The role of strigolactones in stromule formation

Name:

Gilles Vismans

Student#:

900224890110

Supervisors:

Dr. Ir. Sander van der Krol

Laboratory of plant physiology, Wageningen university and research centre

Dr. Ir. Tijs Ketelaar

LAboratory of cell biology, Wageningen university and research centre

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Abstract

Stromules are highly dynamic protrusions of the plastids in plants. Several factors, such as drought and light conditions influence the stromule frequency in a positive or negative way. No evidence has been found for their exact function. It is thought that the increase of plastid surface helps with the exchange of proteins and metabolites from the plastid to the cytosol or other organelles. A relatively recent discovered class of plant hormones are the strigolactones, first found for their role in the germination of parasitic plants. More recently beneficial and regulatory roles for strigolactones were found. Strigolactones inhibit branching of the shoots and promotes beneficial interactions between roots and arbuscular mycorrizal fungi. Here we propose research on the link between the formation of stromules and strigolactones. This research shows on a strong link between strigolactones and the formation of stromules. This is shown by using several strigolactone mutants (*max2 max3*) and inhibitors (D2) and promotors (GR24) of strigolactones.

Contents

Introduction

Why look at stromules in strigolactone mutants?

Strigolactones are a class of phytohormones, which only recently have been recognized to function both in plant development and as signal molecules from plants to arbuscular mycorhizal fungi (AMF) (Ruyter-Spira, Al-Babili et al. 2013)). The induction of strigolactone biosynthesis and secretion by roots of plants under low phosphate conditions is thought to stimulate the interaction with AMF as strigolactones induce branching in AMF (Toh, Kamiya et al. 2012). The higher branched structure of the AMF may aid the plant in the uptake of scarcely available soil phosphate. Phosphate is required for the production of phospholipids, energy production, regulation of protein activity and much of the plant's phosphate is stored in the phospholipids of the plasmamembrane (PM). When phosphate levels really becomes limiting for the plant cell, phosphor may be mobilised from the phospholipids of the PM, in which case the lipids of the plasma membrane are replaced by galactolipids from the plastids (Tjellström, Andersson et al. 2008, Lambers, Cawthray et al. 2012). This exchange of lipids between the plastid and PM occurs via the ER through plastid ER membrane contact sites (Dörmann and Benning 2002, Tjellström, Andersson et al. 2008) and indeed this frequency of these contact sites is increased under low Pi conditions. Stromules are highly dynamic double membrane tubular extensions filled with stroma, which protrude from the plastid. Thus, stromules give a large increase in the potential contact between plastid and ER and indeed stromules seem to be intercalated between ER membrane structures (Schattat, Barton et al. 2011, Schattat, Barton et al. 2011). Although it has been suggested that stromules may be functional in the exchange of galactolipids from the plastid to the PM, this has never been investigated.

Stromule induction by plant hormones

There are several conditions influencing stromule formation. Stress, especially drought or high salt conditions, can strongly induce the formation of stromules. Responses to drought and salt are at least partly regulated by ABA. ABA precursors can stimulate the formation of stromules in wheat, while ABA inhibitors (abamine, NCED) abolish this effect (Gray, Hansen et al. 2012). The ABA and strigolactone biosynthesis pathways have some shared precursors, so the previously mentioned compounds may also affect the strigolactone biosynthesis. However, some conflicting results exist. Some ABA responsive RNA-binding proteins are involved in stromule formation, but no reliable effects of ABA on stromule density were observed (Raab, Toth et al. 2006). Other plant hormones influencing the stromule frequency are jasmonic acid and ethylene. The ethylene inhibitor silver nitrate is able to abolish stromule formation, while methyl-jasmonate increased the stromule frequency (Gray, Hansen et al. 2012). Another factor influencing stromule formation is temperature. High temperatures can induce the formation of stromules (Buchner, Holzinger et al. 2007) (Holzinger, Buchner et al. 2007). Since chloroplasts are the sites of

photosynthesis, it is likely stromules are influenced by sugars and light conditions. Indeed Schattat et al showed that extracellular sugars induce the formation of stromules in *A. thaliana* (Schattat and Klösgen 2011). There are multiple accounts of an increased stromule frequency in dark grown seedlings (Gray, Hansen et al. 2012, Schattat, Griffiths et al. 2012). In relative recent research, the effects of a DNA virus infection on stromule formation has been studied. The results show an increase in stromule frequency after infection with the virus (Krenz, Jeske et al. 2012).

Stromule visualisation

Here we used tobacco and Arabidopsis plants expressing respectively 35S35SAMV-CT(RecA)S65TmGFP4-nos or 35S::FNRtp::EGFP which allowed GFP labelling of plastid stroma and the stroma filled stromules to image stromule activity under different conditions. When such reporterplants were exposed to low Pi conditions, results show indeed an increase in stromule frequency of plastids in the hypocotyl epidermal cells of *A. thaliana*. PFD infiltrated leave discs of *N*. *Tabacum* grown on low nutrient medium show similar results. Because low phosphate conditions also stimulate the production of strigolactones, and because SL biosynthesis starts in the plastids, this raised the intriguing question whether strigolactone production are actually causal to stromule formation. Here we show that the high strigolactone levels in the SL signalling mutant max2 relate to increased stromule formation, while low SL levels in the max3 biosynthesis mutant relate to reduced stromule formation. Also, the synthetic strigolactone GR24 stimulated stromule formation one hour after addition in WT, *max2* and *max3* plants. Similar correlation between SL levels and stromules were found in tobacco using inhibitors of SL biosynthesis and exogenous supplied GR24.

Methods

Plant growth conditions

For all experiments involving stromule visualization, *Arabidopsis thaliana* (Col0, Max2-1, Max3-9), transformed with pGreenII0129::35S::FNRtp::EGFP were used. The eGFP is located to the plastid stroma and stromules. Seeds were grown on 0,5 MS medium containing 0.8% agar (Duchefa bio) and stratified at 4°C in the dark for at least 48 hours. After stratification plates were transferred to a climate chamber, 24°C 16 hours light for 72 hours. For the Phosphate starvation experiment, the samples were grown on 0,5MS with or without phosphate and stratified at 4°C in the dark for 48 hours. After stratification, the plates were transferred to a climate chamber, 24°C 16 hours light for 7 days.

Sample preparations

GR24 experiment and concentration series

For experiments using GR24 seedlings were grown as described previously. One hour before imaging the seedlings were submerged in 100µL perfluordecalin (PFD, Sigma Aldrich) containing the concentration (0µM, 10µM, 25µM) of GR24 diluted from a stock solution in acetone. As indicated images were taken between 1 and 2 hours after submerging in PFD. The control contained 10µL/mL acetone. For imaging the seedlings were mounted in PFD supplemented with the corresponding concentration of GR24

D2 experiments

Seedlings for the D2 (a specific strigolactone biosynthesis inhibitor) experiments were grown as described previously. Approximately 36 hours prior to imaging the seedlings were transferred from 0,5MS medium to 0,5MS medium containing 0µM, 1µM, 5µM or 10µM D2. The control contained 0.5µL/ml acetone. Samples were mounted in MQ water.

Sugar treatment

For the sugar treatment seedlings were grown under previous mentioned condition. Two hours prior to imaging the seedling were vacuum infiltrated with 40mM sucrose or demi water using a 5ml syringe. Vacuum was applied for less than 3 seconds. After infiltration seedlings were placed back on 0.5MS under normal growth conditions until imaging structures (Schattat, Barton et al. 2011, Schattat, Barton et al. 2011).

Phosphate experiment

For the phosphate experiment, seedling were grown on 0.5MS + 0.8% agar with or without phosphate for 7 days at 24°C 16 hours light , after being stratified for 48H at 4°C.

Nicotiana tabacum experiments

For all experiments Nicotiana tabacum transformed with 35S35SAMV-CT(RecA)S65TmGFP4-nos were used, targeting GFP to the plastids' stroma. (Köhler et al. 1997) The Nicotiana tabacum seedlings of which the leaves were

used for the GR24 infiltration experiments were grown on potting compost in the climate chamber (24 oC, 16L/8D). Nicotiana seeds were sterilized before planting by washing them in 70% ethanol for 1.5 min. followed by 10 min. wash in 4% bleach + 0.2% (v/v) Tween-20. Finally the seeds were washed 3 to 4 times in MQ water. Sterilized seeds were stratified for 24h (4 oC, dark) prior to germination in the climate chamber (24 oC, 16L/8D). Seedlings used for the fluridone, D2 and nutrient deficiency experiments were germinated on medium containing 2.3 g/l MS salts + vitamins (Duchefa, Haarlem, NL) and 10g/l agar for two weeks. 36h prior to imaging, seedlings were transferred to agar media supplemented with the appropriate chemicals. Control seedlings were mock treated by transferring them to fresh MS medium. The fluridone media contained 2.3 g/l MS salts with vitamins, 10 g/l agar and the appropriate concentration of fluridone (Sigma-aldrich, St. Louis, MO, USA). 100 mM fluridone and D2 stocks were prepared in acetone. Seedlings used for the D2 experiments were also grown on regular MS media for two weeks before transferring them to D2 containing media. Nutrient poor conditions were created by growing seedlings on 10 g/l agar in water.

Nicotiana tabacum GR24 experiments

To determine the optimal GR24 concentration, leaf discs were incubated in 100 µl PFD supplemented with GR24 1.5h prior to imaging. Three different concentrations of GR24 (prepared using 3.3 mM stock solution in acetone) were used, 0.3 µM, 3.3 µM and 33 µM. Samples used for control measurements were incubated in PFD supplemented with 10 µl/ml acetone. The leaf discs were mounted on a microscope slide in PFD supplemented with the corresponding concentration of GR24.

Nicotiana tabacum Fluridone/D2 experiments

Nicotiana seedlings were grown on MS agar medium. 36 hours prior to imaging the seedlings were transferred to fresh MS media either supplemented with the correct concentration of fluridone (1 µM, 5µM or 100 µM) or with no additional supplements (control treatment). During imaging the roots were mounted in PFD.

The same set-up was used to test the effect of D2, a specific SL biosynthesis inhibitor. The D2 stock (27.6 mg/ml in acetone), kindly provided by Yangxia Xhang, was diluted to a final concentration of 1 µM. Again, seedlings grown on MS media for two weeks were transferred to fresh MS media supplemented with D2 (1 µM and 5 µM) 36h prior to imaging. Other seedlings were transferred to fresh MS media with 0.5 µl/ml acetone to serve as a control.

Microscopy and image processing

Three or seven day old *A. thaliana* seedlings were imaged on a Nikon Eclipse Ti microscope, equipped with CSU-X1 spinning disk module (Yokogawa). A Plan apo 60X oil immersion objective (NA 1,4) with a 491nm laser were used. Z-stacks of 10 slices with 0.5µm intervals were made from the hypocotyl epidermis. The microscope is controlled by Metamorph software. The resulting images are converted to max intensity z-stacks. Short time series were made of

approximately 10 seconds. Images were taken by an Evolve EM-CCD camera (Photometrics). All images were processed by Fiji (ImageJ). Plastids were counted after applying a threshold using the analyse particles function in ImageJ. Stromules and stromule length were determined manually in ImageJ.

Results

Different stromule frequency in different cell types

Since stromules are highly dynamic structures it is important to make sure that growth and imaging conditions are constant throughout an experiment. To check which celltype and growth stage were suitable to observe stromule frequencies, several different cell types were studied. Different stromule frequencies can be found in different cell types in three day old seedlings. Also the homogeneity of the stromule frequency varies between cell types. As seen in figure 1, the stromule frequency is highest in root epidermal cells with a mean stromule frequency of 0.24±0.05 (N=12). Both hypocotyl epidermal cells and guard cells have lower stromule frequencies, with 0.04±0.006(N=15) and 0.13±0.04(N=14) respectively.

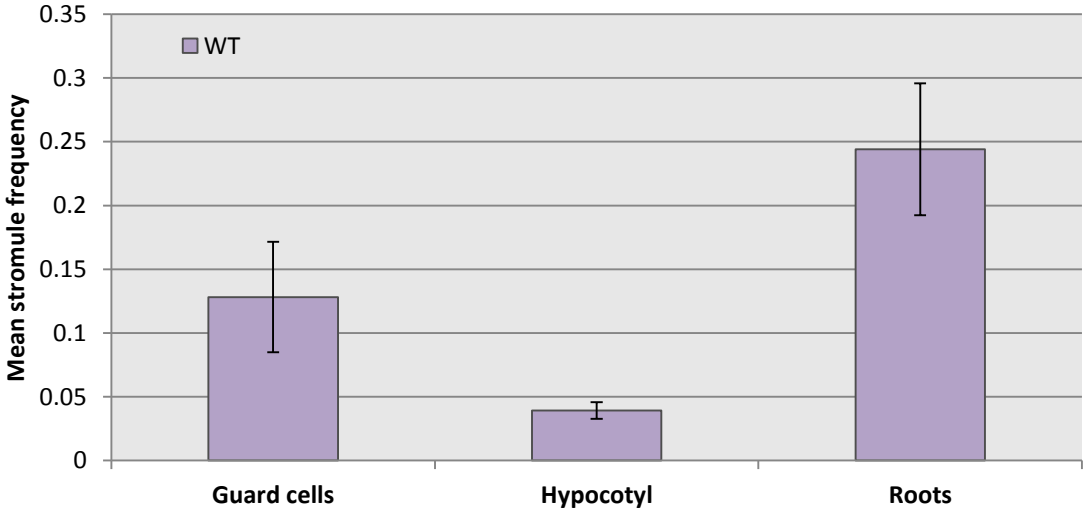

Figure 1: The difference in stromule frequencies in WT plants in different cell types.

Because of the low variance in stromule frequency in hypocotyl epidermal cells and because of preceding literature, hypocotyl cells were chosen for all further experiments (Holzinger, Kwok et al. 2008, Newell, Natesan et al. 2012). Also because of the low stromule frequency, shifts in stromule frequency are expected to be more easily observed in the hypocotyl.

Stromule frequency is increased by low Pi treatment

The biosynthesis and exudation of strigolactones is strongly increased under low nutrient availability. Especially phosphate limiting conditions elicit an increase of strigolactone biosynthesis in *A.thaliana* (Yoneyama, Xie et al. 2007). In plants other than *Arabidopsis* the exudation of strigolactones can increase the interaction of the plant with arbuscular mycorrhyzal fungi to increase phosphate uptake.

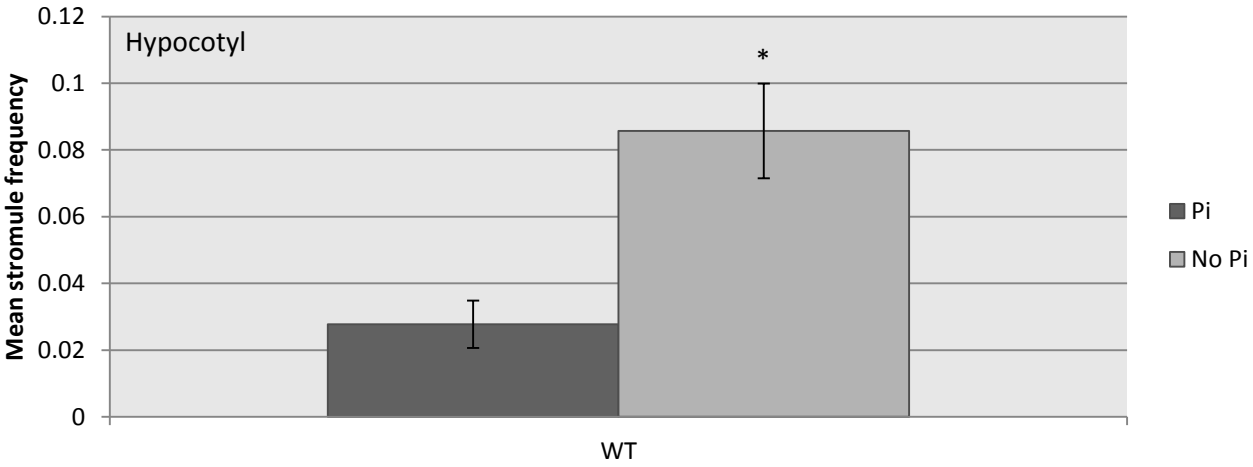

Figure 2: Increase in stromule frequency under phosphate starvation in WT plants.

In figure 2, a threefold increase in stromule frequency is observed in plants grown for seven days on medium without phosphate. The frequency increases from 0.028±0.007 to 0.086±0.01, N= 4 plants, with 5 images/plant p=0.001. Since strigolactones are increased under phosphate limiting condition, we further investigated the link between strigolactones and stromules.

Stromule frequency relates to endogenous SL levels in Arabidopsis

The tree genotypes have different levels of endogenous of strigolactones. Compared to WT, *max3* has lower levels of strigolactones. Because of lack of feedback regulation, the *max2* mutant has higher strigolactone levels than both WT and *max3* (Mashiguchi, Sasaki et al. 2009). Here we tested if the endougenous strigolactone levels correlate with stromule frequencies in all three genotypes. As seen in figure 3 the stromule frequency relates to the endogenous levels of strigolactones. Compared to WT 0.039±0.0066 N=3 plants, 4 images/plant, *max2* has a higher stromule frequency 0.11±0.014 N=3 plants, 4 images/plant, p≤0.001. *Max3* 0.016±0.0044 N=3 plants, 4 images/plant, however has a lower stromule frequency than WT (although not significantly), p=0.095 and *max2* p≤0.001.

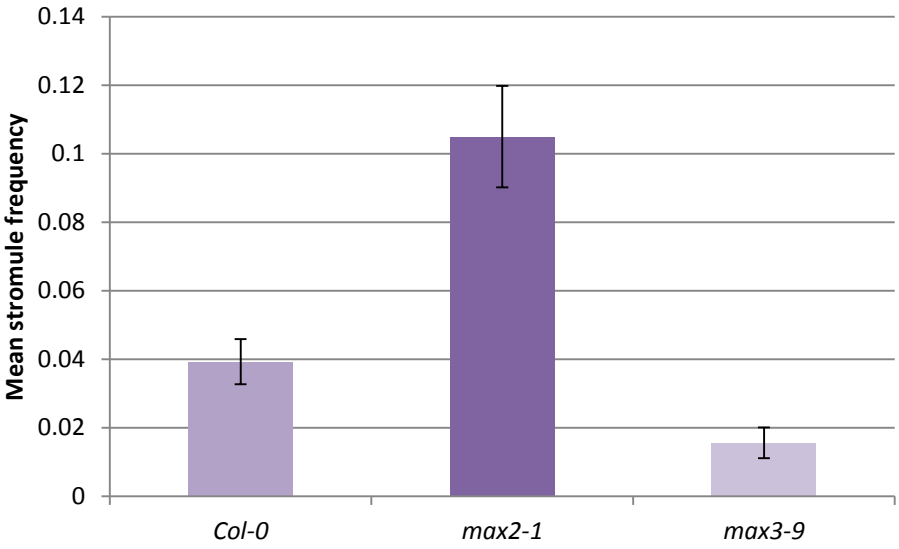

Figure 3: The mean stromule frequency in hypocotyl epidermal cells in the three different genotypes, WT, *max2* and *max3*.

In figure 4, the differences in stromule frequency in the hypocotyl are comparable to figure 3. The stromule frequencies in guard cells and roots show similar trends, although the differences are not

significant. These results show a correlation between endogenous strigolactone levels and stromule frequencies.

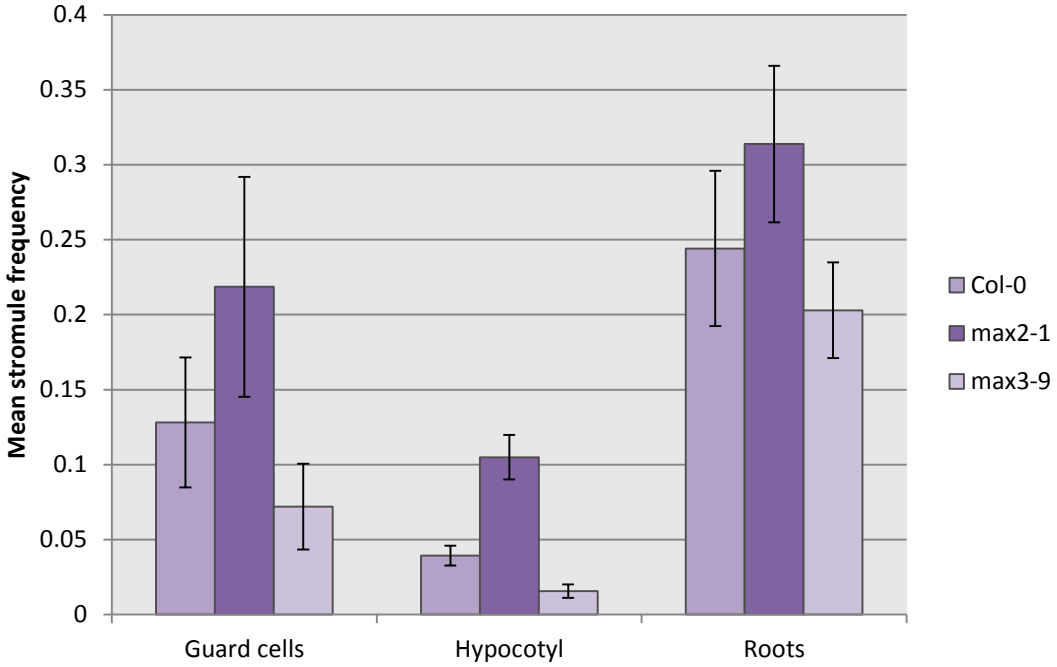

Figure 4: The stromule frequencies in several cell types. No significant differences are found in guard cells or root cells, bit the frequencies show similar trends as in hypocotyl epidermal cells.

Strigolactones stimulate stromules formation independent of *MAX2*

Strigolactone signalling involves several proteins, Max2, D14 and Kai2 (Nelson, Scaffidi et al. 2011). Of these, Max2 is the most extensively studied. Here we show that stromule formation is stimulated by strigolactones independently from max2. In figure 6 all three genotypes show an increase in stromule frequency. WT from 0.03±0.005 to 0.10±0.02 N=3 plants, 5 images/plant, p=0.001. Max2 0.10±0.02 compared to 0.15±0.02 N=3 plants, 5 images/plant, p=0.012. Max3 from 0.02±0.001 to 0.09±0.01 N=3 plants, 5 images/plant, p=0.002.

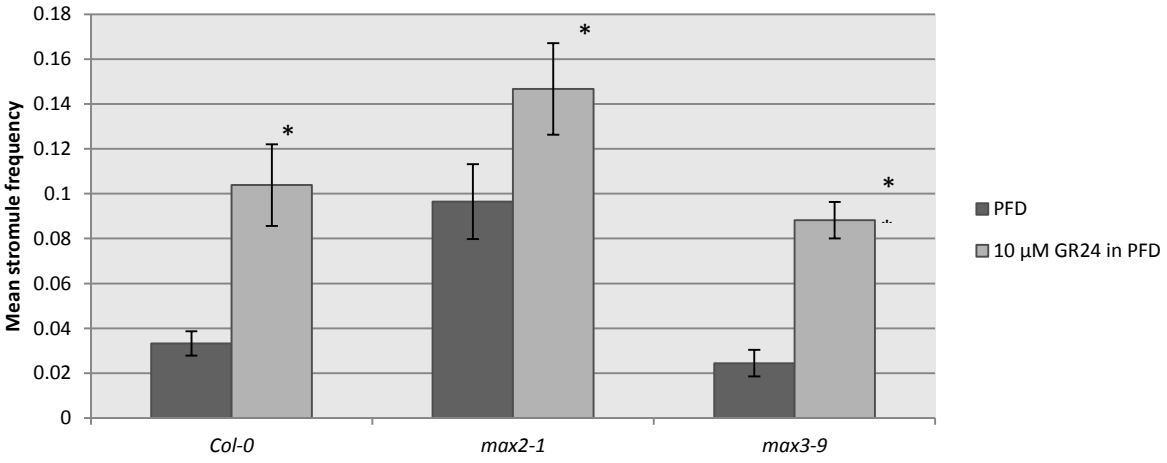

Figure 5: 10µm of the synthetic strigolactone GR24 increases the stromule frequency in WT, *max2* and *max3*.

The fact that *max2* shows an increase in stromule frequency means that the mechanism by which strigolactones stimulate stromule formation is MAX2 independent. Several of other components of strigolactone signalling might be involved.

High stromule frequency in max2 is reduced by SL biosynthesis inhibitor

In contrast to the previous experiment, here we used a strigolactone biosynthesis inhibitor D2, to see if the stromule frequency would decrease. *Max2* was used to test the effect of D2 because of its high levels of endogenous strigolactones. The results support the previous results using GR24. When D2 is supplied in high enough concentrations, the stromule frequency decreases significantly. Both 5µM and 10µM show a significant decrease compared to the control. The stromule frequency in the control is 0.12±0.02 compared to 0.06±0.01, p=0.002 and 0.06±0.004 p=0.001 for 5µM and 10µM respectively. N= 3 plants, 5 images/plant.

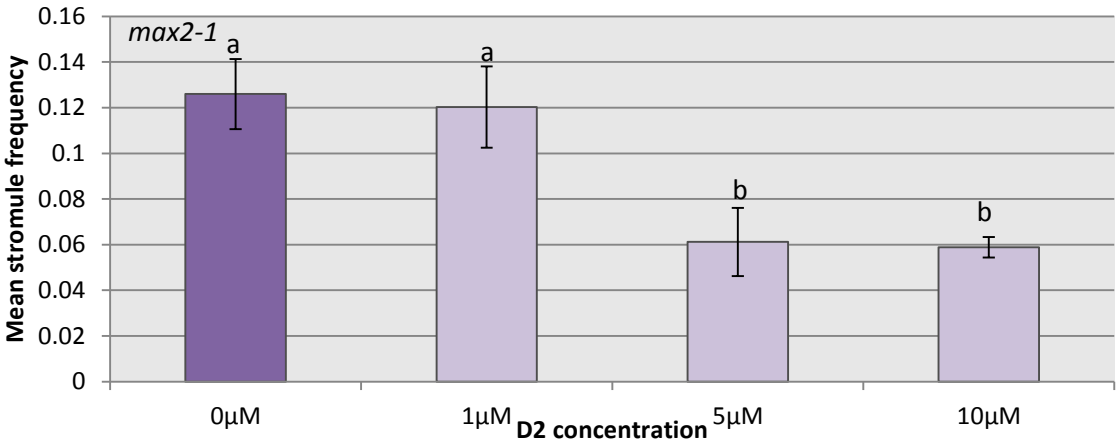

Figure 6: D2 decreases the stromule frequency in *max2* almost back to WT levels.

This shows that the high endogenous stromule frequency can be lowered by inhibiting the production of strigolactones.

Stromule induction by sucrose is absent in SL biosynthesis mutant max3

One of the previously mentioned stimulants of stromule formation, is extracellular sugar. To test if this increase in stromule frequency is dependent on strigolactones, we tested if 40mM of sucrose could increase the stromule frequency of hypocotyl epidermal cells, by vacuum infiltrating the seedlings. A small increase of stromule frequency is observed in WT plants. An increase in stromule frequency of 0.033±0.016, N=25, p=0.041. As expected no significant differences in stromule frequency were observed in *max3*. *Max2* also does not show a significant difference. This might be due to the treatment, or *max2* might be saturated with strigolactones and not be able to produce more stromules. Although not significant, both *max2* and *max3* show a decreasing trend in stromule frequency.

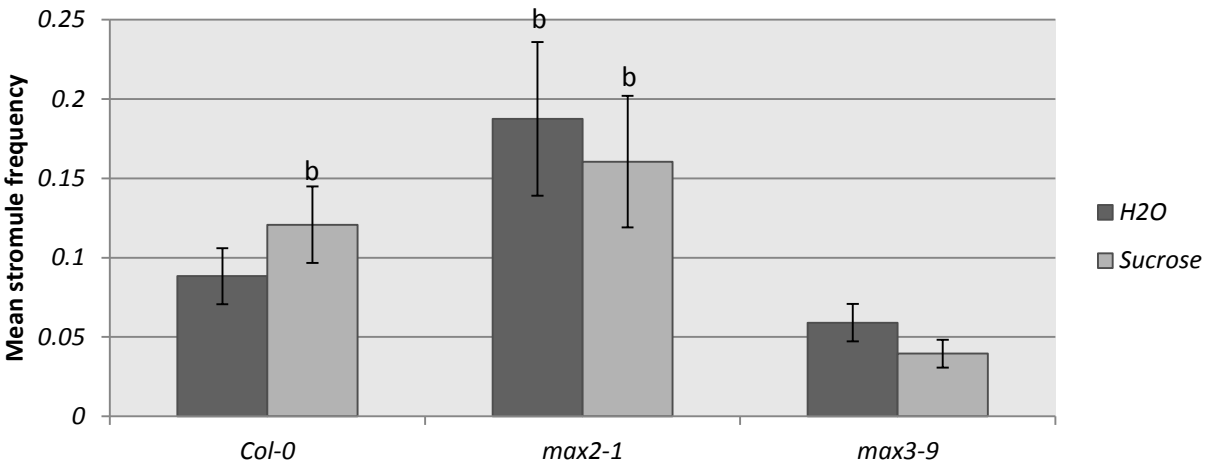

Figure 7: Stromule frequencies of plants receiving 40mM sucrose or H_2O.

Stromule induction by low Pi is absent in SL biosynthesis mutant max3

Since phosphate starvation is one of the main causes of strigolactone production, stromule frequency should increase when seedlings are grown under phosphate limiting conditions if stromule formation is dependent on strigolactones. Here seedlings were fixed in water on the microscope slides.

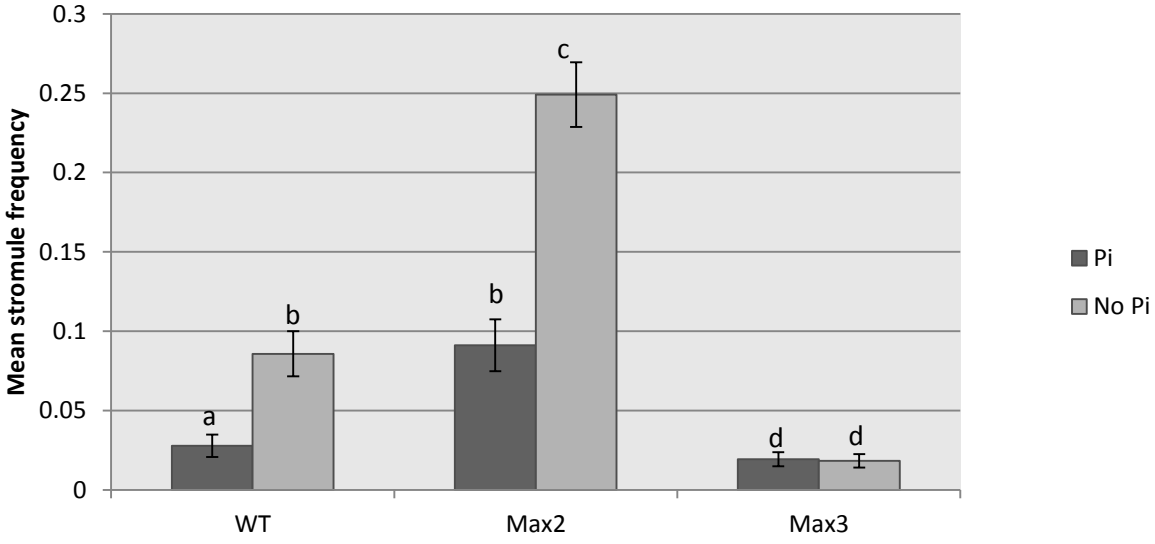

Figure 8: The effect of low phosphate conditions on stromule frequency. In the biosynthesis mutant *max3* low phosphate does not increase the stromule frequency.

As seen in figure 7, phosphate starvation increases the stromule frequency in both WT and *max2*, but not in *max3*. The stromule frequency in WT shows an increase of 0.058±0.016, N=20, p=0.001. The stromule frequency in m*ax2* is increased by 0.16±0.018, N=15, p≤0.001. The fact that the strigolactone biosynthesis mutant *max3* shows no increase in stromule frequency, points out that stromule formation is dependent on strigolactones.

Stromule induction by low Pi and SL in tobacco

Not only in Arabidopsis do low phosphate conditions result in an increase in stromule frequency. In *Nicotiana tabacum,* after 14 days of growth on the media, clear phenotypic differences were observed. The seedlings grown on MS media showed bright green leaves and an elaborate root system. The seedlings grown on minimal media showed a single primary root which was approximately twice as long as the roots of the MS grown seedlings. The leaves were also significantly smaller and paler green in colour.

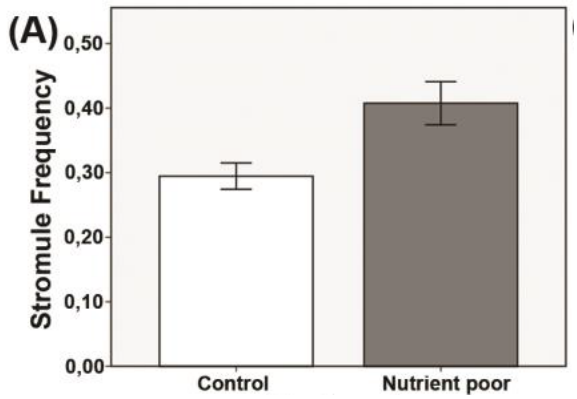

Figure 9: Induction of stromule formation by low nutrient availability in *N. tabacum* roots (differentiation zone). When seedlings are grown on nutrient poor media, the stromule frequency increases from 0.29 in the control to 0.41.

The seedlings grown on the MS agar media show a stromule frequency of 0.29 ± 0.02 compared to a stromule frequency of 0.41 ± 0.03 in the roots of seedlings grown on nutrient poor media (N=15, p=0.008, t-test) (Figure 10). These results are similar to the results obtained using A. thaliana.

Induction of stromules by GR24 in *N. tabacum*

To check if GR24 can induce stromule formation in other plant species than *A. thaliana*, *N. tabacum* is used to test the effects of GR24 on stromule formation (figure 11).

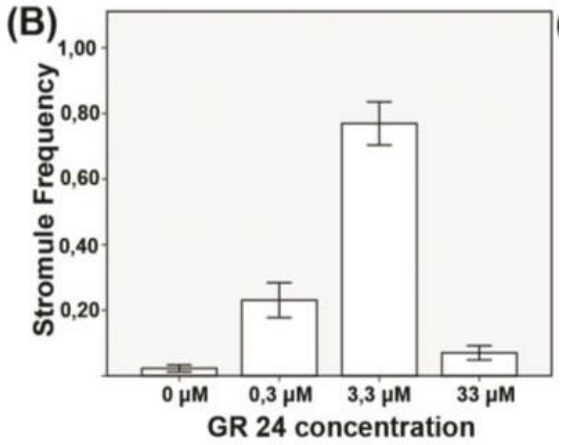

Figure 10: Concentration dependent induction of stromule formation in *N. tabacum* stomata guard cells of 14 days old seedlings. An increase in stromule frequency is observed at concentrations of 0.3 µM GR 24 and 3.3 µM GR 24 compared to 0 µM GR 24. Reduced stromule formation is observed at 33 µM GR 24.

Stomata guard cells in tobacco show a very strong reaction to different levels of GR24 as is shown in figure 11. A clear increase in stromule frequency from 0.02 ± 0.01 to 0.23 ± 0.05 (N=15, p=0,001, post hoc: LSD) is observed between the control and 0.3 µM GR24. An even bigger increase in stromule frequency, from 0.02 ± 0.01 to 0.77 ± 0.07 (N=15, p=0.000, post hoc: LSD) is observed when the GR24

concentration is increased to 3.3 µM. When the concentration is again increased 10-fold to 33 µM, the stromule frequency drops almost to the level of the control treatment (0.07 ± 0.02, N=15, p=0.450, post hoc: LSD). It appears that there is an optimum concentration of GR24, which is not observed in A. thaliana.

Stromule formation in tobacco blocked by Fluridone and D2

As shown previously, the synthetic strigolactone GR24 can induce stromule formation in both *A. thaliana* and *N. tabacum*. The question is now if strigolactone inhibitors can reduce the stromule frequency also in *N. tabacum*.

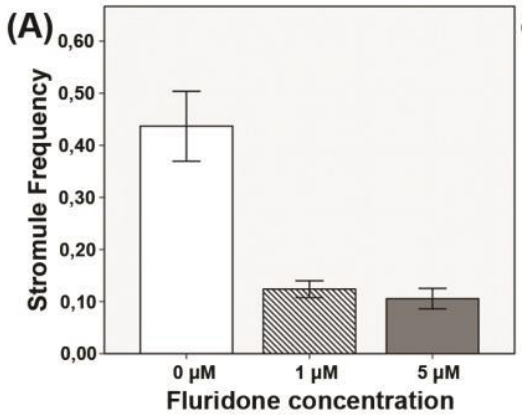

Figure 11: Inhibition of stromule formation by the ABA/strigolactone biosynthesis inhibitor fluridone in *N. tabacum* roots (differentiation zone). A decrease of approximately 0.30 in stromule frequency (A) is observed when fluridone is applied at a concentration of 1 µM and 5 µM.

Also in N.tabacum experiment with strigolactone biosynthesis inhibitors are performed. Using the ABA/strigolactone biosynthesis inhibitor fluridone, a decrease in stromule frequency can be observed. At 1 µM fluridone the stromule frequency is 0.12 ± 0.02 and at 5 µM fluridone it is 0.10 ± 0.02. The control stromule frequency however is 0.44 ± 0.07, which is a 73% higher compared to the stromule frequency of 1 µM (N=13, p=0.000, post hoc: LSD) and 5 µM (N=14, p=0.000, post hoc: LSD) (figure 12). This shows that fluridone is able to lower the stromule frequency. Whether this is due to a decrease in SLs, ABA or both, requires additional experiments.

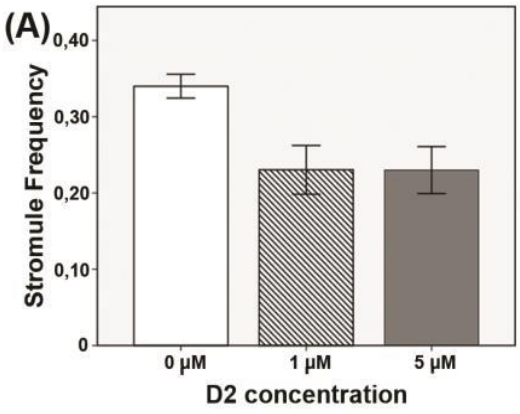

Figure 12: Inhibition of stromule formation by D2 in *N. tabacum* roots (differentiation zone). A decrease of approximately 0.10 in stromule frequency (A) is observed when D2 is applied at a concentration of 1 µM and 5 µM.

A lower stromule frequency can be observed for the D2 (strigolactone biosynthesis inhibitor) treated seedlings. The stromule frequency (Figure 13) for the control seedlings is 0.34 ± 0.02 whereas for both

the 1 µM and 5 µM D2 treated seedlings the average stromule frequency is 0.23 ± 0.03 (N=9, p=0.008, post hoc: LSD). This decrease in stromule frequency, 33%, is not as big compared to the fluridone treatments, which showed a 73% decrease when the seedlings were grown on media containing fluridone.

Stimulation of stromule formation by SL correlates with increased monoterpene emission by leaves

Plastids do not only synthesize SLs but also a wide variety of other metabolites such as monoterpenes and sesquiterpenes. Soaking leaf discs in a solution containing 3.3 µM GR24 resulted in an increase in SLs within two to three hours. By measuring the amount of monoterpene and sesquiterpene volatiles emitted by N. tabacum leaves within four hours it could be tested if infusion with GR24 results in an increase in mono- and sesquiterpene emission. N. tabacum leaves were placed with their petiole in 0.5 ml Eppendorf tubes containing either 0.2 ml H2O or 0.2 ml H2O + 3.3 µM GR24.

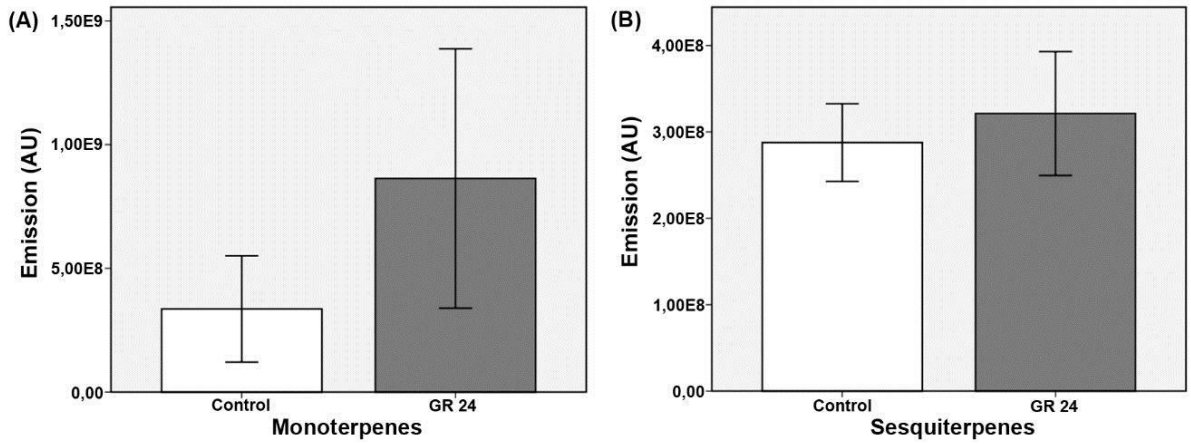

Figure 13: Headspace emission of N. tabacum incubated with GR 24. Panel A shows the emission of limonene and linalool, collectively called monoterpenes. An increase in monoterpenes is observed when leaves are incubated on GR 24. This difference is however not significant. Panel B shows the emission of caryophylene and caryophylene-oxide. No difference in emission was observed. N = 5.

The results show that there is no increase in sesquiterpene emission if GR24 is applied (N=5, p=0.545, t-test) (Figure 12B). The peaks that appeared in the chromatogram are caryophyllene and caryophyllene-oxide. The emission of monoterpenes however does show an increase when leaves are treated with GR24 although not a significant difference (N=5, p=0.618, t-test). The peak of monoterpene emission consist of linalool and limonene.

Discussion

This research has shown a strong link between strigolactones and the formation of stromules. As with all research, more questions are being raised. What is the function of stromules and how exactly are they formed are questions still to be answered.

Function of stromules in molecular transport from cytosol to plastid?

The main function of chloroplasts is still photosynthesis and sugar production. Our experiments showed an increase in stromule frequency when extracellular sugar was applied. This suggests that stromules function to increase the plastid surface area and to regulate the uptake or secretion of sugars from/into the cytosol. As also shown by Schattat (2011) extracellular sugar induces the stromule frequency. Connected to this might be the signalling molecule trehalose-6-phosphate (T6P), formed by a reaction between glucose-6-phosphate and UDP by the enzyme trehalose phosphate synthase (TPS). T6P influences the formation of cell walls, embryo development and photosynthesis, next to its function as signal molecule. As mentioned in the introduction, under low phosphate conditions, phospholipids are exchanged for galactolipids. Stromules might play an important role in this process, by increasing surface area and interaction area between plastids and the endoplasmatic retriculum (Tjellström, Andersson et al. 2008). A true exchange of phospholipids to galactolipids has yet to be observed. This process changes the availability of sugars, which may have an effect on the expression of TPS. TPS is also an important regulator of ABA availability (Avonce et al. 2004). As shown in a study by López-Ráez et al (2010) ABA influences the expression of strigolatones. Our studies show that all different modulators of stromule frequency can be lead back to strigolactones.

Function of stromules in molecular transport from plastid to other cellular compartments

Plastids are important organelles in plant cells and it is likely that they are involved in several important processes.

Plastids are the place where several metabolites, such as monoterpenes and sesquiterpenes are synthesised. And increase of plastid size by an increase in stromule frequency leads to an increase in monoterpene secretion in *N. tabacum*. This has to be studied in *A. thaliana* but so far results point to a role for stromules in monoterpene production or excretion.

Another possible role for stromule might be related to the production of strigolactones themselves. Strigolactones are (partly) produced in plastids, before being secreted. A positive feedback loop in the production of strigolactones and stromules is one of the possibilities.

The function of SL in stromule formation

SL signalling through MAX2 involves both complex formation with D14 and with Kai2. D14 has been identified as receptor of canonical strigolactones while Kai2 is sensitive to karakins and presumably an unknown endogenous unknown compound.

It has been described that the artificial strigolactone GR24 consists of a racemic mixture of two isomer forms, one of which mimics canonical strigolactones (perception by D14/MAX2 complex), while the other stereoisomer happens to bind to Kai2, which also requires MAX2 for signalling.

The stromule formation is independent of the higher actin bundling in max2 and max3 mutants versus WT plants (Koltai 2014). At present it is not clear where the GR24/endogenous SL signal is perceived that affects stromule formation by SLs. Although independent of the F-box protein MAX2, it is still possible that the racemic mixture of GR24 stimulates stromule formation through D14, D14L or Kai2 in combination with an F-Box protein other than MAX2. To test a putative role of other F-box proteins in D14, D14L or Kai2 mediated signalling we considered testing the induction of stromule formation by GR24 in the presence of the general protease inhibitor MG132, but literature shows that protease inhibitors result in collapse of the cytoskeleton and blocks cytoplasmic streaming, while cytoplasmic streaming through myosinXI is required for stromule formation(Sheng, Hu et al. 2006, Natesan, Sullivan et al. 2009). We therefor expect that stromule formation will be blocked by MG132 regardless of the SL signalling or biosynthesis background.

Testing a selective role of the individual D14 type proteins in Arabidopsis in stromule formation will be subject of future investigations.

Conclusion

Here we have shown that Strigolactones link to the formation of stromules on plastids. Several factors, such as sucrose content, low phosphate condition can be linked brought back to the availability of strigolactones. Allthough this research elucidated an important key player in stromule formation, more research needs to be done to find the function(s) of stromules. Moreover, the cellulair mechanisms by which the stromules are formed are still a mystery.

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Supplemetal data

GR24 concentration line

In this experiment we tested if the synthetic strigolactone GR24 can induce stromule formation. Because of the low endogenous level of strigolactones in max3, this mutant was used to see if we could increase the stromule frequency by applying the synthetic strigolactone GR24.

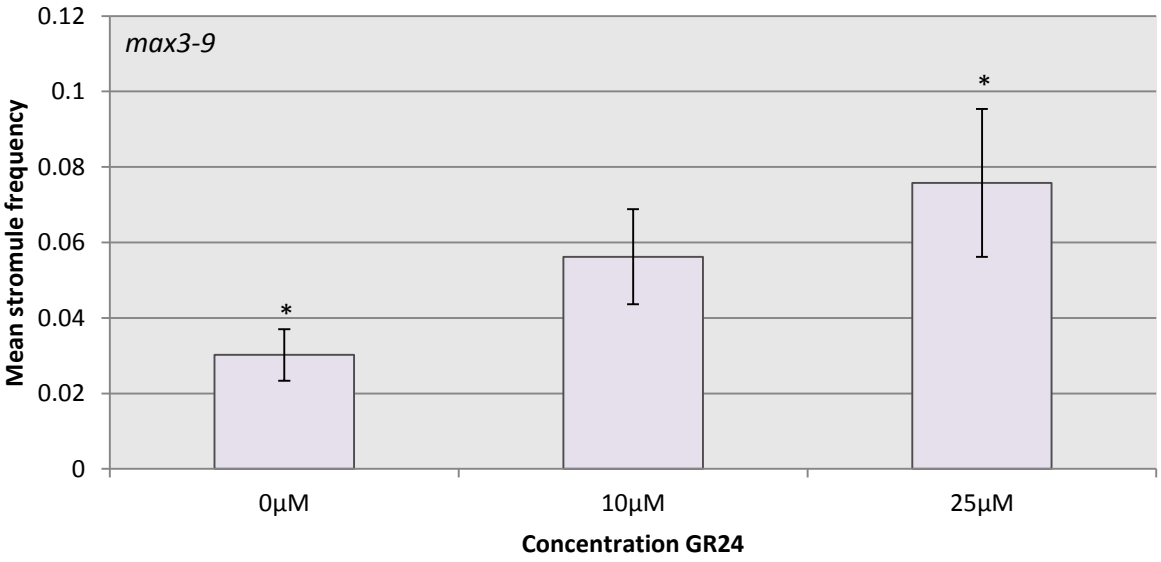

Figure 14: A concentration line of GR24. The stromule frequency of *max3* is increased by applying 10µM GR24 1 hour before imaging.

Although an increase is seen when 10µM GR24 was applied for 1 hour, the difference is not significant (p=0.06). The difference between the control and 25µM is significant however. This shows an increase in stromule frequency from 0.03±0.01 to 0.08±0.02, N= 4 plants, 5 images/plant, p=0.003.