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Fusarium comparative genomics reveals lineage-specific chromosomes related to pathogenicity

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Fusarium species are important phytopathogenic and toxigenic fungi, having significant impact on agriculture. Distinctively, strains of *F. oxysporum* exhibit wide host range and are pathogenic to both plant and animal species, reflecting remarkable genetic adaptability. To understand the mechanism underlying such genetic plasticity and rapid pathogenic development, we compared the genomes of three economically important and phylogenetically related, yet phenotypically distinct phytopathogenic species, *F. graminearum*, *F. verticillioides* and *F. oxysporum* f. sp. *lycopersici*. Comparative analysis revealed diverse and co-ordinately transcribed secondary metabolite biosynthetic clusters in *F. graminearum* and *F. verticillioides* as well as greatly expanded lineage-specific (LS) genomic regions in *F. oxysporum* that include four entire chromosomes that account for more than one-quarter of the genome. LS regions are rich in transposons and genes involved in host-pathogen interactions, including known effectors, enzymes targeting plant substrates or processes, and genes involved in lipid signalling and gene silencing. We found evidence for the acquisition of the LS chromosomes through horizontal transfer, which may explain the polyphyletic origin of host specificity in *F. oxysporum* and the rapid emergence of new pathogenic lineages in distinct genetic backgrounds.

Exploring Lineage-specific chromosomes in F. oxysporum species complex

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The Fusarium comparative genomes of *F. graminearum (Fg)*, *F. verticillioides (Fv)* and *F. oxysporum (Fo)* revealed greatly expanded lineage-specific (LS) chromosomes in *Fo*. These mobile LS chromosomes contribute to fungal pathogenicity and host–specificity, providing an explanation for the polyphyletic origin of host specificity and the emergence of new pathogenic lineages in the *F. oxysporum* species complex (FOSC). Following this discovery, a comparative study focusing on the members of FOSC was developed to: 1) examine genome structural variation and confirm the presence of LS chromosomes among different isolates using optical mapping; 2) determine gene content variation among these selected isolates using next-generation sequencing (NGS); 3) identify all lineage-specific genes using targeted sequencing of the LS chromosomes and RNA sequencing via whole transcriptome approaches. One human isolate and 11 plant pathogenic isolates that represent eight *formae speciales* were included in the study. Preliminary results from the optical mapping confirm the existence of LS chromosomes in different isolates. Genomic data generated using NGS detects genome-wide patterns of mutation among isolates during their brief time of evolutionary divergence. RNA-seq data shows great promise in detecting novel genes encoded in the LS chromosomes and for determining gene expression profiles under different conditions.

The identification of a virulence factor-enriched micro-region in the Fusarium graminearum genome

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Initial studies using a novel bioinformatics and statistical approach, identified a genomic micro-region in *Fusarium graminearum* that appeared to be enriched for homologues of verified pathogenicity genes in the other pathogenic species. Detailed analysis of this micro-region by a combination of bioinformatic and reverse genetics approaches has confirmed this micro-region has a role in *F. graminearum* pathogenicity and has lead to the identification of a novel virulence determinant.

This micro-region which is also found in other *Fusaria* genomes appears to be distinctly different from the virulence-associated biosynthetic and secreted protein clusters identified so far in other pathogenic fungi. Further investigation will reveal more about the properties of this small genomic region.

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Variation in sequence and location of the fumonisin mycotoxin biosynthetic gene cluster in Fusarium

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Several *Fusarium* species in the *Gibberella fujikuroi* species complex (GFSC) and rare strains of *F. oxysporum* can produce fumonisins, a family of mycotoxins associated with multiple health disorders in humans and animals.

In *Fusarium*, the ability to produce fumonisins is governed by a 17-gene fumonisin biosynthetic gene (*FUM*) cluster. Here, we examined the cluster in *F. oxysporum* strain O-1890 and nine other species (e.g. *F. proliferatum* and *F. verticillioides*) selected to represent a wide range of the genetic diversity within the GFSC. Flanking-gene analysis revealed that the *FUM* cluster can be located in one of four genetic environments.

Comparison of the genetic environments with a housekeeping gene-based species phylogeny revealed that *FUM* cluster location is correlated with the phylogenetic relationships of species; the cluster is in the same genetic environment in more closely related species and different environments in more distantly related species. Additional analyses revealed that sequence polymorphism in the *FUM* cluster is not correlated with phylogenetic relationships of some species.

However, cluster polymorphism is associated with production of different classes of fumonisins in some species. As a result, closely related species can have markedly different *FUM* gene sequences and can produce different classes of fumonisins.

The data indicate that the *FUM* cluster has moved within the *Fusarium* genome during evolution of the GFSC and further that sequence polymorphism was sometimes maintained during the movement such that clusters with markedly different sequences moved to the same genetic environment.

Novel pathways of regulation of deoxynivalenol production in Fusarium graminearum

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Fusarium head blight of wheat, caused by *F. graminearum*, is one of the most important diseases of wheat not only because of yield losses but also the contamination of grain with trichothecene toxins such as deoxynivalenol (DON).
An intriguing aspect of the pathogen's biology is that the production of DON occurs at much higher levels during the infection process than during axenic culture, even on plant-derived media such as autoclaved grain. Presumably, the fungus produces toxins in response to unknown signals of plant origin. We have used a reporter strain of *F. graminearum* carrying a *TRI*-gene promoter linked to the green fluorescent protein gene to identify compounds that induce high levels of DON production in culture.
Through this system, we have identified a number of amines and polyamine compounds that induced the genes involved in the biosynthesis of DON to levels equivalent to those observed during infection, and resulted in high concentrations (>1500 ppm) of DON being produced in culture filtrate. Polyamines and other inducers increase in concentration in heads following inoculation suggesting that they may act as *in planta* DON inducers.
The Affymetrix *Fusarium* GeneChip® was used to compare gene expression during culture under DON-inducing conditions, to that under non-inducing conditions.
The polyamine inducer agmatine differentially regulated a large number of fungal genes, including both known and uncharacterised putative secondary metabolite biosynthetic gene clusters.

In silico prediction of binding sites for the transcriptional regulator (TRI6) controlling *TRI* gene expression and gene expression analysis in a *TRI6* mutant of *F. graminearum* showed that three of the differentially regulated genes were under the control of TRI6. Gene knock-out mutations of two of these genes resulted in mutants with massively increased production of deoxynivalenol and, under our infection conditions, increased aggressiveness towards wheat.

Our results identify a novel mechanism of negative regulation of DON production in *F. graminearum*.

Characterization of a novel regulatory gene involved in virulence in the phytopathogen Fusarium graminearum

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A study performed by Alexander et al. (2004), published in Current Genetics 45, pp 157-162, suggested *Tri15* may be negatively regulating some of the genes in the trichothecene biosynthetic pathway in *F. sporotrichioides*.
In contrast, disruption of *Tri15* in *F. graminearum*, neither affected its ability to synthesize 15-ADON nor its pathogenicity.

This study explores the role of *Tri15alt*, a homologue of *Tri15*. *Tri15alt* encodes for a protein that has three zinc fingers, two of which are highly homologous to the zinc fingers found in Tri15.

Targeted disruption of *Tri15alt* in *F. graminearum* did not compromise the biosynthesis of 15-ADON. However, pathology studies performed on a susceptible variety of wheat (Roblin) revealed that *Tri15alt* disrupted strain is more virulent than the wildtype strain.
We have performed microarray analyses on this mutant and results will be presented to identify genes involved in virulence.

Incidence of Fusarium graminearum and Fusarium poae from a 2-year wheat monitoring: factors promoting infection and mycotoxin contamination

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In a 2-year investigation, wheat samples and respective information on cultivation techniques were collected from Swiss growers. Wheat kernels were examined for incidence of Fusarium head blight (FHB) causing species and mycotoxin content (LC-MS/MS). From a total of 248 samples originating from 16 out of 26 cantons, three FBH species were dominant: *F. graminearum*, followed by *F. poae* and *F. avenaceum*. The average deoxynivalenol (DON) content was 940 ppb and thus barely below the European limit for unprocessed cereals (1250 ppb). With pre-crop maize and conservation tillage versus ploughing, an average DON content of 2670 ppb or 470 ppb, respectively, was obtained. We also measured the content of other trichothecenes and zearalenone (ZEA). Nivalenol (NIV) and ZEA contents in samples from the same two cropping systems showed a similar pattern as those of DON (NIV: average of 30 and 14 ppb for the two cropping systems; ZEA: 190 and 12 ppb). However, no correlation was found between *F. poae* incidence and the NIV content. Thus, we assume for *F. graminearum* the presence of NIV chemotypes in certain geographic areas. Current fungal incidence and toxin measurements from a third year of monitoring, chemotype investigations as well as in-depth analyses of the cultivation data should contribute to elucidate factors that influence the occurrence and toxin contamination by the most prevalent *Fusarium* species on wheat. The hypothesis of *F. graminearum* NIV chemotypes is in line with recent observations from other European wheat surveys. Hence, it would be worthwhile to discuss the establishment of a concerted initiative assembling data on fungal prevalence and toxins from various geographic areas in order to establish a European map on FHB chemotypes.

Fusarium species, chemotypes and toxins in wheat from Luxembourg

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Seventeen wheat production sites in Luxembourg were chosen to represent the 3 different climatological conditions in Luxembourg. Grains sampled during the years 2007 and 2008 from all the 17 locations were analysed for the presence of Deoxynivalenol (DON), Nivalenol (NIV), T-2 and HT-2 and Zearalenone (ZON).
Seventy-five percent of the investigated fields were contaminated by DON (range 0-8111 µg/kg). Eight fields were also contaminated by NIV. Our study represents the first report of fusariotoxines in harvested grains in Luxembourg. Species determination of Fusarium populations isolated from the same grains used for toxin analysis was carried out according to morphological criteria and confirmed by species-specific PCR. Major species found were *F. graminearum*, *F. poae*, *F. avenaceum* and *F. culmorum*.
In order to verify if chemotype may have an effect on toxin accumulation, *F. graminearum* and *F. culmorum* were screened by using chemotype-specific primers. NIV chemotype was the less frequent one and distributed non-homogenously. Investigating factors that may favour the presence of NIV chemotype in wheat grains, maize as preceding crop showed a significantly positive effect, suggesting its biological role as an ecological niche for the nivalenol chemotype. Nivalenol presence in grains was correlated to the number of *F. culmorum* with NIV chemotype detected in grains (and not to *F. poae* nor to *F. graminearum* with NIV chemotype). Our finding suggests the potentiality of prediction of toxin content by analysing the genetic chemotype of *Fusarium* population from the fields.

A molecular diagnostic for tropical race 4 of the banana

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This study analysed genomic variation of the translation elongation factor 1α(TEF-1) and the intergenic spacer region (IGS) of the nuclear ribosomal operon of *Fusarium oxysporum* f. sp. *cubense* (Foc) isolates, from different banana production areas, representing strains within the known races, comprising 20 vegetative compatibility groups (VCG). Based on two single nucleotide polymorphisms present in the IGS region, a PCR-based diagnostic tool was developed to specifically detect isolates from VCG 01213, also called tropical race 4 (TR4), which is currently a major concern in global banana production. Validation involved TR4 isolates, as well as Foc isolates from 19 other VCGs, other fungal plant pathogens and DNA samples from infected tissues of the Cavendish banana cultivar Grand Naine (AAA). Subsequently, a multiplex PCR was developed for fungal or plant samples that also discriminated Musa acuminata and M. balbisiana genotypes. It was concluded that this diagnostic procedure is currently the best option for the rapid and reliable detection and monitoring of TR4 to support eradication and quarantine strategies.

Characterization of fatty acid regulating transcription factors of Fusarium graminearum

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F. graminearum is a major pathogen of cereals worldwide. Recently, we identified secreted lipases as general virulence factors. To study the regulation of lipase genes we investigate the role of so called cutinase transcription factors. The cutinase transcription factor protein family is extensively present and conserved among filamentous fungi. We identified several putative cutinase transcription factor genes in *F. graminearum* and characterized them by gene disruption. Disruption of *Far1 (fatty acid regulator1)*, a homolog of *Aspergillus FarA* gene, indicates that it is important for long chain fatty acid utilization. Disruption of *Far2*, a homolog of the *Aspergillus FarB* gene, demonstrates that *Far2* is required for very short chain fatty acid assimilation by the fungus. *Lr1 (lipase regulator1)*, which belongs to the *Far1* clade, leads to reduced total extracellular lipolytic activity and transcriptional repression of several lipase genes in culture. These results suggest that *Lr1* mediates expression of genes involved in fatty acid hydrolysis. In summary, our results show that transcription factors of the plant pathogen *F. graminearum* are involved in regulation of genes important for fatty acid assimilation and lipid hydrolysis.

Infection cushions and mycotoxin induction of Fusarium graminearum on wheat florets

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The mycotoxin producing pathogen *Fusarium graminearum* is the causal agent of Fusarium head blight (FHB) of small grain cereals on fields worldwide. Although *F. graminearum* is one of the best investigated phytopathogens, detailed information about fungal development on host surfaces and the penetration strategy of the pathogen is limited. We established a bioassay that allows a comprehensive investigation of the inoculated host surfaces. Detection of mycelium was facilitated by constitutive expression of a *dsRed* reporter gene, thereby allowing bioimaging with white light and fluorescence stereomi-

croscopy, as well as confocal laser microscopy. Additionally, a *GFP* coupled *TRI5*-promotor allows monitoring of the mycotoxin desoxynivalenol production during infection. Combining bioimaging with scanning electron microscopy we identified penetration structures and alterations of the host surface on a three-dimensional level. For the first time we demonstrate the formation of infection cushions during *F. graminearum* infection on host tissues. We discovered that the infection cushions are attended by an intensive subcuticulary growth stage of the pathogen and exhibit a high mycotoxin induction. Surprisingly, a *TRI5*-k.o. mutant exhibits the same infection strategy and efficacy. We conclude that mycotoxin production is specifically induced in infection structures but not necessary for penetration.

Loss-of-function of the avirulence gene, SIX4, by transposon-insertion in tomato wilt pathogen Fusarium oxysporum f. sp. Lycopersici

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Fusarium oxysporum f. sp. *lycopersici* (*FOL*) is the soilborne pathogen of tomato wilt. In the pathogen, three races 1, 2, and 3 have been determined based on the specific pathogenicity to tomato varieties. The compatible or incompatible relationships between races and varieties can be explained by the interactions between the avirulence genes carried by *FOL* and resistance genes carried by tomato varieties according to gene-for-gene theory (Flor, 1956). For example, race 1 carrying *AVR1* is avirulent to tomato cultivars with a resistance gene *I*, and races 2 and 3 carrying no *AVR1* is virulent to the tomato cultivars with *I*. Houterman et al. (2008) reported *SIX4* corresponding to *AVR1* in *FOL* race 1. In 2008 a strain of *FOL* (KoChi-1), overcoming *I*-mediated resistance, emerged in Japan. Although KoChi-1 is not race 1, PCR revealed that KoChi-1 carried *SIX4*. Sequence analysis showed that *SIX4* ORF in KoChi-1 was truncated by a transposon (759 bp). The inserted transposon is non-autonomous and belongs to *hAT* family (Hua-Van et al., 2000). According to the Genome Databases of Broad Institute, 72 copies of the identical transposon exist in *F. oxysporum*. Integration of an intact *SIX4* derived from a race 1 isolate into KoChi-1 genome complemented avirulence to a tomato cultivar possessing *I*. This is the first report of an avirulence gene truncated by transposon-insertion in *F. oxysporum*.

Fungal virulence and host susceptibility genes in the Fusarium oxyporum-Arabidopsis interaction

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The interaction of the root infecting fungal pathogen *Fusarium oxysporum* with Arabidopsis is a highly tractable system for a molecular analysis of fungal virulence and host susceptibility and immunity. We have completed a rigorous analysis of 6868 T-DNA insertion mutants of Arabidopsis Col-0 ecotype, selected lines with altered disease phenotype (P=<0.01) and re-tested these to identify mutants with significant & reproducible increased resistance or susceptibility. Second allele insertions are currently being tested to provide certainty on specific gene functions. These studies have identified ~100 novel genes with previously unidentified roles in immunity and susceptibility to this pathogen. To complement this we have identified a small range of fungal mutants with altered pathogenicity and virulence. One of these includes mutants in the SIX4 gene which is required for full virulence. Experiments are underway to attempt to match putative functions in the host that are necessary for susceptibility with functions in the pathogen required for virulence. Initial experiments are focusing on the role of host jasmonate signalling in susceptibility and how the pathogen may intervene in this.

Role of a mucin-like membrane protein in signalling and pathogenicity of Fusarium oxysporum

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The soilborne fungus *Fusarium oxysporum* causes vascular wilt in a wide range of plant species by penetrating roots, invading the cortex and colonizing the vascular tissue. Fmk1, a mitogen activated protein kinase (MAPK) orthologous to *S. cerevisiae* Fus3 and Kss1, is essential for plant infection. The signalling components upstream of the Fmk1 cascade are currently unknown. In yeast, the membrane mucin Msb2 functions at the head of the filamentous growth MAPK cascade. We identified a gene from *F. oxysporum* whose predicted product has sequence homology with yeast Msb2 and shows a similar domain structure, including an N-terminal signal sequence, a predicted serine-threonine rich mucin region, a transmembrane domain and a short cytoplasmic tail. Western analysis using an HA-tagged Msb2 version showed that *F. oxysporum* Msb2 is an integral membrane protein which is expressed during vegetative growth and tomato root infection. Deletion mutants lacking *msb2* showed reduced phosphorylation levels of Fmk1, suggesting that Msb2 may function upstream of this MAPK. In contrast to △*fmk1* strains, △*msb2* single and △*fmk1*/△*msb2* double mutants exhibited enhanced sensitivity to the cell wall-targeting compounds Congo Red and Calcofluor White, suggesting that Msb2 also signals in an Fmk1-independent pathway functioning in the cell wall stress response. The △*msb2* strains showed delayed invasive growth across cellophane membranes and significantly reduced virulence on tomato plants.

Our results suggest that Msb2 is a mucin-like membrane protein that contributes to invasive growth and virulence of *F. oxysporum* by signalling partly via the Fmk1 MAPK cascade.

Transcriptional analysis of the response to extracellular pH changes in Fusarium graminearum Pac1 mutants and effect on trichothecenes B accumulation

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Fusarium graminearum infects wheat and maize and produces type B trichothecenes. These mycotoxins cause serious problems when consumed via contaminated cereals. *Tri* genes, located in the "*Tri* cluster", are responsible for the biosynthesis of trichothecenes B. *In vitro*, *Tri* genes of *F. graminearum* strain CBS 185.32 are expressed at day 3 with the toxin starting to accumulate one day latter. Strikingly, the induction of *Tri* genes expression always seems concomitant with a sharp pH drop in the media. Acidic pH seems a determinant factor for induction, as neither the toxin nor the *Tri* genes are detectable at neutral pH. The pH regulation of gene expression in fungi is mediated by the Pac1 transcription factor involved in various secondary metabolites regulation. An *FgΔPac1*deletion mutant and a strain expressing a constitutively active form (*FgPac1C*) were constructed in *F. graminearum*. Expression of this constitutive Pac1^{C} factor strongly reduces expression of *Tri* genes and toxin accumulation at acidic pH. Unexpectedly, deletion of *Pac1* does not induce toxin production at neutral pH. However, it causes an earlier *TRI5* induction and toxin accumulation at acidic pH. In order to determine the interference with other *Tri* genes regulatory mechanisms, exploring general transcriptional response to pH variation for mutants and wild-type strains were also performed using microarrays. Preliminary results will be presented.

Characterization of the serine-/threonine protein kinase gad8 in the phytopathogenic fungus Fusarium graminearum

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Fusarium graminearum is the causal agent of Fusarium head blight and a highly destructive disease of all cereals. A knock-out of the serine-threonine protein kinase *gad8* in this fungus leads to a severe inhibition of growth *in vitro* as well as of virulence *in planta*.

To determine the role of this gene in the regulatory pathways involved in fungal development, a complementation study in yeast was accomplished.
The aimed homologue of *gad8* in *S. cerevisiae*, is coding for the AGC-type protein kinase. Ypk1 and is part of a signalling module which activates a phosphorylation cascade.

This pathway is stimulated by sphingolipid base phytosphingosine, a metabolic product of sphingolipids which are upregulated by several stimuli and serve as second messenger in signal transduction pathways and controls a wide range of cellular processes including growth, cell wall integrity, stress resistance, endocytosis and aging.

Ypk1 is a high copy suppressor gene that allows growth when the synthesis of sphingolipids is inhibited. We could show that the Δpks1 strain in *S. cerevisiae* is less stress tolerant than the wt in regard to temperature and toxic agents.

Additional tests with the complemented yeast as well as studies with the *gad8* knock-out in *F. graminearum* will be presented.

The tetraspanin FgPls1 is involved in fitness and pathogenicity of Fusarium graminearum

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Tetraspanins are a a group of membrane proteins with four transmembrane domains common among different species like mammals, fish, insects, and fungi.

Their capacity is complex and involved in a broad range of physiological processes where they function as “molecular facilitator”, interacting with proteins from different families like integrins, proteoglycans, growth factors and growth factor receptors as well as members from the Ig superfamily.

In fungi, three different families of tetraspanins have been characterized so far: Pls1, which is found in both ascomycetes and basidiomycetes, Tsp2, which is unique to basidiomycetes, and Tsp3, which is exclusively found in ascomycetes.

Pls1 knock out mutants in three appressorium producing plant pathogenic fungi, *Magnaporthe grisea*, *Botrytis cinerea*, and *Colletotrichum lindemuthianum* were non pathogenic on their respective host plants which proves that Pls1 is a pathogenicity factor in these fungi.

In this study, we identified a tetraspanin Pls1 like protein, named FgPls1 in the wheat scab fungus *F. graminearum*. Results show that FgPls1 is important for vegetative growth and the production of macroconidia as well as pathogenicity of *F. graminearum*.

RNA interference in Fusarium graminearum using intron containing hairpin vectors

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RNA interference is a posttranscriptional gene silencing mechanism. In multinucleate filamentous fungi, RNAi is an efficient way to silence multiple gene copies at the same time. We used transgenic strains of *Fusarium graminearum* expressing the GFP gene from *Aequorea victoria* and the DsRed gene from *Discosoma sp.*, respectively, under control of the gpdA promoter. To maintain a permanent down regulation of the target genes, we used an intron containing hairpin vector carrying fragments of the DsRed/GFP gene. To monitor the effects of silencing, we investigated the down regulation on the transcriptional level through RT-PCR and quantitative real-time PCR, and on translational level through western blotting, fluorescence microscopy, and measurement of the fluorescence level in 96 well plates. Most of the transformants showed a down regulation compared to their respective wild type, albeit to various degrees. Our experiments confirmed the results concerning siRNA based silencing by hairpin constructs in *Fusarium graminearum*. We report here that 400bp sense and antisense fragments are sufficient to maintain silencing. The applied method will be an efficient means to down regulate lethal genes and create new insights in the genome of *Fusarium graminearum*.

De Koninklijke Nederlandse Plantenziektekundige Verenging roept kandidaten op voor de functie van

Voorzitter KNPV

Omdat de laatste zittingstermijn van onze huidige voorzitter Gert Kema eind dit jaar afloopt is de KNPV op zoek naar een nieuwe voorzitter. Vanwege de spilfunctie die een voorzitter heeft binnen onze vereniging is onderstaand functieprofiel opgesteld:

De voorzitter (m/v) is:
- Een netwerker en samenbinder.
- Representatief voor de gehele KNPV, met sterke voeling voor de praktijk.
- In staat buiten de eigen werkomgeving te kijken en te denken.
- Een professionele procesbegeleider.
- Een strategische denker met visie op de rol van gewasbescherming in de samenleving.
- Een natuurlijke leider met een krachtige en energieke uitstraling.

De voorzitter van de KNPV is in staat om bruggen te bouwen en draagt actief bij aan het profiel en de visie van de vereniging. Hij/zij is in staat om scholieren en studenten te interesseren voor de gewasbescherming en initieert activiteiten met een nationale uitstraling. Hij/zij geeft leiding aan het bestuur en de staf van de KNPV en vormt met de secretaris en de penningmeester het dagelijks bestuur. Hij/zij is nauw betrokken bij de organisatie van bijeenkomsten en is het gezicht en de vertegenwoordiger van de vereniging in internationaal verband.

De zittingstermijn voor elk KNPV-bestuurslid is in principe drie jaar; een termijn kan eenmaal, en in bijzondere gevallen tweemaal, worden verlengd. Het bestuur van de KNPV roept kandidaten op om te reageren.
Indien u interesse hebt in deze functie kunt u dit kenbaar maken aan onze secretaris Jan Bouwman (Jan.Bouwman@syngenta.com). Voor verdere informatie kunt u contact opnemen met de huidige voorzitter Gert Kema (tel. 0317-480632).