Plant Genomics and Gene Regulation

GENOMICS IN AGRICULTURE RESEARCH

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1. Introduction

The rapid growing progress that has been made in modern biology during the last few years, represents a big challenge for agriculture research. This is especially true for Genomics, a new area, which was unknown until some years ago, and is growing at a great pace. Genomics is a technology platform aimed at unravelling the totality of all genes and their functions in an organism, with extremely rapid, miniaturized and automated methods. As a consequence, an overwhelming mass of information is accumulated, which is stored and analysed with the help of Bioinformatics, and is then made available at any time to help solve biological problems, e.g. in agriculture research for the improvement of germplasm or the discovery of new agrochemicals. In order to keep pace with these developments, new genomics centers are being built up by both, industry and academia.

2. Goal of Agriculture Research

The main goal of agriculture research is the improvement of the main crops (rice, wheat, corn, oilseeds, vegetables etc.) for the production of high quality food in appropriate quantity. This means, on the one hand, crop growth has to be optimized (protection against insects, diseases and weeds) using either new genetic methods ('Input Traits'), or chemical crop protection. In addition, agronomic traits like higher tolerance against drought- and salt-stress are typical 'Input Traits' too. Yet, all these are improvements which are mainly beneficial for the farmer. On the other hand there is a lot of innovation and a concerted effort today to produce high value crop varieties with new traits and improved quality ('Output Traits') for processors and consumers further along the food chain. These plants either produce food with better processing quality (e.g. for milling-, brewing-, baking) or higher nutritional value (e.g. oil content, vitamins, flavour). Especially attractive is the production of food with completely new functions (functional food), e.g. for human health (antibodies against infections, drugs for stroke prevention, etc.). In addition, crops can be developed to produce either feed with improved traits for animals or industrial products (bioplastics, fibers etc.)

3. Use of Genomics in Agriculture Research

In order to meet these high expectations of agriculture and the food chain, genomics, as an integrated technology platform, will play a key role in the future. It will help to find new solutions for improved germplasm and chemical crop protection, and will be involved in various research areas like biology, genetics, molecular biology, gene technology, breeding, chemistry, biochemistry, analytics etc. (i) Genomic solutions for improved germplasm in crop plants means: optimization of endogenous genes or introduction of new ones, in order to improve crop traits. (ii) Genomic solutions for chemical crop protection means: Validation of genes and their proteins, from insects, fungi and weeds, to test their suitability as molecular targets of novel insecticides, fungicides or herbicides ('Target Based Discovery', a method already used for several years for drug discovery in medical research). An especially attractive solution for agriculture research represents the synergistic combination of chemical, genetic and genomic methods, e.g. chemical regulation of genes for controlled expression of genes and traits (induced resistance, production of toxic compounds, production of male or female sterile plants for hybrid seeds etc.)

4. Genomics in Seeds Research

There are two biotechnological methods to improve traits in crop plants: (i) By inserting foreign genes. (ii) As attractive alternative, by introducing natural traits of closely related species or varieties with the help of efficient, genomic assisted breeding methods (Marker Assisted Breeding, MAB). In this case, genomics is used as analytical method to identify the genes which are responsible for the new trait. This means that the new crop trait - mostly characterized by multiple genes - can rapidly be identified on the basis of a typical pattern of the molecular markers. Thus, the time consuming assessing of phenotypes in the field is not necessary anymore. In addition, Marker Assisted Breeding is especially attractive, since it enables the use of the natural genetic diversity of related species (e.g. resistant ancient varieties) in order to produce germplasm with new traits. It should be noted, that after these modern research methods, classical breeding is of main importance to develop new seed varieties for the market.

5. Genomics in Crop Protection Research

Genomic technologies can also be used to find new targets for crop protection chemicals - and with this to discover the chemicals themselves, novel insecticides, fungicides and herbicides. In a first step, the genes of the potential targets in the pests, pathogens or weeds are knocked-out. Depending on the organism, this is done using suitable methods, like homologues recombination, RNA antisense technology, silencing, mutation tagging etc. Knock-outs of genes, giving raise to lethal or impairing effects in the organism, simulate with biotechnological means the inhibiting effect of potential insecticides, fungicides or herbicides. In a next step, these validated targets (proteins) are developed into ***in-vitro*** micro-assays for 'High Throughput Screening', i.e. testing of hundred thousands of chemical coumpounds in minute amounts for their activity as crop protection agents. For the development of these compounds to market products, proven methods of crop protection like organic synthesis in chemical lead programs as well as greenhouse and field screening still have to be used.

6. Cooperation

In these processes - from gene discovery to new traits or to novel agrochemicals - many centers of excellence are involved in a complex network: academic institutes with relevant biological systems, biotechnology boutiques with specific front-end technologies, companies with fully automated high throughput robots, genomic and bioinformatic centers for bundling technologies and information and finally, potent international agribusiness concerns with the breadth for worldwide development of new germplasm and agrochemicals. Only with all these in place will we be able to introduce new modern agricultural product solutions to the market.

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GENOME-WIDE VARIATION OF THE SOMATIC MUTATION RATE IN PLANTS

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Summary

In order to analyse the rate of point mutations in whole plants a new reporter system was established. Several constructs containing different single nonsense mutations in the b?-glucoronidase (***uidA***) gene (Jefferson, 1987) were used to generate transgenic ***Arabidopsis thaliana*** plants. Upon histochemical staining of transgenic plants sectors indicative of transgene reactivation appeared. DNA amplification and sequencing of the ***uidA*** gene from these sectors proved that reversions to the original nucleotide had taken place. All three stop-codons showed significant levels of reversions, which were in the range of 10^{-7} - 10^{-8} events per base pair (bp) in the haploid genome. The rate of point mutations varied within two orders of magnitude between different lines containing different mutations and up to thirteen times between sub-lines carrying the identical constructs at different positions in the genome. This is the first estimation of point mutation rate of a non-essential gene in somatic plant tissues.

The influence of different DNA damaging factors such as UV-light, X-rays and methyl methanesulfonate (MMS) on the mutation frequency was studied. Induction of reversions was highest (3 to 56 fold, in different mutation lines) after UV-C irradiation, whereas irradiation with X-rays (25Gy) and treatment with MMS (50m?M) led to only moderate increase in the reversion frequency (3.1 and 2.2 times, respectively).

This new plant mutation-monitoring system may be used for the identification of genes involved in DNA repair. Very little is known about how the mutation repair machinery is regulated in plants (Britt, 1996; Culligan and Hays, 1997) To address these questions we propose a genetic screen to identify mutations that result in increased frequencies of point mutations in ***Arabidopsis thaliana***. We planed to perform T-DNA mutagenesis experiments (Azpiroz-Leehan and Feldmann, 1997) with transgenic "mutation" plants in order to generate "mutation-up" mutants. The particular T-DNA we have used has a 35S promoter as an enhancer element. Two outcomes from T-DNA tagging mutagenesis can be obtained. First, inactivation by T-DNA insertion of genes involved in repair processes. And second, an enhancer element in the T-DNA can activate expression of neighboring genes involved in mutation.

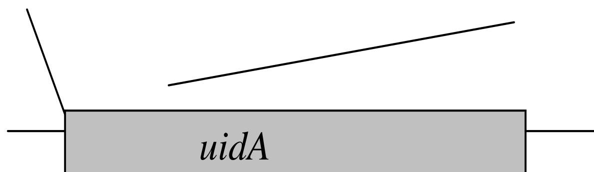
This screen should identify genes and activities involved in several steps of the regulation of mutation repair. Genetic, molecular and biochemical analysis of these mutations will enhance our understanding of how plants can sense environmental stimuli and translate these into a genetic response.

Fig. Mutations generated in the ***uidA*** gene

Position of mutated nucleotide	Mutation	Reversiontype	Reversion rate / bp
49C→T	CAG→TAG	T→C	1.2×10^{-8}
112G→T	GAA→TAA	T→G	2.9×10^{-8}
118A→T	AAA→TAA	T→A	5.3×10^{-8}
166G→T	GGA→TGA	T→G	1.4×10^{-7}
166G→A	GGA→AGA	A→G	2.0×10^{-7}
424G→T	GAA→TAA	T→G	3.3×10^{-8}

1 49 112 118 166 424

...gATG…G**C**AGG…T**G**AAATC**A**AAA…T**G**GAA…T**G**AAG…



Nucleotides shown in bold were mutated by site-directed mutagenesis. Numbers represent the position of the nucleotides in the ORF starting from ATG. In-frame triplets are underlined.

References:

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FUNCTIONAL GENOMICS IN SOLANACEAE: FORWARD AND REVERSE GENETICS IN *PETUNIA* USING D*TPH*1 TRANSPOSABLE ELEMENTS.

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The line W138 of ***Petunia hybrida***, a solanaceous species most closely related to tobacco, contains more than 200 copies of the transposable element d***Tph***1 (284 bp). Activity of d***Tph***1 in W138 leads to very high mutation frequencies of on average 1 insertion in a given gene per 1000 plants (Koes ***et al.*** Proc. Natl. Acad. Sci. USA 92 (18): 8149-53, 1995). With its economic growth habit (200 plants/ m^2), W138 is probably the best solanaceous plant for insertion mutagenesis.

Saturated d***Tph***1 insertion libraries have been constructed by groups in Amsterdam, Gent, Wageningen and recently in Berne. Together, these libraries contain approximately 17'000 plants, corresponding to roughly 6 times genome saturation. We have used collections in Amsterdam and Berne for reverse genetic analysis of shoot apical meristem (SAM) associated expansin genes and for genes from the ethanolic fermentation pathway. Using 3-dimensional PCR based screens, all of 7 genes were found to be hit at least once (table 1).

In the course of constructing an insertion library, several new mutations were identified that affect the maintenance of the SAM and processes of pattern formation and lateral organ initiation (table 2, fig.1). The best characterized mutant, ***eph***, is fully dominant and leads to ectopic leaf formation in a broad domain of the young seedling, including the cotyledonary petioles (fig1a). Leaves appear at random positions and may be laterally, dorsally or ventrally fused. This suggests that ***eph*** mutants initiate ectopic leaves independently of a zonally organized SAM. The normal function of EPH may be to control organ initiation. Revertants to wild-type occur at frequencies of ~1%, suggesting transposon insertion at the *EPH* locus. Some revertants show a new dominant phenotype of severe growth retardation and consistent maternally induced embryo abortion ~10 days after pollination. The *EPH* locus was cloned by transposon tagging. Identification of the protein encoded by *EPH* is in progress. Genetic characterization of additional mutations (table 2) is underway.

To induce new unexplored genetic variation into an active d***Tph***1 background, several ecogenetically highly variant wild ***Petunia*** species ***(P.inflata, P.violacea, P.axillaris, P.parodii***) have been hybridized with W138. Recombinant inbred lines are being developed through single seed descent.

Table 1: insertion frequencies in expansin genes (PhExp), pyruvate decarboxylase (PhPDC) and aldehyde dehydrogenase (PhALDH).

gene	#hits	library
PhExp1	6	A
PhExp2	6	A
PhExp3	4	A
PhPDC3	1	A
PhExp4	1	B
PhALDH1	1	B
PhALDH2	2	B

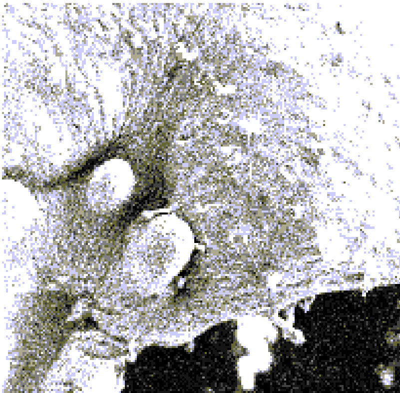
B = 2400 plants Berne

A = 6000 plants Amsterdam

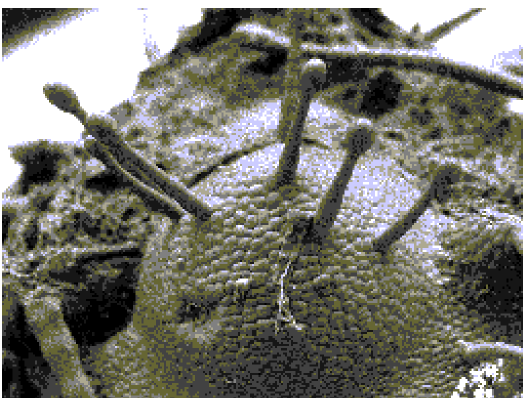
Table 2: phenotypes of 3 mutations in SAM, patterning and organ initiation

locus	mutant phenotype
quadrilateral (*QDL*)	3-4 variably fused cotyledons. Leaf- and floral shape distorted.
lanceolate (*LAN*)	Thick lanceolate leaves. Differentiation of cells in the central zone of the SAM to leaf-like characteristics, leading to trichome bearing SAMs and aborted apical growth. Flowers abort after initiating a variable number of organs in any of the 4 whorls.
epiphylla (*EPA*)	see text

Figure 1: phenotype of ***eph*** and ***lan*** mutants.



a) ***eph*** mutant seedling initiating leaf primordia at random locations at the border between the cotyledonary petiole and blade.



b) ***lan*** mutant inflorescence showing differentiation of the central zone of the SAM.

THE MAINTENANCE AND PROPAGATION OF POST-TRANSCRIPTIONAL GENE SILENCING

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Posttranscriptional gene silencing (PTGS), i.e, the interaction ***in trans*** of genes with similar transcribed sequences resulting in sequence-specific RNA degradation, frequently occurs in transgenic plants. It is now recognized to be a more general epigenetic phenomenon described for fungi, protozoa, nematodes, and insects. The mechanism for PTGS is not known. Most current models include an autoregulatory component to account for stability, production of special or aberrant RNAs for sequence specificity, and a mechanism for increased degradation of specific transcripts. We are using *N. sylvestris* transformed with tobacco class I chitinase (*CHN*) and â-1,3-glucanase (*GLU*) genes as a model system for studying the maintenance and propagation of PTGS.

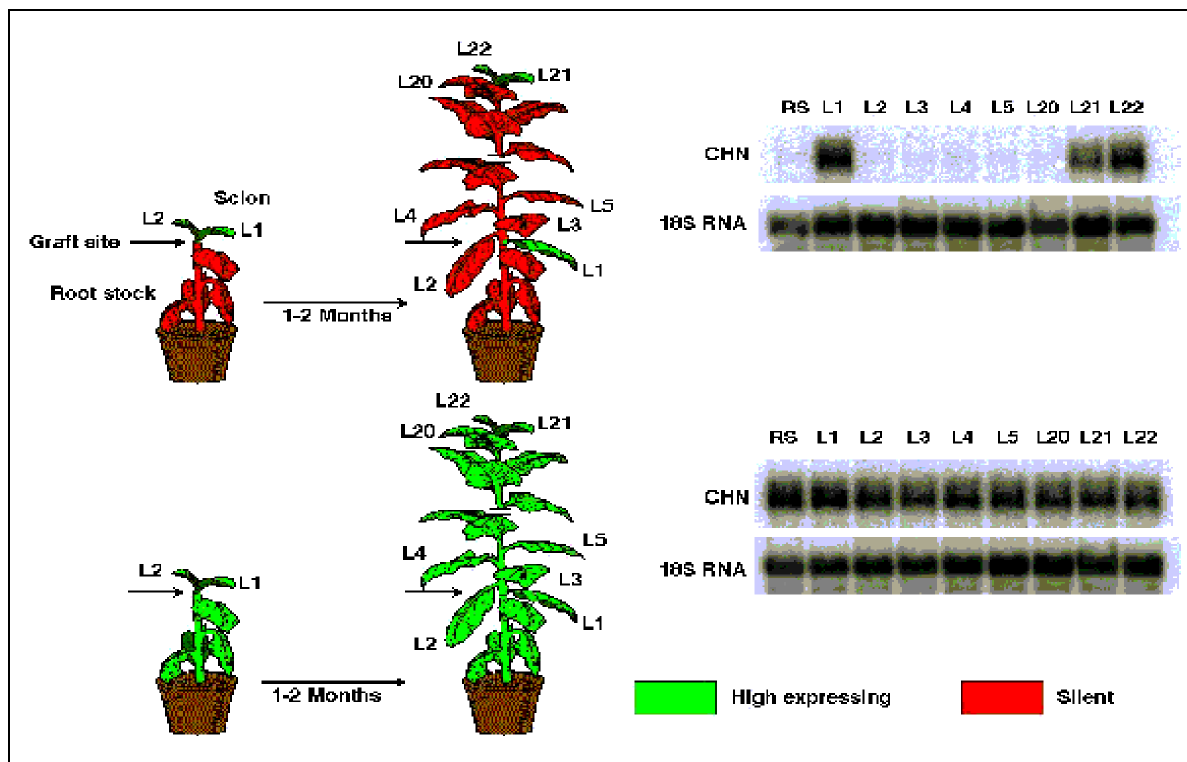
CHN and GLU gene silencing is results from increased RNA degradation rather than from decreased transcription. Silencing of *CHN*- and *GLU*-transgenes is triggered at the 6-10 leaf stage of development, the silent state persists throughout vegetative growth, and is then reset to the high-expressing state during seed development. The results of nuclear run-on transcription experiments show that although steady-state levels of RNA are reduced >10-fold in silent tissues, levels of nascent transcripts are not appreciably altered. Because nuclear run-on transcription may not faithfully reflect transcription rates *in vivo*, we also confirmed by RNase protection assays that primary nuclear transcripts were not reduced in silent tissues. Therefore, *CHN*- and *GLU*-gene silencing is a posttranscriptional phenomenon.

Inhibitor-chase experiments with α-amanitin, actinomycin D and cordycepin showed PTGS increases the degradation rate of *CHN* and *GLU* RNAs, but not that of RNA encoded by the *NPTII* marker gene in the same T-DNA. Degradation of *CHN* RNA targeted for PTGS was not inhibited in tissues treated with the protein synthesis inhibitors cycloheximide, which blocks chain elongation, and verrucarin A, which dissociates ribosomes from mRNA. Thus, PTGS-related degradation of *CHN* RNA may involve a novel mechanism which does not depend on either rapidly turning-over protein factors or the association of this RNA with ribosomes.

Evidence for a CHN-PTGS specific Silencing-Inducing Principle in tobacco.

Figure 1 shows grafts of tobacco plants made with silent (upper panel) and high-expressing (lower panel) *CHN* transformants as root stocks and high-expressing *CHN* transformants as scions. Several weeks after grafting, RNA was prepared from leaves and assayed for *CHN* RNA by RNA-blot hybridization. The results show that *CHN* PTGS can be transmitted by grafting from a silent root stock to a high-expressing scion. Spontaneous PTGS as well as systemic PTGS triggered by biolistic bombardment of additional *CHN48* gene copies were graft transmissible (29% of 101 grafts) and strongly depended on the grafting method used. No PTGS of the scion was detected with wild-type (20 grafts) or high-expressing (8 grafts) root stocks. These results confirm earlier reports for nitrate reductase, nitrite reductase, and a-glucuronidase PTGS in tobacco and green-fluorescent-protein PTGS in *N. benthamiana* suggesting that graft-transmission may be a more general feature of PTGS.

Analyses of spatial pattern of PTGS and grafting experiments support the view that *CHN* PTGS is propagated by a sequence-specific, silencing-inducing principle (SIP) that can move over long distances in plants.



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A PLANT GENE REQUIRED FOR TRANSCRIPTIONAL GENE SILENCING BUT NOT FOR DNA METHYLATION

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Epigenetic modifications change transcription patterns in multicellular organisms to achieve tissue-specific gene expression and inactivate alien DNA such as transposons or transgenes. In plants and animals, the involvement of DNA methylation in heritability and flexibility of epigenetic states is widely accepted, although its function is far from clear. We have isolated an ***Arabidopsis*** gene, MOM, whose product is required for maintenance of transcriptional gene silencing (TGS). Mutation of this gene or depletion of its transcript by expression of antisense RNA results in the rapid release of TGS at several previously silent, heavily methylated loci. Despite the transcriptional reactivation, the dense methylation at these reactivated loci is maintained even after nine generations, suggesting that transcriptional activity and the methylation pattern are inherited independently. The predicted MOM gene product is a novel nuclear protein of 2001 amino acids containing a region of similarity to one part of the ATPase region of the SWI2/SNF2 family involved in chromatin remodelling. The MOM protein is the first known molecular component essential for TGS that does not affect methylation level. Thus, it may act downstream of methylation in epigenetic regulation or it is part of a novel pathway not requiring methylation marks.

Figure 1 Schematic structure of the MOM protein repetitive region

1 repetitive region

2 binding region of tensin homology to the actin region of SWI2/SNF2 proteins homology to the ATPase transmembrane domain repetitive region

3 NLSNLSATP binding motif

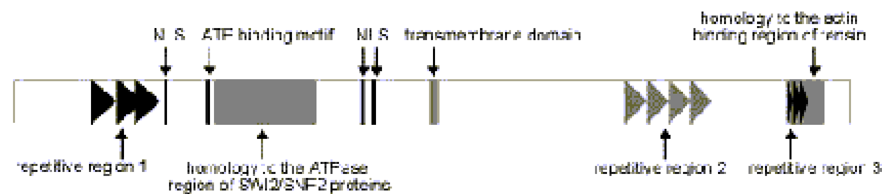

Figure 1 Schematic structure of the MOM protein

Poster G1

DOUBLE-STRANDED RNA-MEDIATED GENE SILENCING IN CEREALS

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In a recent report, Fire et al. (1998) described the rapid and efficient gene silencing in ***C. elegans*** by double-stranded RNA (dsRNA) and called the phenomenon „RNA interference“ (RNAi). In plants, a growing body of evidence suggests that posttranscriptional gene silencing is also mediated by double-stranded RNA, also called „aberrant RNA“ (Kooter et al., 1999). In an attempt to develop a versatile, transient gene silencing system for functional testing of defence-related genes, we bombarded leaves of maize, barley and wheat with tungsten particles coated with dsRNAs of different target genes, followed by direct or indirect assessment of target-gene expression. Three approaches have been taken:

1. Co-transformation of cells with a plasmid containing a reporter gene under the control of the CaMV 35S promoter and with dsRNA corresponding to this reporter gene.

2. Co-transformation of cells with dsRNA corresponding to enzymes of the anthocyanin biosynthetic pathway and with ***B-Peru*** plus ***C1*** that are transcriptional regulators of the pathway. Transformed cells turn red within 3 days. The degree of RNAi of the target enzyme of the pathway is assessed by the reduced anthocyanin accumulation.

3. Co-transformation of cells with the ***GUS*** or ***GFP*** reporter gene and with dsRNA corresponding to defence-related genes, followed by inoculation with the powdery mildew fungus. The resistance phenotype of transformed (***GUS***- or ***GFP*** expressing) cells is assessed under the microscope.

Co-transformation of wheat with the ***GUS*** gene and with ***GUS*** dsRNA resulted in weaker ***GUS*** expression. The effect was gene specific, since dsRNAs corresponding to other genes did no significantly reduce ***GUS*** expression. Moreover, the effect was dsRNA specific, since sense RNA as well as antisense RNA did not reduce the expression of ***GUS***. These data suggest a rapid triggering of RNAi in wheat leaves. By contrast, no effect of dsRNA corresponding to ***GFP***, ***oxalate oxidase*** or ***glucose oxidase*** on the expression of the corresponding target genes was found. However, the accumulation of an instable TaGLP2a::GFP fusion protein was inhibited by dsRNA corresponding to ***GFP*** as well as by dsRNA corresponding to ***TaGLP2a***. This suggests that RNA and/or protein stability might be critical for detection of RNAi in this experimental system.

Co-transformation of maize and barley with dsRNA corresponding to the maize ***A1*** gene and the barley ***Ant18*** gene, respectively, with the ***B-Peru*** and ***C1*** genes, resulted in an inhibition of anthocyanin accumulation. As with the ***GUS*** gene, the effect was gene specific and only observed with dsRNA.

We have started RNAi experiments for testing the impact of defence-related genes, which are expressed in the powdery mildew-attacked, epidermal cell layer of wheat and barley, on these plant/pathogen interactions. Genes under evaluation include ***TaGLP2a*** encoding a germin-like protein of wheat, genes encoding members of the phenylalanine-ammonia lyase family in barley, as well as members of the ***Mla***1-mediated signal transduction chain in barley.

Literature:

Fire A et al. (1998) Nature 391: 706-811.

Kooter JM. et al. (1999) Trends Plant Sci 4: 340-347.

Poster G2

SEARCH FOR PLANT COMPONENTS INVOLVED IN HOMOLOGOUS RECOMBINATION: A MUTANT APPROACH.

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Homologous recombination is an important process both in meiosis and during somatic development. It is striking that this process is involved both in genome flexibility, which is important for evolution, and genome stability by participating in DNA repair processes.

Although it was clearly shown that a variety of environmental factors affect the frequency of somatic recombination events, there is rather poor knowledge about the regulatory cascades involved and their connections to the recombination process itself. DNA damage for example induces homologous recombination, but also salt stress, which is ***a priori*** not directly related to DNA damage. There are also recent indications that plant-pathogen interactions might induce recombination.

Fig.1: Restoration of the functional luciferase gene by homologous recombination

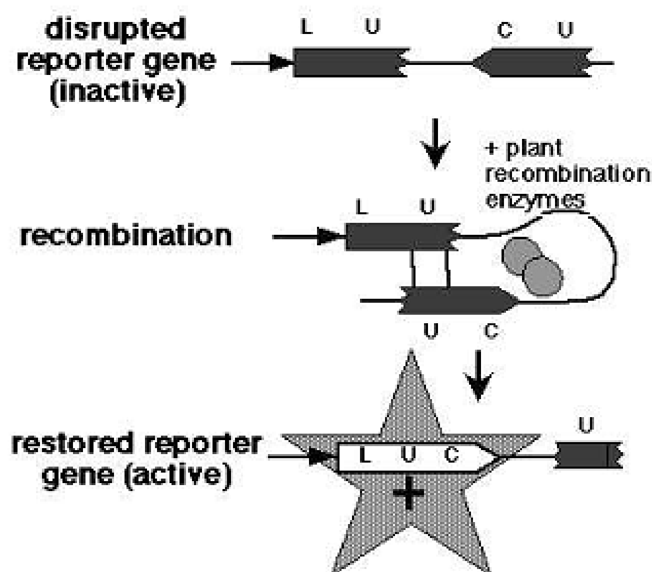

Fig.2: Recombination-up mutant phenotype.

The white sectors show luciferase activity indicative for recombination.

How does the plant cell integrate environmental factors and internal signals to maintain the subtle equilibrium between genome flexibility and stability? To address this question, we used a mutant approach as a powerful tool. We wanted to look more specifically for plant components involved in the regulatory pathways related to homologous recombination and in the recombination process itself.

Using homologous recombination as a marker for genome dynamic, we decided to establish and screen a mutant collection of ***Arabidopsis thaliana*** ecotype Columbia for altered recombination phenotypes in the absence of external stresses. Monitoring somatic recombination phenotypes directly ***in vivo*** is one of the key point for such approach. For this, we used a luciferase reporter line designed for detection of intrachromosomal recombination. The T-DNA construct in this line harbours two truncated but partially overlapping segments of the luciferase gene in opposite orientation (Fig.1). This reporter line was then mutagenised by T-DNA activation tagging, giving rise to a collection of about 20000 independent transformants which were directly screened for dominant recombination-up phenotypes. This first round of screening yielded 46 candidates with a significant increase in the number of luciferase sectors (See example fig.2). All transformants were grown up to set seeds, and the second generation screening for recessive mutant phenotypes has been started.

The 46 candidates displayed an increase in the number of luciferase sectors raging from 10 to more than 100 fold. Strikingly, these sectors were found to be not equally distributed throughout the plant, leading to several distinct patterns which have now to be analysed further. Although in most of the cases the candidate plants and their progeny seem not be developmentaly affected, several plants displayed dramatic effects ranging from bushy phenotypes to sterile or non viable plants.

The genetic characterisation of the mutations and the molecular analysis of the putatively activated genes are on the way. Our project now is to characterise and analyse further some of the recombination-up candidates by crossing them with other ***Arabidopsis*** mutants and recombination lines and cloning of affected genes. The knowledge brought by these results might give new powerful insights into the relation between the genome and it's environment

Poster G3

INTERACTION OF CAMV TRANSACTIVATOR (TAV) WITH COMPONENTS OF TRANSLATIONAL MACHINERY

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The yeast two-hybrid method applied to an Arabidopsis cDNA library has allowed the characterization of several TAV interacting partners. The screening was done either with full length TAV, the N-terminal part of TAV (N-TAV) or the C-terminal part of TAV (C-TAV). I identified a set of proteins specifically interacting with either C-TAV or N-TAV and most of them also show interaction with full length TAV. Terminal and full length TAV interacting protein (CtTIP1 and FTIP1) was found many times as nearly identical clones and exhibits high homology with yeast and mammalian initiation factor 3 (eIF-3) subunit 5 (eIF35). The interacting region of CtTIP1 has been mapped to a central region of the protein which contains Zn finger motif. CtTIP2 interacts with C-TAV, and was identified as ribosomal protein RL24 of the 60S large ribosomal subunit and its N-terminal part is responsible for the interaction. The interactions has been confirmed ***in vitro*** by using GST-fusion system. The effect of their interactions with TAV has been tested in N. Plumbaginifolia protoplast. Over-expression of eIF35 inhibits reinitiation activity of TAV. Thus, eIF35 alone is not functional, but it competes with eIF3 complex for TAV binding. While the interaction of TAV with the ribosomal protein L24 enhances reinitiation activity of TAV.

We are planning to identify further the interacting regions by point mutations in TAV and eIF35. The yeast three hybrid system will be applied to analyse the competition between eIF35 and RL24 for binding to TAV.

Poster G4

*AT*GRP7 A CLOCK-REGULATED PROTEIN IN *ARABIDOPSIS THALIANA*

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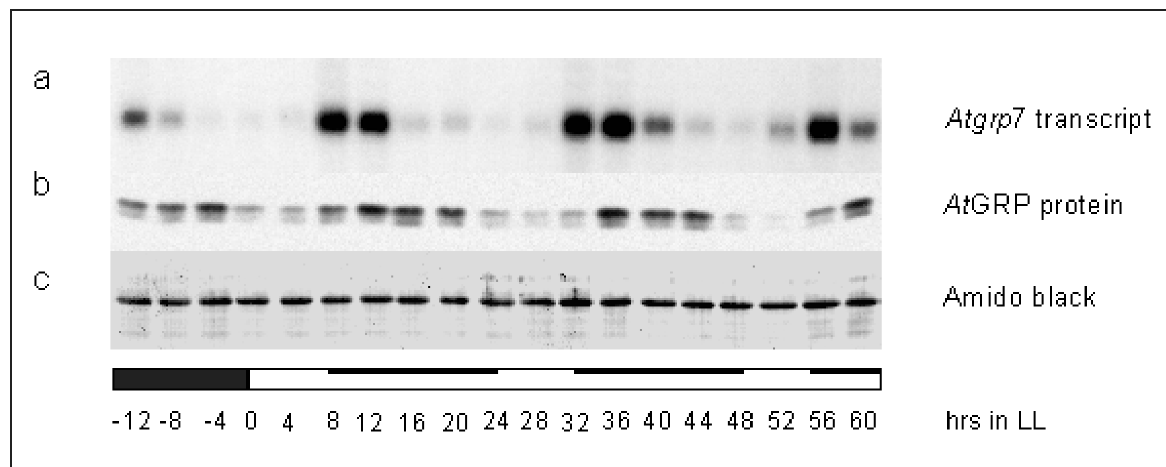
In an differential cDNA screen for clock-regulated transcripts a cDNA encoding a glycine-rich putative RNA-binding protein in *Arabidopsis thaliana* was isolated.



The *Atgrp7* (= *Arabidopsis thaliana* glycine-rich protein 7) transcript oscillates with maximal concentration at the end of the daily light phase. Oscillations of the protein *At*GRP7 are delayed relative to the oscillations of its transcript *Atgrp*7 (see Fig. 1; Heintzen et al., PNAS 94: 8515-8520).

Fig. 1 *Atgrp*7 mRNA and protein cycling in *Arabidopsis*: delay of the protein peak relative to the transcript peak.

Plants were harvested at 4-hour intervals in a light/dark cycle and subsequently kept in

continuous light for the indicated number of hours.(**a**) RNA gel blot hybridised with a gene-specific *Atgrp*7 probe derived from the 5' untranslated region.(**b**) Immunoblot of the same plants reacted with an antibody against *Sa*GRP, followed by chemiluminescence detection.(**c**) Amido black staining of total protein to confirm equal loading and even transfer (the major band corresponds to the ribulose-1,5-bisphosphate carboxylase-oxygenase large subunit).Light and dark periods are represented by open and solid bars, respectively. The inserted bars correspond to subjective night. LL, continuous light.

In transgenic plants constitutively overexpressing *At*GRP7 the oscillations of the endogenous *Atgrp7* transcript are severely depressed (Fig.2). Overexpressing a mutated *Atgrp7* transcript that does not produce a functional protein has no effect, indicating that both the *Atgrp7* transcript and the *At*GRP7 protein are linked in an autoregulatory circuit.

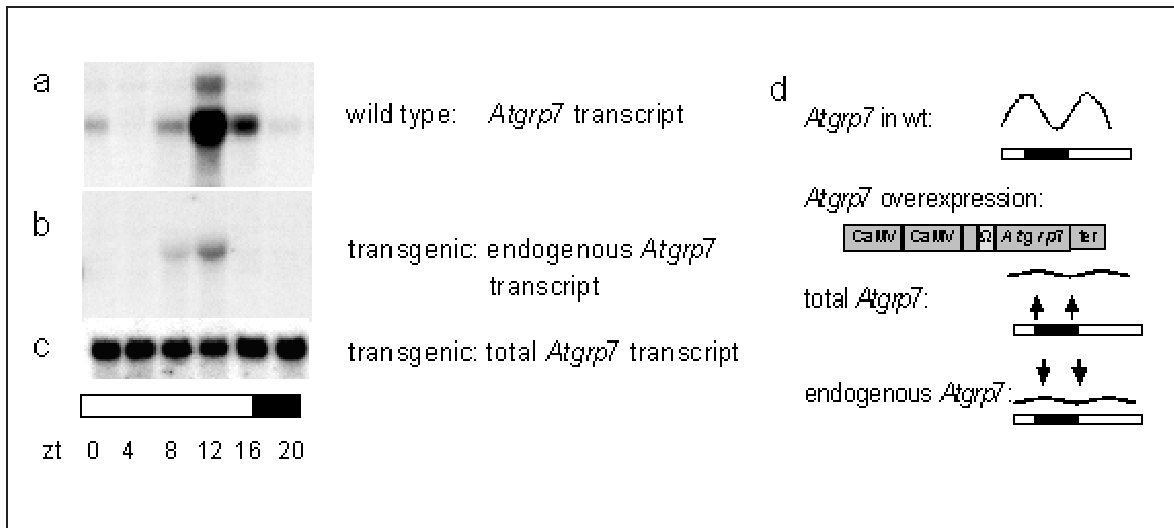


Fig. 2 *At*GRP7 overexpression depresses oscillations of the endogenous *Atgrp*7 transcript.

Wild type plants and transgenic plants overexpressing *At*GRP7 were harvested at 4-hour-intervals in a light/dark cycle. Expression of the endogenous *Atgrp*7 transcript was monitored with a gene-specific probe derived from the 5' untranslated region in wild type plants (**a**) and *At*GRP7 overexpressors (**b**). (**c**) Total *Atgrp*7 transcript level in the transgenic line was monitored with the *Atgrp*7 cDNA probe. (**d**) experimental design. Light and dark periods are represented by open and solid bars, respectively. zt, zeitgeber time.

To determine whether sequence elements mediating *At*GRP7-dependent mRNA cycling would reside within the region mediating rhythmic *Atgrp7* transcription, a representative transgenic line constitutively overexpressing *At*GRP7 was crossed to a line harboring a transcriptional fusion of the *Atgrp7* promoter to the gus reporter gene. Gus mRNA levels are not depressed in the crosses in contrast to the endogenous *Atgrp7* transcript. This indicates that the promoter by itself does not mediate the negative feedback of *At*GRP7 on its own transcript .

The feedback may operate at the posttranscriptional level. As *Atgrp7* codes for a putative RNA-binding protein, this protein may regulate its concentration by direct interaction with its own transcript. Therefore we tested whether an *At*GRP7 fusion protein is able to bind to the *Atgrp7* mRNA *in vitro*. RNA band shift assays show a sequence specific binding of the fusion protein to defined fragments of the *Atgrp7* transcript.

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