Interaction of Strigolactone with polar auxin transport in roots of *Arabidopsis thaliana*

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Abstract: One of the major regulators of root architecture is the plant hormone auxin. According to recent investigation strigolactone is also involved in this process and is supposed to act by regulation of polar auxin transport mediated by PIN efflux carriers. To choose an optimal concentration for the subsequent study the influence of 12 nM to 50 μM strigolactone GR24 on Arabidopsis root system was evaluated. 242 ecotypes of *Arabidopsis thaliana* were analyzed by genome-wide association study (GWAS) under the influence of 100 nM of strigolactone. Results of the study are analyzed for a search of candidate genes which participate in strigolactone signaling pathways. From obtained results we have chosen 5 candidate genes (AFB4, F-box, BEN2, F-box associated, MPK) for analysis of their expression levels dependent on strigolactone concentration in Arabidopsis roots and simultaneously auxin efflux (PIN1,2,3,4,7) and influx (AUX1) carriers were analyzed by Real-Time PCR.

Key-Words: - Strigolactone, root architecture, PINs, GWAS analysis, gene expression

Introduction

Intensive research activity on the new plant hormone strigolactone started with its description as a regulator of shoot branching [1, 2] and revealed much broader influence on regulation of development throughout the whole plant body [3]. The existence and signal molecule function was elucidated by the analysis of a series of shoot branching mutants, cloning and grafting experiments in *Arabidopsis*, pea and petunia. Strigolactone is transported acropetally from the root system into above ground plant body where it causes inhibition of axillary bud outgrowth [1, 2]. Strigolactone is further connected with various functions in plants like regulation of root architecture, inhibition of branching, control of cambium secondary growth, stimulation of parasitic seed germination and initiation of mycorrhiza

Strigolactone perception and signal transduction are poorly understood. An F-box leucine-rich repeat protein (referred to as AtMAX2 F-box) that is a component of a SCF complex [4] is involved in strigolactone signaling which has been identified from mutation studies in several species. It is related to the TIR1 and COI1 F-box receptors for auxin and jasmonic acid, and they are suggested to be involved in ubiquitin-mediated protein degradation [5].
Basipetal auxin transport in stem and acropetal in the root is realized in floem and parenchyma cells surrounding vascular strands. This characteristic polar auxin transport is facilitated by specific auxin carriers located at the plasma membrane of the cells – AUX and LAX (Like AUX1) ensuring influx into the cell and family of PIN proteins (PIN1-8) which facilitate auxin efflux from the cell [6, 7]. Initiation of primary root growth and branching is dependent on auxin transport and formation of its local maxima. Control of root architecture [8] and adventitious root formation are related to auxin-dependent processes, and strigolactone acts at least in part by regulation of auxin transport via the efflux carrier PIN1 [9].

The study of changes of root architecture caused by strigolactone application is an optimal system for elucidation of the influence of strigolactone on polar auxin transport.

Material and Methods

GWAS analysis

Preliminary experiment to assess strigolactone influence on root architecture was realized to obtain an optimal concentration for the GWAS experiment and included 6 different ecotypes of *Arabidopsis thaliana* [10].

For the Genome-wide association study (GWAS) seeds of 242 ecotypes of *Arabidopsis thaliana* were selected, sterilized by bleach and chloride gas,

stratified and grown on sterile agar medium for 3 weeks. Based on the preliminary experiment 100 nM concentration of GR24 was chosen and control variant with only DMSO were prepared. Individual Petri dishes with plants were scanned for 3 weeks and obtained images were evaluated by BRAT program – Busch Lab Analysis Toolchain [11] for required parameters of root system of individual ecotypes. GWAS analysis followed.

Genome-wide association study (GWAS) is a useful technique for identifying genetic loci responsible for natural variation in *A. thaliana*. Based on common genetic variability of individual ecotypes specific differences associated with investigated trait are studied. How significantly SNPs – single nucleotide polymorphisms – are associated with required traits like root length, root tortuosity, root growth rate, root angle etc. can be determined. Previously genotyped accessions (natural inbred lines) are grown in replicate under different conditions and phenotyped for different traits.

Experimental material

Seeds of *Arabidopsis thaliana* were sterilized by adding 70% ethanol to prevent possible contamination and stratification was carried out in sterile water in dark and cold conditions in a fridge for 3 days. After that the seeds were transferred by pipette onto Petri dishes with media. Plants were grown for 6 days in vertical position in a growth chamber Klimacel.

Four different variants of cultivation media were prepared – 10 nM, 100 nM, 1000 nM GR24 and control medium (5 µl of DMSO, the same amount in which GR24 was dissolved and added). Strigolactone was added into the medium directly after sterilization during the preparation process in sterile conditions. On the surface of the medium the sterile nylon sieve (Uhelon) was placed for better manipulation during harvesting of plants. Seeds were sown on the sieve in two horizontal lines and after 6 days were cut by scalpel in the height of hypocotyls, thus divided into above and below ground parts and collected separately in Eppendorf tubes.

Gene expression

For the assessment of expression of selected genes RNA was isolated from roots by RNAeasy Plant Mini Kit (Qiagen, Germany). Concentration of obtained RNA was measured on Picodrop Pico100 (Picodrop,UK). Reverse transcription was realized with the Enhanced Avian HS RT-PCR Kit (Sigma-Aldrich, USA). Enzyme Reverse transcriptase was used for generating cDNA from isolated RNA.

Transcription levels of selected genes by Real-Time PCR reaction with specific primers – *AtPIN1,2,3,4,7, AtAUX1, AtAFB4, AtFbox, AtBEN2, AtFbox associated, AtMitogenPK (MPK)* was used. Final volume of PCR reaction was 10 µl. For all reactions 5 fold diluted cDNA was used. At first all primers pairs were optimized, specific temperature conditions for efficient amplification process were defined and standard curves were created by evaluation of the selected genes against 2 constitutive genes – *UBQ10* and *UBC*. Two negative controls - sterile water and reverse transcribed water instead of cDNA which was used. For real-Time PCR thermal cycler C1000TM Thermal Cycler/CFX96TM Real-Time System (BIORAD, USA), Intercalation dye Syber Green included in Syber Green MasterMix (Light Cycler 480 SYBER Green, Roche, Diagnostics GmbH, Germany) were used. The experiment was done with 2 biological replicates and 3 technical replicates. Standard deviation of the mean was calculated.

Results and discussion

Influence of strigolactone on root architecture

Preliminary experiment has proven that the dependence of primary root length on concentration of GR24 is non-linear and strongly genotype dependent (Fig. 1).

Fig. 1 Primary root growth of ecotype Ren-11 after GR24 treatment

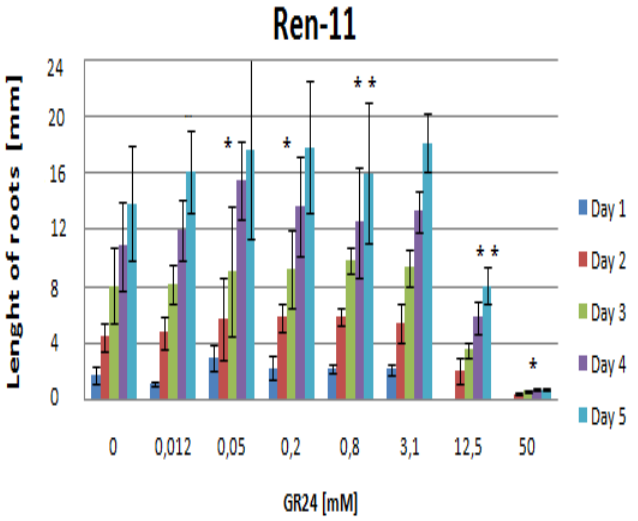


Table 1 Summary of primary root growth dependence on GR24 concentration for individual ecotypes

GR24 conc. (µM)	VOU-5	Wilcox-4	DraIII-1	Bå1-2	Ts-1	Ren-11
0.012	**	*		*		
0.05			*			*
0.2						*
0.8						**
3.1	**	*	**	**		
12.5	*	*	*	*	*	**
50	*	n/a	*	n/a	*	*

Legend	
	Variance less than 5%
	Shortening 5-10%
	Shortening more than 10%
	Elongation 5-10%
	Elongation more than 10%
*	*Statistically significant data*
**	*Statistically very significant data*

The randomly chosen 6 genotypes have shown a wide range of sensitivity caused by changes in the genotype. The data for ecotype Ren-11 (Tab. 1) represent an ecotype in which concentrations till 3.1 µM cause significant elongation of the primry root and only 2 highest conctentration of GR24 reverse the effect to signicicant inhibition of root growth. Ecotype VOU-5 represents another extreme situation where all used concentrations of GR24 caused primary root inhibition. The other analysed ecotypes form a continues transition between VOU-5 and Ren-11.

This results corespond with observed stimulation of primary root growth by 1 and 2.5 µM and inhibition by 5 and 10 µM [8] and by 27µM of GR24 [12]. Considering these results 100 nM concentration was chosen for GWAS study of GR24 influence on root architecture of *Arabidopsis thaliana* ecotypes.

GWAS analysis

For our experiment 242 ecotypes were selected for studying strigolactone influence on *Arabidopsis thaliana* root architecture.

Fig. 2 SNPs associated with primary root elongation by GR24 in selected ecotypes are marked in red area whereas in series of ecotypes under red line with root length shortening by GR24 SNPs are missing. Evaluated by Genome browser on 1001 Genomes site (http://signal.salk.edu/atg1001/3.0/gebrowser.php).

16 different traits in 9 time points were investigated and in the control-strigolactone ratio dataset 158 significant associations were found in 30 associated traits (Tab. 2). These 158 significant associated SNPs (P-value higher than 6) are located in promtor, coding or flanking regions (10 kb before and behind gene were considered) (Fig.2).

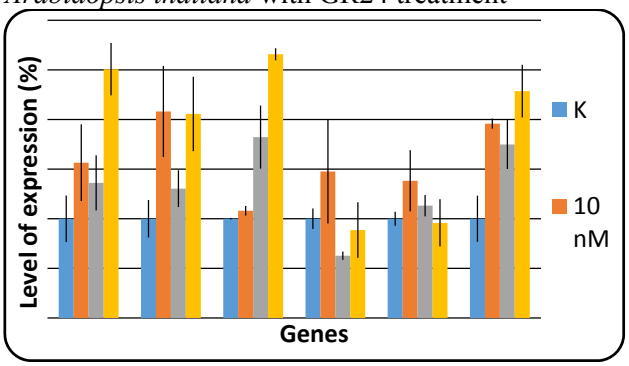
Table 2 Summary of associated traits found in the analysis

TRAIT	DAY
Total length	1, 2, 3, 4, 5
Euclidian length	1, 2, 3, 4, 5
Root tortuosity	4, average
Root growth rate	1-2, 4-5
Relative root growth rate	2-3, 4-5
Root angle	2, 3, 4, 5
Root directional equivalent	2
Root horizontal index	1
Root vertical index	1, 2, 3, 4, 5
Root linearity	1, 4
Average root width	-
Root width 20	-
Root width 40	-
Root width 60	-
Root width 80	1
Root width 100	-

Phenotypic variability of roots under strigolactone influence analyzed by GWAS revealed candidate genes involved in processes that caused the changes. The results were verified using comparison of SNPs associated with the traits by a genome browser on 1001 Genomes site (http://signal.salk.edu/atg1001/3.0/gebrowser.php) (Fig. 2). This analysis includes extreme phenotypes with associated trait and can be suggested as significant if this SNP is present only in one of the two extremes.

According to GWAS analysis results of 242 ecotypes of *Arabidopsis thaliana* and subsequent evaluation of significant associated SNPs 5 candidate genes were chosen for gene expression experiments. *AFB4* (AT4G24390) F-box protein involved in negative polar auxin transport regulation [13], *F-box* (AT5G27920), *F-box associated* (AT2G18780) and *Mitogen-activated Proteinkinase* (AT4G36450) may be considered to be involved in signaling pathway of strigolactone, and cooperate with auxin transport thereby regulating root growth. *BEN2* (AT1G77140) – vacuolar protein sorting 45 – is necessary for polar PIN localization [14].

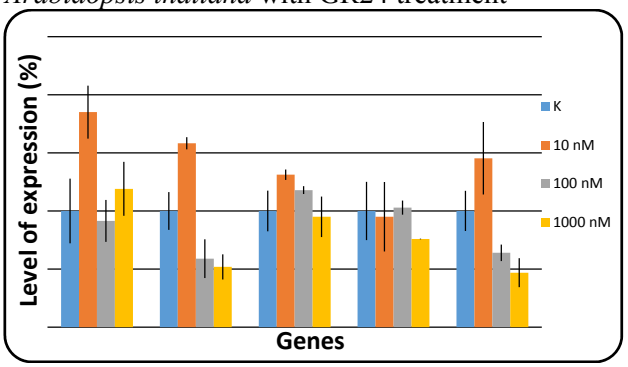
Fig. 3 Gene expresion of auxin transporters in roots *Arabidopsis thaliana* with GR24 treatment



Strigolactone effect on gene expression

The results demonstrate that strigolactone treatment led to non liner response of several genes as well as the primary root lenght response. In some cases the expression has typical bell-shaped response (*AFB4, PIN1, PIN2, PIN4, AUX1*) (Fig. 3,4). With the exception of *F-boxAs*.10 nM GR24 caused mostly significant increase in gene expression. Increase to 100 nM GR24 decreased the gene expression against the lower concentration and only in *PIN3* an increase was observed. Most differences were observed with the highest used concentration (1000 nM) where (*AFB4, PIN1, PIN2, PIN4, AUX1*) after increase of gene expression display the bell-shaped response. *PIN3* expression increases steadily. *PIN7 F-box, BEN2, MPK* and *F-boxAs* show a decrease at the highest concentration thus leading to the steadily decreasing character of response.

Fig. 4 Gene expression of GWAS genes in roots of *Arabidopsis thaliana* with GR24 treatment



Several data were published on influence of strigolactone on gene expression of genes directly involved in polar auxin transport but the conditions of the experiment diverge immensely.

Microarray analysis of gene expression after 90 minutes GR24 treatment [15] demonstrate that 76% of genes which prove increased expression due to GR24 influence are auxin-inducible genes [9]. Also they showed that cycloheximide (proteosynthesis

inhibitor) pretreatment doesn´t lead to changes of *PIN1:PIN1::GFP* fluorescence signal decline after GR24 which suggest that it is independent on the proteosynthesis. They assume that PIN1 decrease is transcription independent [9].

Similarly like our data for several *PINs* threefold induction in *PIN2* expression was detected in the WT but not in *max2-1* upon GR24 treatment, suggesting that the increase in *PIN2* signal under these conditions is at least partly a result of *PIN2* expression induced by the GR24 treatment in a MAX2-dependent fashion [16].
Other genes in our study were until now not analyzed.

Conclusion

Study of GR24 influence on polar auxin transport was realized throught analysis of changes of *Arabidopsis thaliana* root architecture.

According to genome-wide association study of 242 *Arabidpopsis thaliana* ecotypes 5 candidate genes that could be involved in strigolactone action were taken into gene expression analysis together with auxin tramsporters gene family (*PINs, AUX1*).

Taken together data have shown that expression of most of the auxin transporter genes show typical U-shaped response on strigolactone concentration and from the chosen genes only *AFB4* phenocopies this behavior. The other genes have steadily decreasing gene expression pattern which might be due to the different sensitivity to GR24 treatment and only further increase in GR24 could cause a U-shaped- response.

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