10% of GR24, preferably a biological activity of 50 to 200% of GR24 (the biological activity being determined according to the method of Mangnus (1992)).

Preferred embodiments for administering strigolactones to plants according to the present invention are

- spraying a strigolactone containing solution or suspension onto the plants;
- bringing a strigolactone containing carrier into contact with the plants; and
- watering the plants with a strigolactone containing solution or suspension.

These methods may be applied exclusively or in simultaneous or subsequent combination with each other and be combined with other methods for plant breeding, growth and production suitable for the specific plant.

The strigolactones are applied in a concentration wherein

the compounds are effective in enhancing lateral growth. This can easily be adapted based on the activity tests known to the skilled person in the art and the biological activity compared to the biological activity already reported. Administration of strigolactones may be carried out by any suitable method for providing plant growth aiding substances to plants, such as applying the strigolactones as a strigolactone containing solution or suspension (e.g. by spraying or humidifying) or with assistance of a carrier material (e.g. paraffin, lanolin or similar substances or a sponge or sponge-like structure containing strigolactones which can be released to the plants). The strigolactones can be directly applied at the location on the plant where the effect should be obtained (e.g. at the stem, at the leaves, at the root or at the sprout) or can be applied as a growth solution or suspension via the roots. The treatment according to the present invention may be carried out once (with a higher concentration of strigolactones) or more than once (e.g. once in a day or once in a week over a longer period (e.g. over 2 to 10 weeks or 3 to 12 months); with a lower concentration). In many cases, the strigolactone administration according to the present invention is sufficient in an early stage of life of a plant (i.e. before the plant is grown in industrial (natural) soil on the field), because induction of an enhanced secondary growth is already initiated by an early treatment. For example, the plants may be cultured first in the laboratory incubator or greenhouse using strigolactone-containing nutrient bases (solutions, suspensions or soil) and then be planted on the field without further strigolactone administration (or only with a sponge material soaked with strigolactones on the stems). Strigolactone administration via carrier substances can be performed e.g. on a weekly or monthly basis by exchanging the previous (already drained) strigolactone containing carrier by a new one.

Typical administration concentrations of the strigolactones according to the present invention are - on the basis of GR24 and therefore on its specific biological activity - from 1 nM to 1 mM, preferably 10 nM to 100 µM, especially 100 nM to 10 µM, GR24 for nutrient solutions. If directly applied via a carrier, suitable concentrations of strigolactones may be - again based on a GR24 basis - 10 ng to 10 mg, preferably 100 ng

to 1 mg, especially 1 to 100 microg, strigolactone per mg of carrier. Concentration of other strigolactones may be calculated on the basis of these concentration ranges weighed with the specific germination activity of the other strigolactone to be applied in comparison with GR24. Strigolactone concentrates may be used for preparing these nutrient solutions e.g. by mixing the strigolactone concentrated with typical plant growth media. Strigolactone containing concentrates for stimulating lateral growth or accumulating biomass is therefore another aspect of the present invention. These may be concentrated by the factor 2 to 1000, preferably 10 to 100, compared to the planned concentration to be used at the plant (e.g. in a spray suspension or a nutrient solution).

According to another aspect, the present invention relates to an apparatus for spraying a liquid onto plants comprising a spraying device and a container for the liquid, characterised in that the liquid is a strigolactone containing liquid, especially a strigolactone solution or suspension. The strigolactones may be formulated for the present invention as liquid formulations or formulations intended to constitute liquid compositions when applied. Typically, strigolactone solutions are provided, in particular water-soluble concentrates, emulsifiable concentrates, emulsions, concentrated suspensions and wettable powders (or powders for spraying). The wettable powders (or powders for spraying) are usually prepared such that they contain 20% to 95% of active materials, and they usually contain, in addition to the solid support, from 0% to 30% of a wetting agent, from 3% to 20% of a dispersant and, when necessary, from 0.1% to 10% of one or more stabilizers and/or other additives, such as penetration agents, adhesives, anticaking agents, dyes, etc.. As spraying device, any device applicable in plant technology is suitable. In practice, the device will be chosen according to the specific needs and scales of spraying. The spraying devices can be as simple as manually operated vaporisers, of course, also more sophisticated devices can be applied, such as automatic sprays driven by external pressure generators (electric or hydraulic pumps or combustion engines, etc.).

According to another aspect, the present invention relates to a carrier material applicable on plants for releasing active

compounds to plants comprising a carrier whereto the active compound is adsorbed and an active compound, characterised in that the carrier is a strigolactone adsorbing material and that the active compound is a strigolactone. The carrier material may be a solid carrier, such as a sponge or textile fabric or a porous synthetic material wherein the strigolactones are adsorbed in the cavities of the sponge, fabric or material. Examples for other carrier materials are organic carriers, such as waxes, linolin, paraffin, dextrose granules, sucrose granules and maltose-dextrose granules. Examples for anorganic carriers are natural clays, kaolin, pyrophyllite, bentonite, alumina, montmorillonite, kieselguhr, chalk, diatomaceous earths, calcium phosphates, calcium and magnesium carbonates, sulphur, lime, flours or talc. The carrier material itself does not have any significant effect on the plant but enables the release of the active substance to the plant surface so that the active substance may be taken up by the plant.

According to another embodiment, the present invention relates to the use of a strigolactone containing culture medium for culturing plants or plant cells for promoting secondary growth, accumulation of biomass, enhancing shoot stability and/or increasing transport capacity in plants or plant cells. Although strigolactone containing culture media for culturing plants or plant cells as such are known from the prior art, especially for determining the ability of strigolactones to stimulate germination (e.g. Mangnus et al., 1992), the use of such media for the above mentioned purposes is a novel and surprising new application for such media.

The present invention is further described in the following examples and the drawing figures, yet without being limited thereto.

Fig.1 shows the principle of the in vitro system usable for the present invention. (A) Collection strategy of stem fragments for the in vitro system. 1.5 cm long fragments were taken from lowermost internodes which were at least 5 cm in length. The distance from the uppermost rosette leaf was 2.5 cm avoiding the region where the interfascicular cambium had been established at this stage (labelled in red). (B) Experimental setup of the in vitro-system. Stem fragments were surface sterilised and incu-

bated for 5 or 7 days on split-plates.

Fig.2 shows the phenotype of the max mutants. (A, B) max1-1 mutants (A) do not show secondary growth initiation in the stem as observed in wild type plants (B). Arrows in B: cambial zone in wild type which is missing in the section from max1 (A). (C) Quantitative analysis of the longitudinal extension of interfascicular cambium formation at the base of 30 cm tall stems, comparing wild type and max mutants. The uppermost rosette leaf is taken as zero. (D) Comparison of lateral extension of the cambium-derived tissue 3 mm above the rosette in interfascicular regions. The lateral extension of the cambium and the cambium-derived tissue together was measured in cross-sections as shown by the bracket in Fig. 3B.

Fig.3 shows an analysis of the effect of local GR24 treatment. (A, B) A comparison of max1-1 plants which were mock-treated (A) or treated locally with GR24 (B) shows that fascicular and interfascicular cambial activity can be induced by SL treatment (arrows). (C) Quantitative analysis of the effect in Arabidopsis wild-type and max1-1 stems. The lateral extension of the cambium and the cambium-derived tissue together was measured in cross-sections as shown by the bracket in Fig. 3B.

Fig.4 shows the effect of strigolactone treatment on isolated stem fragments. Isolated Arabidopsis stem fragments were incubated in the split-plate system on standard growth medium without (A) and in the presence of GR24 (B) as the only additional hormone. Arrows in B indicate the cells which originated from cambial activity in interfascicular regions and which are not present in untreated stems. Incubation time was 7 days.

Fig.5 shows the effect of GR24-spraying on cambium activity in Arabidopsis thaliana. A + B: Indication of cambium-derived tissue in interfascicular regions on cross sections from mock-treated (A) and GR24-treated (B) stems. Size bar in A: 0.1 mm. Same magnification in A + B. Sections taken from 4 mm above the uppermost rosette leaf (see C). C: Quantification of the cambium-derived tissue as shown in A and B at different position along the stem. Plants were sprayed with 0 (white circles) or 5  $\mu$ m (black triangles) of GR24. Shown are the results for max1 mutant plants.

Fig.6 shows the effect of GR24 treatment on cambium activ-

ity in *Solanum lycopersicum*. A + B: Indication of cambium-derived tissue on cross sections from mock-treated (A) and GR24-treated (10  $\mu$ m, B) stems. Size bar in A: 0.4 mm. Same magnification in A + B. C: Quantification of the effect of different GR24 concentrations on the production of cambium-derived tissue as shown in A and B. Seedlings were treated with 0, 5 or 10  $\mu$ m of GR24.

Fig.7 shows the effect of GR24 treatment on cambium activity in *Brassica napus*. A + B: Indication of cambium-derived tissue on cross sections from mock-treated (A) and GR24-treated (10  $\mu$ m, B) hypocotyls. Size bar in A: 0.1 mm. Same magnification in A + B. C: Quantification of the effect of different GR24 concentrations on the production of cambium-derived tissue as shown in A and B. Seedlings were treated with 0, 5 or 10  $\mu$ m of GR24.

Fig.8 shows the effect of GR24 treatment on cambium activity in *Eucalyptus globulus*. A + B: Indication of cambium-derived tissue on cross sections from mock-treated (A) and GR24-treated (5  $\mu$ m, B) stems. Size bar in A: 0.4 mm. Same magnification in A + B. C: Quantification of the effect of GR24 treatment on the production of cambium-derived tissue as shown in A and B.

#### Examples:

Strigolactones in *Arabidopsis thaliana* are synthesised and perceived by a pathway which was described to consist of four genes, MAX1, MAX2, MAX3 and MAX4.

In wild-type plants, secondary growth is observed in the elongating shoot, almost exclusively at the very base (in a region of ~0.5 cm above the rosette; Fig. 2B). In contrast, all four max mutants (max1 - max4) are nearly devoid of secondary growth (Fig. 2A, 3C). Local applications of strigolactones mixed with lanolin directly on stems induces secondary growth in wild-type and max1 mutants even when it is applied when side shoots have grown out already (Fig. 3B, GR24, 5  $\mu$ m, 16 days treatment, results shown for max1). This shows that strigolactones can be used as agents to positively influence secondary growth and that side shoot development and secondary growth can be separated experimentally.

### Methodological approaches

In an attempt to specifically address the role of different factors in the initiation of secondary growth, especially to analyse the effect of strigolactones on isolated stem fragments, an inducible in vitro system was established. Adapting an experimental setup described before (Chatfield et al., Plant J. 24(2000), 159), internodal stem fragments were incubated in a split-plate system (Fig. 1). By applying auxin exclusively apically, it was possible to induce fascicular and interfascicular secondary growth in these fragments. On plates without auxin or on plates with auxin but in addition with the auxin transport inhibitor NPA, no secondary growth is observed. Interestingly, on plates where auxin is only applied in the basal half, no secondary growth was induced, suggesting that stem polarity is maintained and that the situation in intact plants is recapitulated by the present system. In summary, these analyses show that a system was developed that allows the induction of secondary growth in Arabidopsis stems in a highly specific and controlled manner. Incubation of stem fragments with the addition of GR24 (1  $\mu$ m) as the only added hormone for 7 days induced secondary growth (Fig. 2D) and shows that also strigolactone application via growth media has a positive effect on secondary growth.

### Materials and Methods

*Arabidopsis thaliana* (L.) Heynh. plants of the accession Columbia were used for all experiments. wild type and max1-1, max2-1, max3-9, and max4-1 mutants (Booker et al., Dev. Cell 8 (2005), 443-449); Stirnberg et al., Plant J. 50 (2007), 80-94; Booker et al., Curr. Biol. 14 (2004), 1232-1238; Sorefan et al., Genes Dev. 17 (2003), 1469-1474) were used for all experiments. The background Wester was used for *Brassica napus* and the background Money Maker for *Solanum lycopersicum*. All SL treatments were performed by applying GR24. Plants were grown under standard greenhouse/growth chamber conditions in standard potting soil at 21 °C in long day conditions (16 h light, 8 h dark). Arabidopsis plants were grown first for 3 weeks in short day conditions (8 h light, 16 h dark) and then shifted to long day conditions. For histological analyses, stem fragments were fixed

in EtOH:acetic acid (3:1) and embedded in paraffin. Subsequently, 10 µm sections were made using a microtome, stained with toluidine blue (AppliChem) and fixed with Entellan (Merck) on microscope slides. For quantitative analyses, at least 5 plants were evaluated for each data point. The standard error of means was used to visualise variation.

For local GR24 treatments, stems or hypocotyls were treated in a region of 0.5 cm three times a week using a paint brush for a period of three weeks with 0, 5 or 10 µm of GR24 dissolved in water supplemented with 0.5% acetone and 0.1% surfactant. For *Solanum lycopersicum*, treatments were started when plants were 15 cm tall. For *Arabidopsis thaliana*, treatments were started when plants were 10 cm tall. Hypocotyls of *Brassica napus* were treated when plants were 10 cm tall. For *Eucalyptus globulus*, treatments were started when plants were 12 cm tall.

For spraying experiments, plants were sprayed with a standard vaporiser for three weeks starting 3 weeks after germination 3 times a week with 0 or 5 µm of GR24 dissolved in water supplemented with 0.5% acetone and 0.1% surfactant.

## Results and discussion

In wild type *Arabidopsis thaliana* plants, secondary growth is observed in the elongating shoot, almost exclusively at the very base (in a region of ~0.5 cm above the rosette; Fig. 2B, C). In contrast, all mutants affected in SL biosynthesis (max1, max3, max4) or perception (max2) show a strong reduction in secondary growth (Fig. 2A, C, D). This observation indicates that the SL signalling pathway plays a role in the promotion of cambium initiation and activity.

To prove this conclusion, local applications of SLs directly on stems were performed. Histological analyses showed that the treatment induces secondary growth in wild type and max1 mutants even when it is applied when side shoots have grown out already (Fig. 3). This effect demonstrates that SLs promote cambium activity directly and that they can be used as agents to positively influence secondary growth. Furthermore, it argues that side shoot development and secondary growth can be separated experimentally and, thus, that both processes represent



independent responses of the SL signalling pathway.

To confirm that cambium activity represents a distinct and direct SL response, an in vitro-system (see Material and Methods) was employed to incubate isolated stem fragments with and without GR24. Histological analyses showed that incubation of stem fragments with GR24 as the only added hormone induced the production of cambium-derived tissues (Fig. 4). This result demonstrates that SLs act directly on stems and, additionally, that also strigolactone application via growth media has a positive effect on secondary growth.

In order to see whether also SL treatments of whole plants had a positive effect on cambium activity, a GR24 solution was sprayed over an extended period on growing *Arabidopsis* plants. As a result, a clear increase in cambium-related cell divisions was observed (Fig. 5), demonstrating that SLs do not have to be applied locally to obtain a positive effect on cambium activity.

To further proof that SLs function as positive regulators of secondary growth in a broader range of species, *Solanum lycopersicum*, *Brassica napus* and *Eucalyptus globulus* stems or hypocotyls were treated locally with GR24. Histological analyses showed that also in these distantly related crop species a positive effect of cambium activity was obtained (Fig. 6, Fig. 7, Fig. 8). Different concentration of GR24 (5 and 10  $\mu\text{M}$ ) were also tested and a more pronounced effect was obtained with higher concentrations demonstrating that the effect is dose-dependent (Fig. 6, Fig. 7).

In summary, a positive role of the SL signalling pathway in secondary growth regulation was shown. This conclusion is based on the observation that mutants for SL biosynthesis and perception display a reduced cambium activity and that SL treatments enhance the production of cambium-derived cells. Importantly, this effect does not depend on other SL-responses like apical dominance and biotic interactions of roots because it is also observed in stems fragments taken out of the whole-plant context. Therefore, a direct influence of SLs on cambium activity is likely. Furthermore, the effect does not depend on the way of SL application as three different methods (local application, media-based application and spraying) had the same effect indicating that SLs are taken up by various tissues and, possibly,

distributed within the plant. Similar as other SL responses, it was shown that SLs play a role in secondary growth regulation in distantly related species, among them one tree species, suggesting that manipulation of SL levels can be used to modulate cambium formation and the production of secondary vascular tissue in a wide range of species.

## Claims:

1.: Use of strigolactones for promoting secondary growth of plants.

2.: Use of strigolactones for accumulation of biomass in industrial plant production.

3.: Use of strigolactones for enhancing shoot stability in plants.

4.: Use of strigolactones for increasing transport capacity in plants.

5.: Use according to any one of claims 1 to 4, characterised in that the strigolactones are strigol, strigyl acetate, orobanchol, orobanchyl acetate, 5-deoxystrigol, sorgolactone, alec-trol, 3-[[ (2,5-Dihydro-3-methyl-2-oxo-5-furanyl)oxy]methylene]-3,3a,6,6a-tetrahydrocyclopenta[b]furan-2-one (GR7), 3-[[ (2,5-Dihydro-3-methyl-2-oxo-5-furanyl)oxy]methylene]-3,3a,4,8b-tetrahydroindeno[1,2-b]furan-2-one (GR24), Methyl 2-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-3-[4-methyl-5-oxo-2,5-dihydrofuran-2(R)-yloxy]acrylate (Nijmwegen 1), demethylsorgolactone, or mix-tures thereof.

6.: Use according to any one of claims 1 to 5 characterised in that the plants are trees, especially oak, chestnut, black lo-cust, mulberry, osage-orange, sassafras, maple, ash, hickory, hackberry, beech, pine, buckeye, poplar, willow, catalpa, elm, walnut, mahogany, balsa, black ironwood, birch, eucalyptus, fir or alder.

7.: Use according to any one of claims 1 to 6 characterised in that the plants are flax (*Linum usitatissimum*), jute (*Corchorus capsularis* and *Corchorus olitorius*), hemp (*Canabis sativa*) or ramie (*Boehmeria nivea*).

8.: Use according to any one of claims 1 to 7 characterised in that the plants are kohlrabi, potato, carrot, sugar beet or gar-

den beet.

9.: Use according to any one of claims 1 to 8 characterised in that the plants are wheat, barley, Miscanthus, switchgrass, maize, sugarcane, rape seed, tomato, peas, grape or ornamental flowers.

10.: Use according to any one of claims 1 to 9 characterised in that the strigolactones are administered to plants by spraying a strigolactone containing solution or suspension onto the plants.

11.: Use according to any one of claims 1 to 10 characterised in that the strigolactones are administered to plants by bringing a strigolactone containing carrier into contact with the plants.

12.: Use according to any one of claims 1 to 11 characterised in that the strigolactones are administered to plants by watering the plants with a strigolactone containing solution or suspension.

13.: Apparatus for spraying a liquid onto plants comprising a spraying device and a container for the liquid, characterised in that the liquid is a strigolactone containing liquid, especially a strigolactone containing solution or suspension.

14.: Carrier material applicable on plants for releasing active compounds to plants comprising a carrier where to the active compound is adsorbed and an active compound, characterised in that the carrier is a strigolactone adsorbing material and that the active compound is a strigolactone.

15.: Use of a strigolactone containing culture medium for culturing plants or plant cells for promoting secondary growth, enhancing shoot stability and/or increasing transport capacity in the plants or plant cells.

1/8

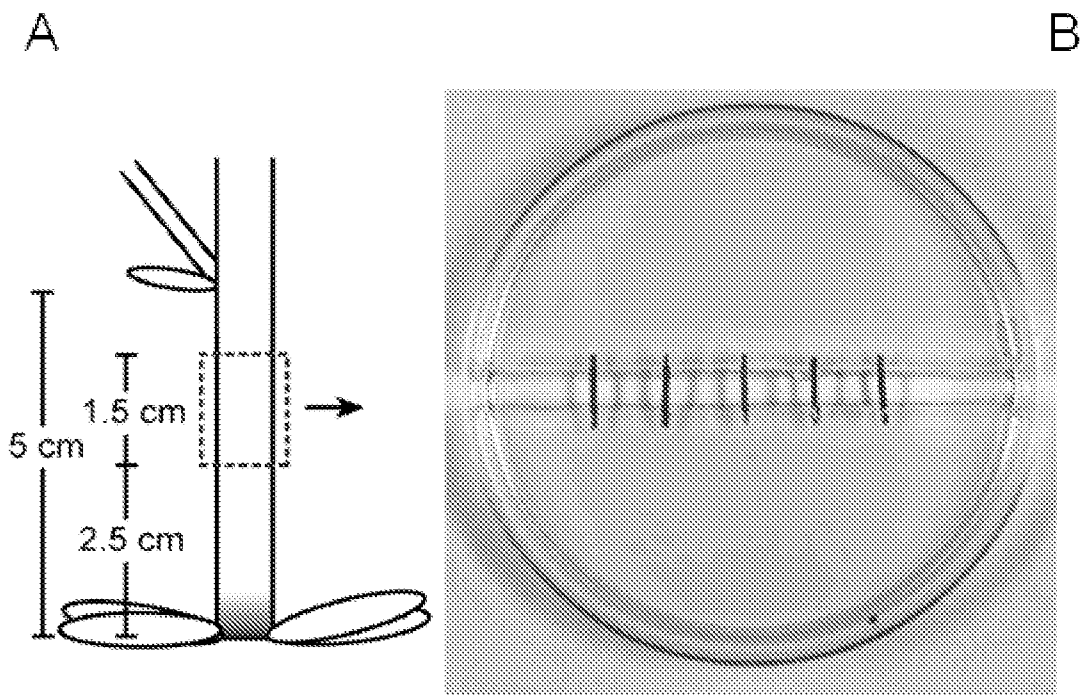


Fig.1

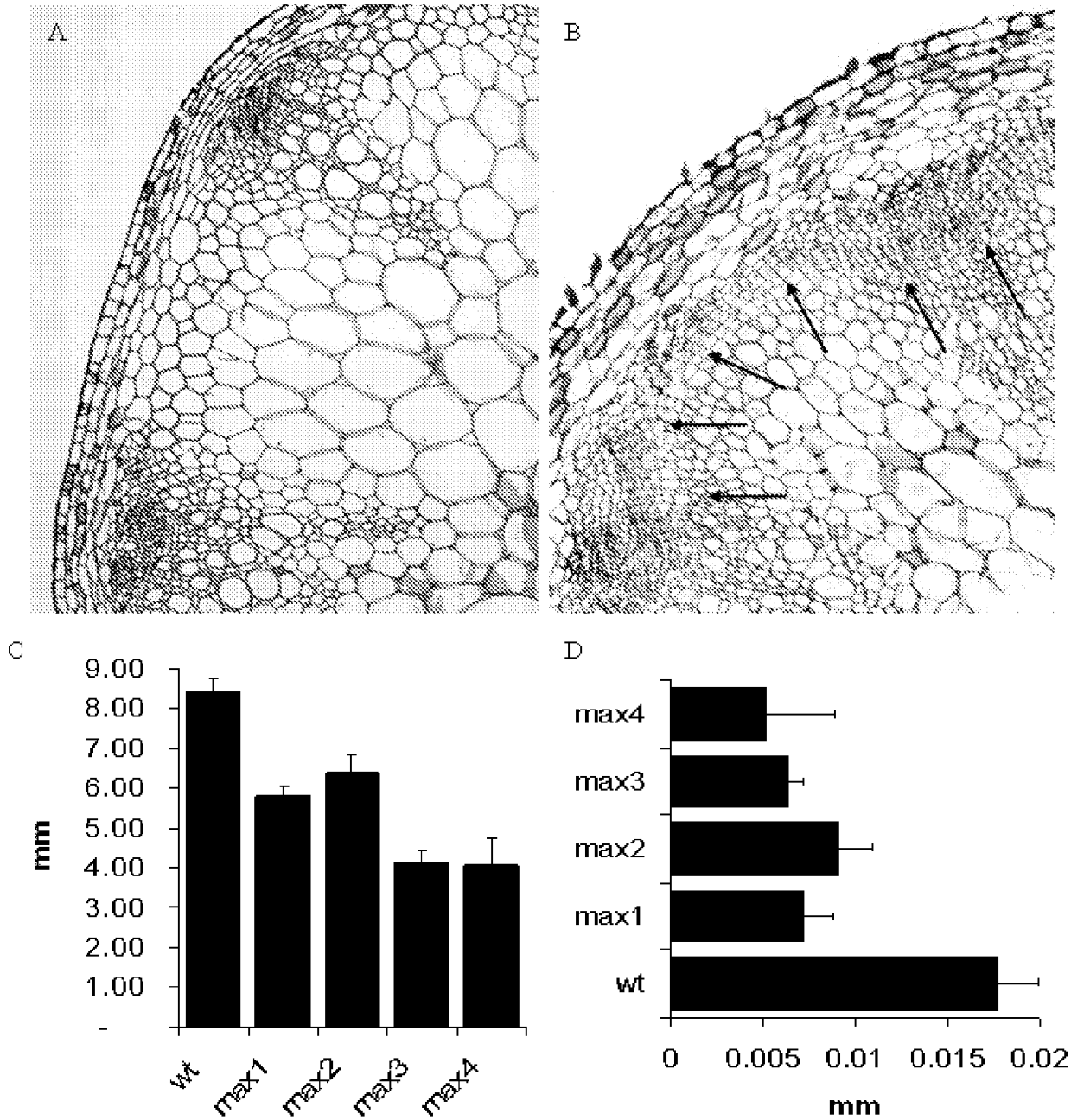


Fig.2

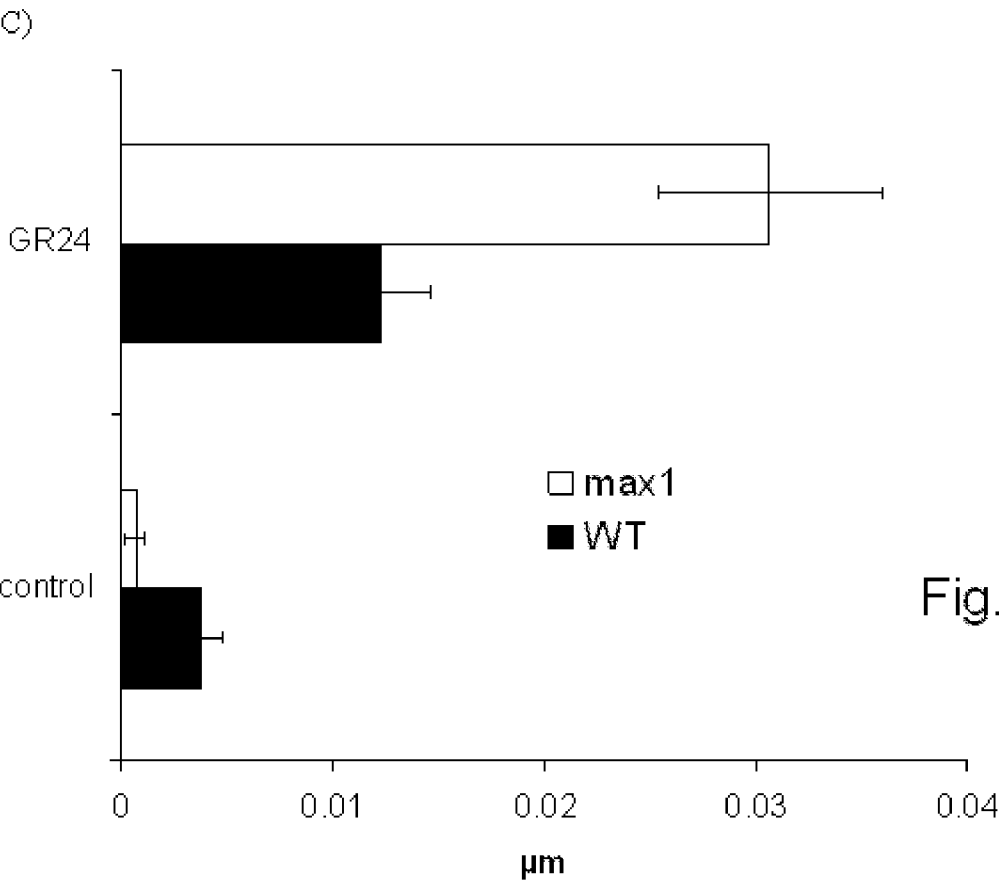
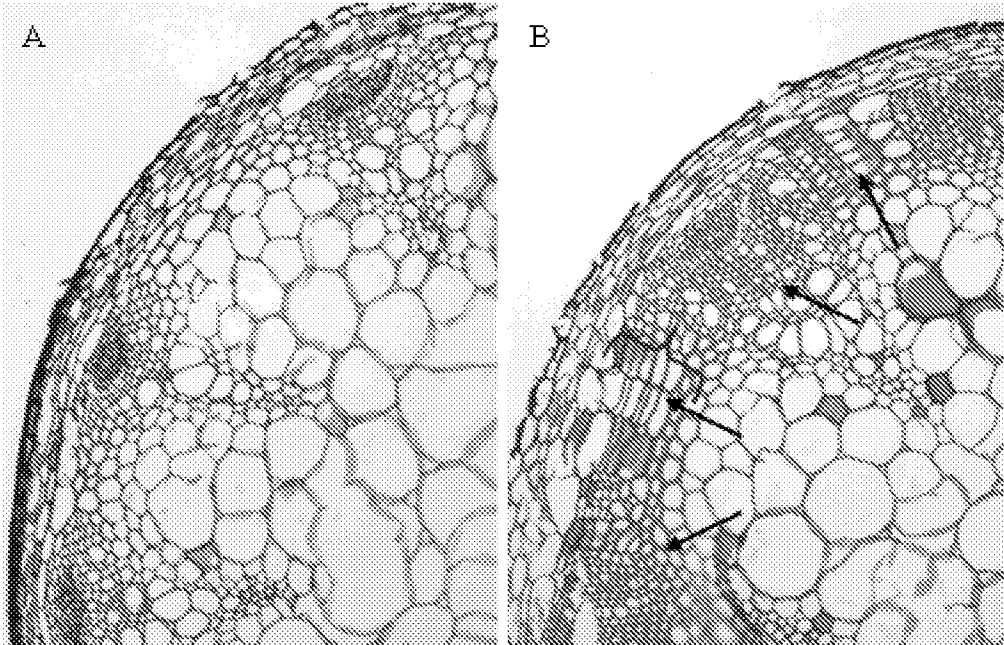


Fig.3

4/8

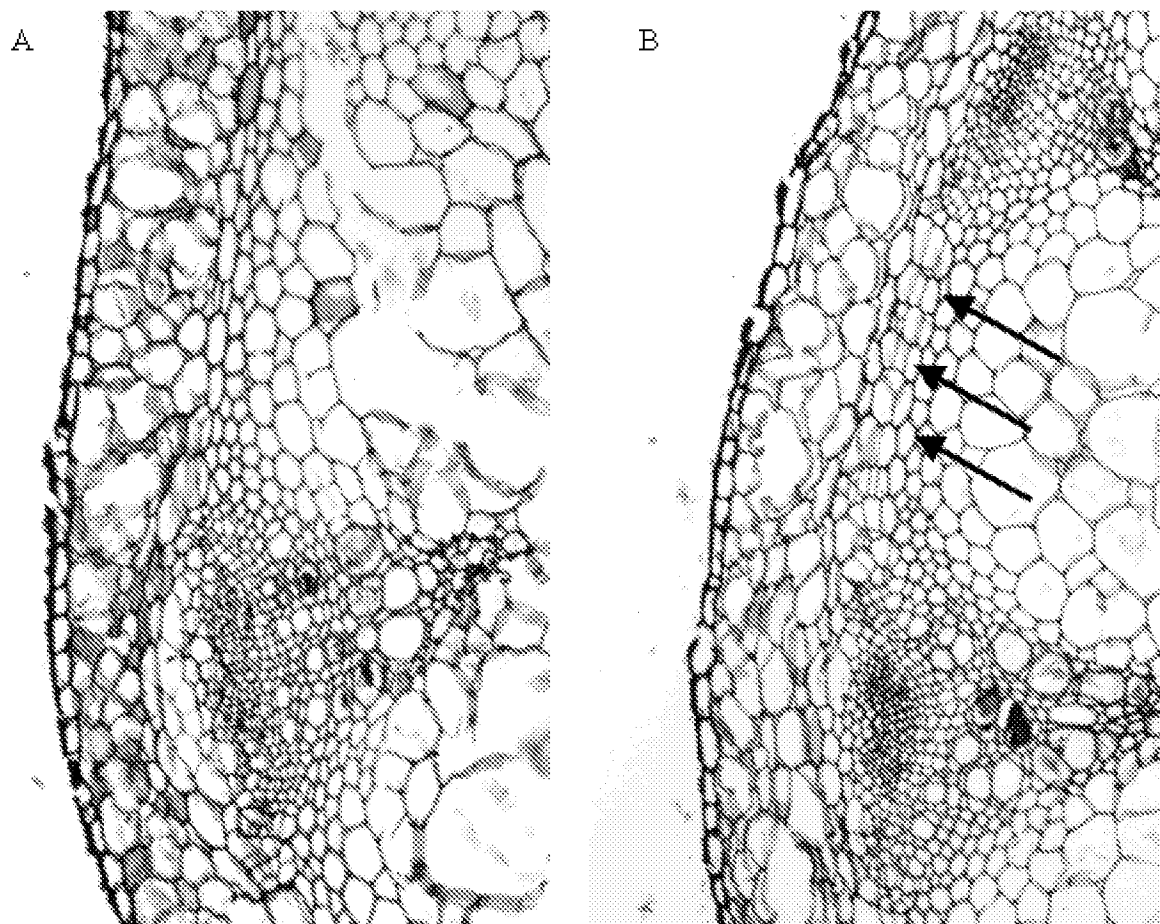
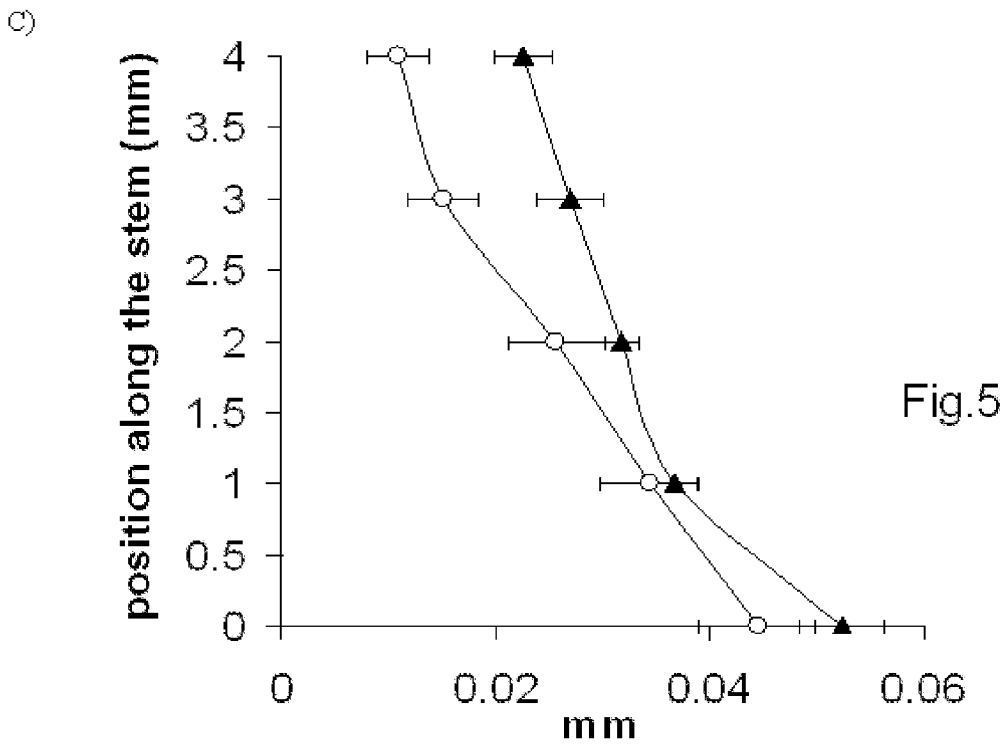
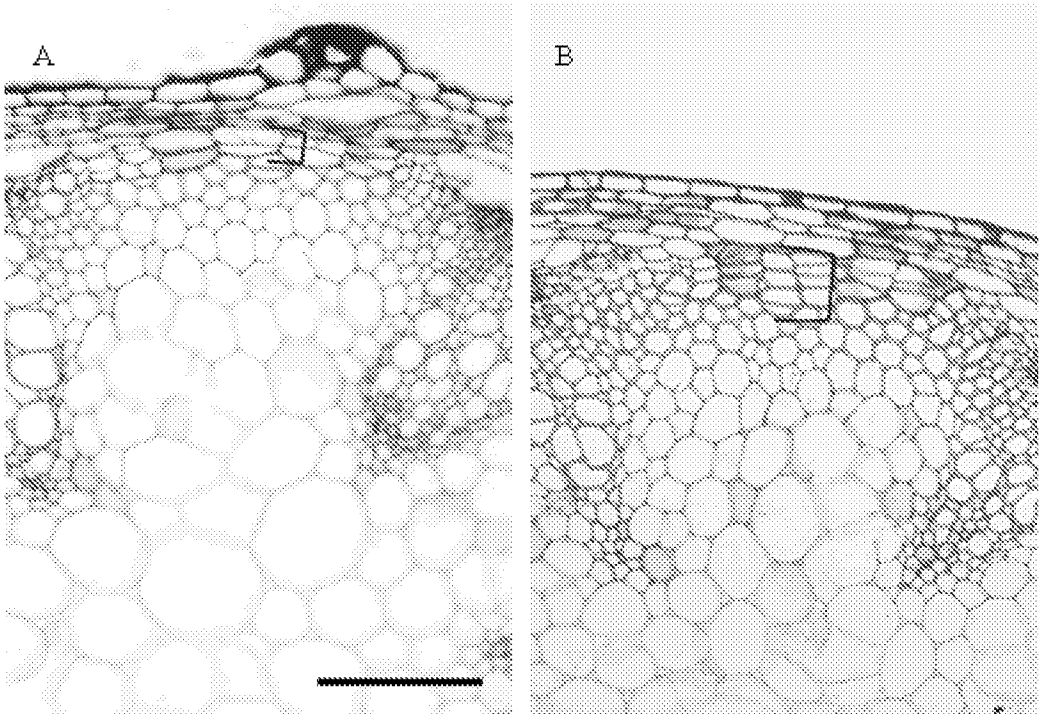
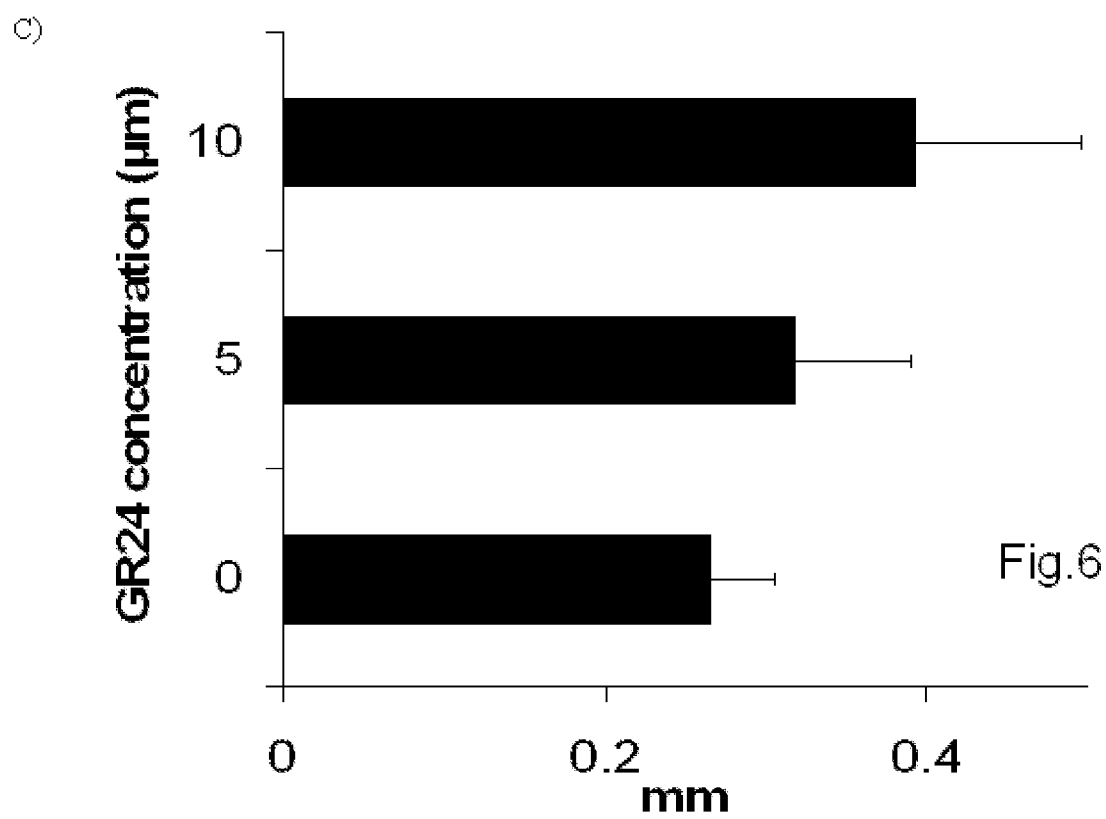
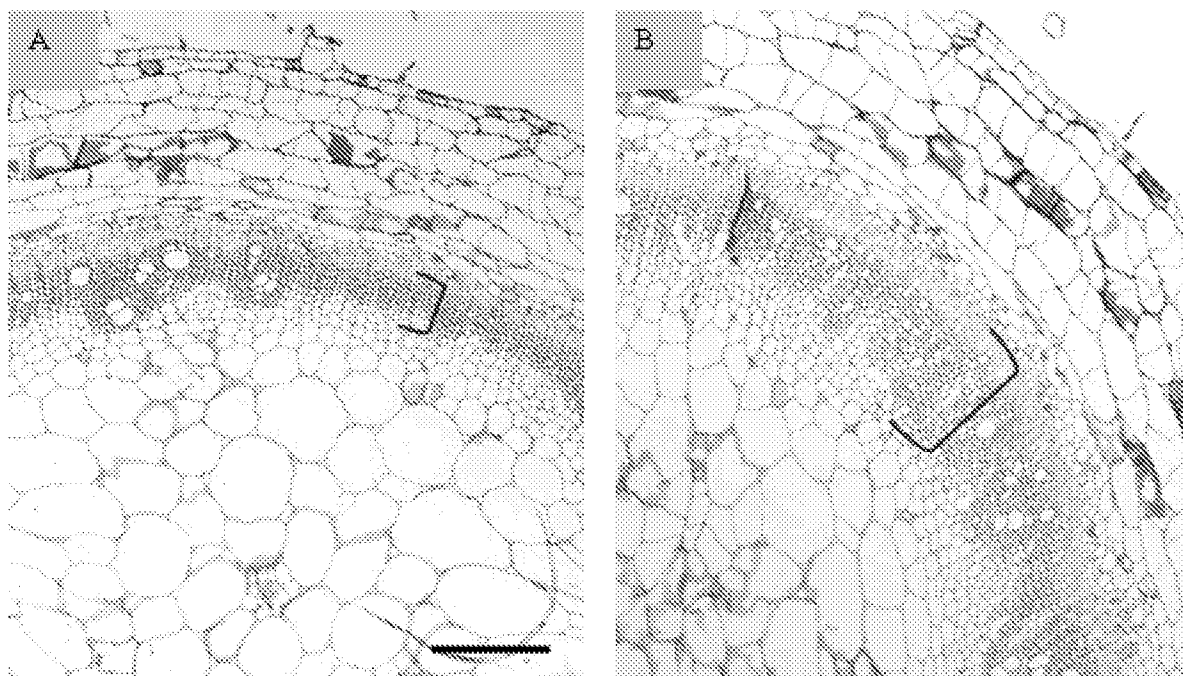


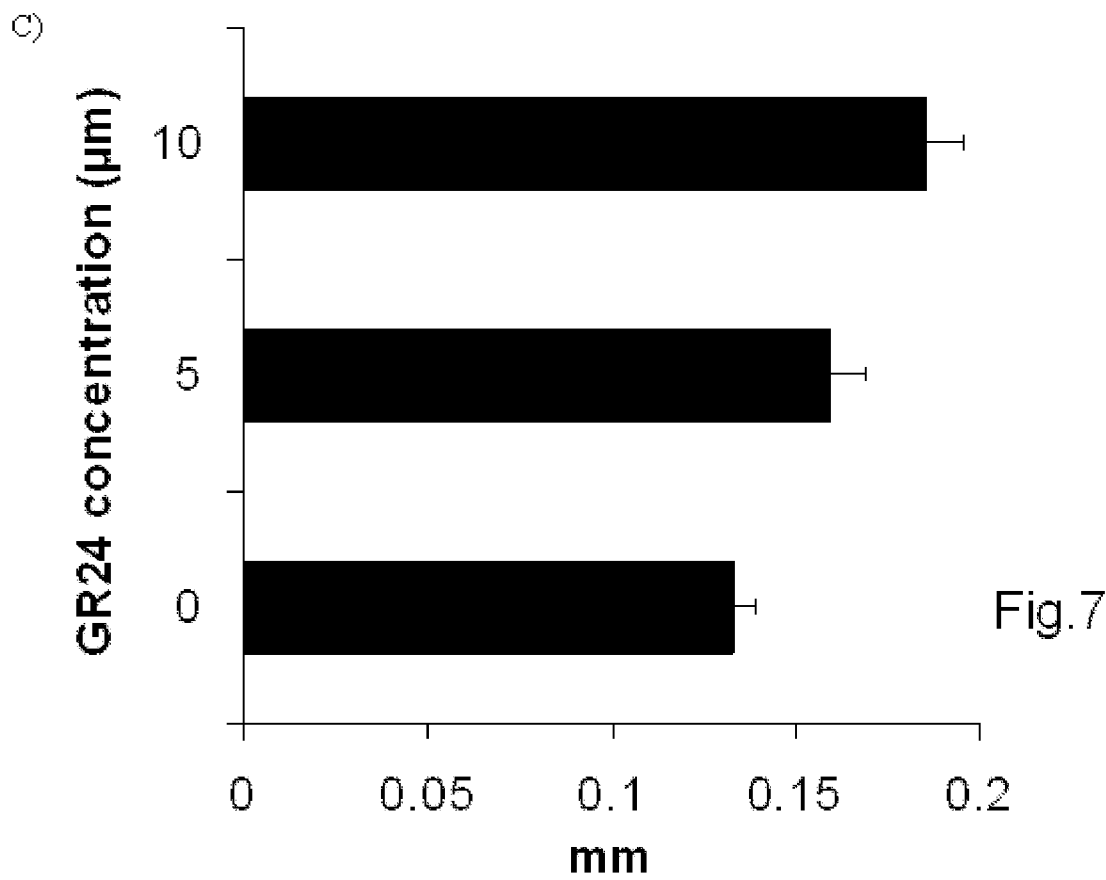
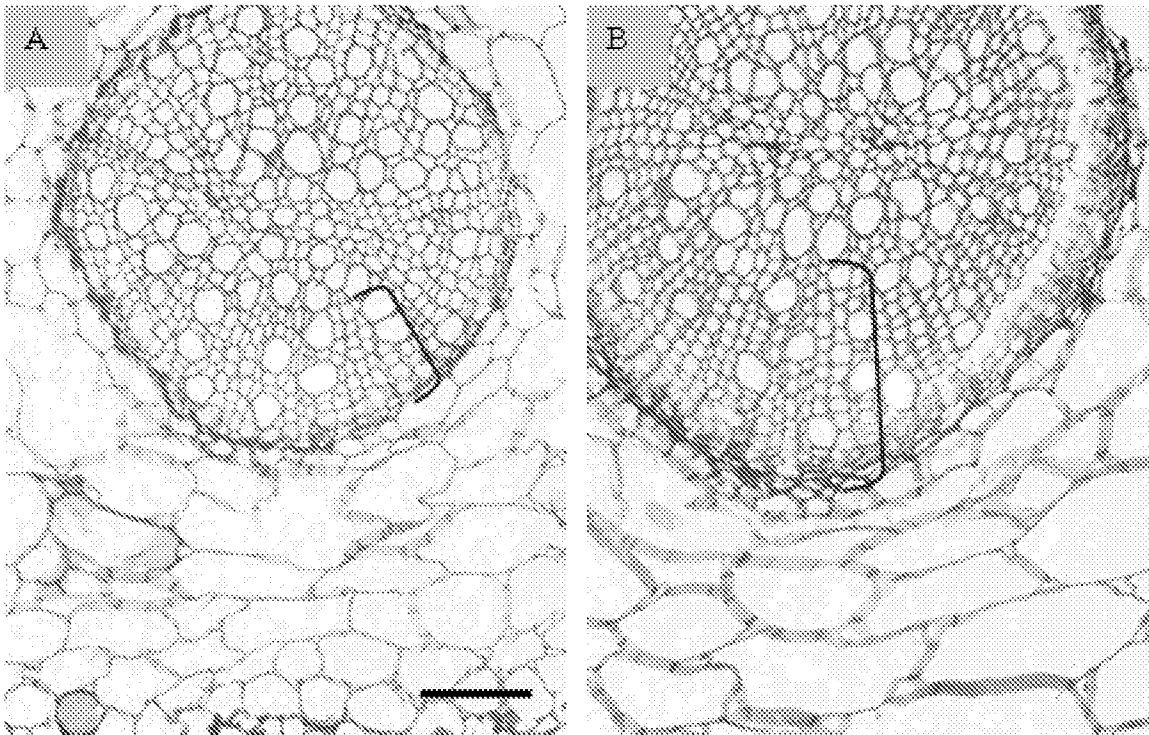
Fig.4





6/8





8/8

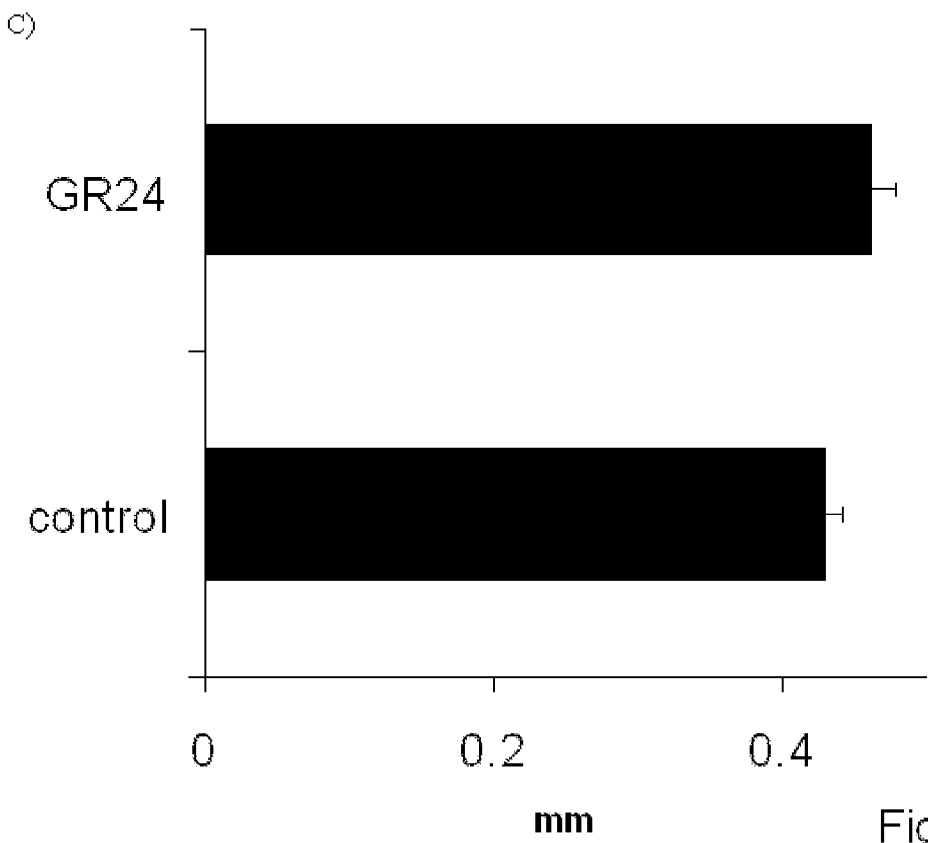
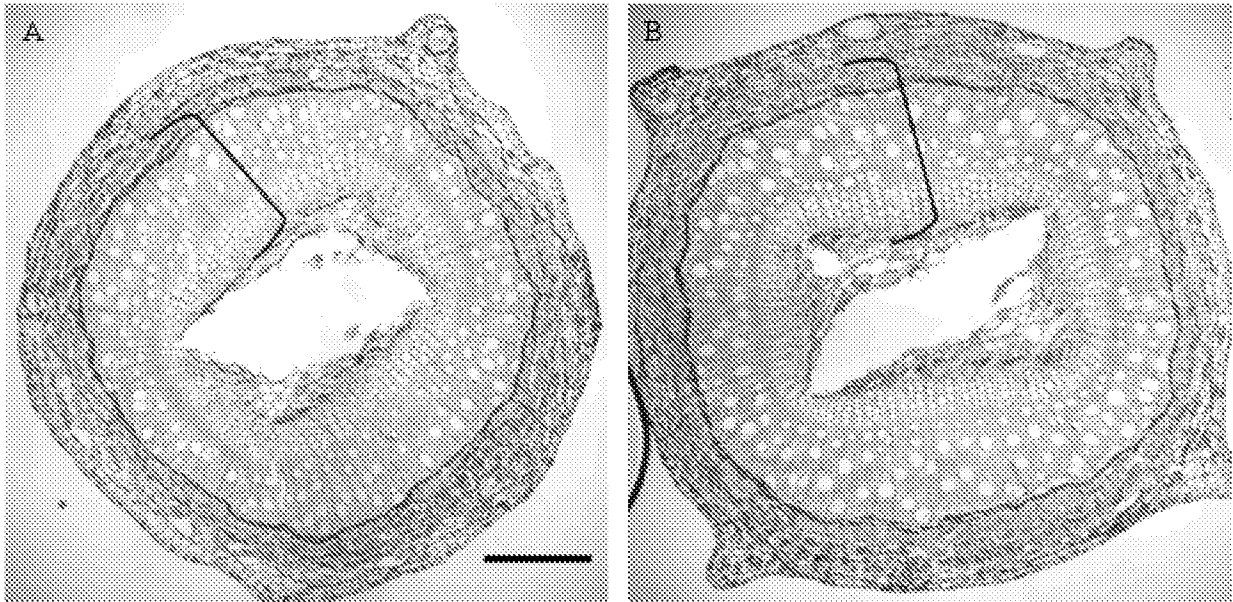


Fig.8