Sit4-associated protein is required for pathogenicity of *Leptosphaeria maculans* on *Brassica napus*

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Abstract

An insertional mutant with reduced pathogenicity on *Brassica napus* was identified in the plant pathogenic fungus *Leptosphaeria maculans*. The transfer-DNA molecule from *Agrobacterium tumefaciens* inserted into a gene encoding a protein with similarity to SIT4-associated proteins (SAP). In contrast to *Saccharomyces cerevisiae* which has four members of the SAP family, there is a single copy of the gene in *L. maculans*. The mutant had normal spore production and spore germination, but altered hyphal branching, suggesting that nutrient signaling is impaired in the strain. This is the first time that a SAP gene has been mutated in a filamentous fungus and links the function of SAP proteins to plant pathogenesis and hyphal branching.

Keywords: canola, insertional mutagenesis, *sap1*, target of rapamycin

Introduction

The Dothideomycete *Leptosphaeria maculans* is a major burden on the canola (*Brassica napus*) industry worldwide because it causes the disease “blackleg”, which results in severe yield loss [10]. Antifungal targets in this species, in particular those genes that are required by the fungus during pathogenesis, are being sought by techniques that include insertional mutagenesis [28].

Despite significant effort, only nine genes required for pathogenicity on *B. napus* have been identified in *L. maculans*. These encode a predicted isocitrate lyase [14], a gene of unknown function [15], 3-ketoacyl-CoA thiolase [8], a plasma membrane H⁺-ATPase [24], a GPI-anchor biosynthesis pathway gene [25], UDP-glucose-4-epimerase [26], a gene affecting cell wall integrity [35], a homolog of the SNF1 protein kinase [9] and the StuA transcription factor [31]. The majority of these genes have been identified using insertional mutagenesis. The slow rate of gene discovery using insertional mutagenesis in part relates to the fact that the inserted DNA often occurs between rather than within genes and multiple insertions may occur simultaneously [3, 5, 15]. This complicates the analysis of resulting pathogenicity mutants. For example, a reduction in pathogenicity may result from misregulation of gene expression, as in the case of 3-ketoacyl-CoA thiolase [8]. Furthermore, another problem encountered when using an insertional mutagenesis approach is that the loss of pathogenicity may not be due to the disrupted gene itself but due to background mutations at other positions in the genome. Blaise *et al.* found that only 50% of mutants had loss of pathogenicity phenotypes segregating with the T-DNA insert [3]. Another consideration is that a second gene may be disrupted elsewhere in the genome by a second T-DNA insertion. Analysis of gene function using targeted gene disruption is equally problematic, with targeting being at rates lower than 0.25% of transformants [16]. However, as more pathogenicity genes are discovered, the types of processes involved in the pathogenicity of *L. maculans* will become clearer, potentially leading to new strategies to control blackleg disease.

In many plant pathogenic fungi, genes involved in signalling in response to the external environment are of key importance to pathogenesis. An example of a well studied family of such genes is the mitogen activated protein kinases. Mutants in these genes have reduced pathogenicity in a number of species including *Bipolaris sorokiniana* [20] and *Magnaporthe oryzae* [37].

Another type of signalling kinase, sucrose non-fermenting protein kinase 1 (SNF1), has been identified as being required for virulence in *L. maculans* [9]. Feng *et al.* found that in *L. maculans* SNF1 expression was induced by the non-preferred carbon source pectin, which agrees with the function of the *Saccharomyces cerevisiae* homolog that is to derepress genes that are repressed in the presence of the preferred carbon source

glucose [6, 9]. This finding indicates that signalling in response to environmental stimuli, in particular nutrient availability, might be important to the ability of *L. maculans* to cause disease.

In this paper we describe an insertional mutant in a Sit4-associated protein homolog (SAP) that shows markedly reduced pathogenicity. SAPs are part of the Target of Rapamycin (TOR) signalling pathway and associate with the protein kinase, Sit4.

Materials and methods

***Leptosphaeria maculans* isolates and culture conditions**

The *L. maculans* isolate D5 (M1; IBCN18) was used for insertional mutagenesis and isolate D9 (05MGPS049) was used for mating experiments. *L. maculans* was subcultured on V8 agar plates [10% V8 juice (Campbell's), 2% agar, pH 6] at 22°C with a 12 h night/day cycle. Hyphal and colony morphology was observed on both V8 agar and on water agar. Asexual pycnidiospores from sporulating cultures of *L. maculans* were harvested by scraping the surface of V8 agar plates with sterile water, and subsequently filtered through Miracloth (Millipore). Mycelial cultures for DNA extraction were set up in liquid V8 juice medium and incubated for one week at room temperature.

Agrobacterium*-mediated transformation of *L. maculans

A plasmid containing a hygromycin resistance gene was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. This plasmid, pLAU12, is modified from pPZPHygHindX [8] for the overexpression of genes in *L. maculans* (manuscript in preparation). These additional sequences do not modify the T-DNA transfer capabilities or selection system. *A. tumefaciens*-mediated transformation of *L. maculans* was carried out and transformant were selected using an agar overlay method described previously [11]. Antibiotics in the overlay were cefotaxime (200 µg/ml) and hygromycin (100 µg/ml).

Pathogenicity tests on *Brassica napus* cotyledons

Cotyledons of *B. napus* cultivar Westar were inoculated 10 days after seed germination by first wounding the leaves with modified tweezers (four inoculations per plant) then applying 10 µl of pycnidiospores (~5×10⁶ spores/ml); 150 transformants were screened. After 14 days cotyledons were photographed and disease was assessed by measuring the area of necrosis. Image J software [30] was used to assess disease for 70, 76, 85 and 80 inoculation points for the wild type D5 isolate, the T-DNA insertional mutant, and complementation strains

SAPC1_1 and SAPC9_1, respectively. Student's *t*-tests were used to determine statistical significance for $P < 0.05$.

Trypan blue staining of infected cotyledons

Hyphae in infected cotyledons (14 days post inoculation) were stained with trypan blue [18]. Cotyledons were immersed in staining solution (10 ml lactic acid, 10 g phenol, 10 ml glycerol, 10 mg trypan blue (Sigma-Aldrich T0776), 10 ml H₂O mixed 1:1 with ethanol immediately prior to use) and boiled for several minutes. Cotyledons were destained in chloral hydrate (70% w/w in H₂O) for one week then observed under a compound microscope.

Genomic DNA extractions from *L. maculans* mycelia

Mycelia (~100 mg) were freeze-dried then ground on a MM 300 mixer mill (Retsch Instruments) using tungsten carbide beads at 30 Hz for 30 s. Homogenized tissue was mixed with DNA isolation buffer (0.5 M NaCl, 10 mM EDTA, 1% SDS, 10 mM Tris-HCl) in a phase lock-tube. Phenol:chloroform (1:1) was added and the samples were centrifuged for 15 min at 13000 rpm. Chloroform was added to the supernatant and the samples were centrifuged for 10 min at 13000 rpm. DNA in the supernatant was precipitated by adding 0.6 volume of isopropanol and pelleting by centrifugation at 13000 rpm for 5 min.

Inverse PCR

Inverse PCR [23] was used to identify the site of T-DNA insertions in the genome of the T-DNA insertional mutant. Genomic DNA was digested with PstI restriction enzyme (Promega) at 37°C for 3 h and the digested fragments circularised using T4 DNA ligase (Promega). PCR reactions were set up off the circularised template using ExTaq polymerase (Takara) and the primer pairs M13F (5' GTAAAACGACGGCCAG 3') / ai76 (5' AACAGTTGCGCAGCCTGAATG 3') and M13R (5' AACAGCTATGACCATG 3') / ai077 (5' AGAGGCGGTTTGCGTATTGG 3'). The location of the insert was confirmed by a second PCR using primers AU130 (5' ACAACAGGCCAGGGACTGG 3') and ai077. Amplicons were sequenced and the sequences were matched to the *L. maculans* genome ([29]; available through <http://genome.jgi.doe.gov>) using BLAST [1].

cDNA cloning and sequencing

Mycelium of isolate D5 was cultured in a defined minimal medium [34] with 2% glucose as the carbon source. RNA was isolated using Trizol reagent following the manufacturer's guidelines (Invitrogen). cDNA

was synthesized using SuperScript IV (Invitrogen). The full length open reading frame was amplified with primers MAI0152 (5' GGATCCATGTTTTGGCGCTTCGGC 3') and MAI0153 (5' GGATCCTTATCGATAGTCCATGTCAGC 3') and amplicons were cloned into the pCR™ 2.1-TOPO® TA vector (Invitrogen). The cloned fragments were sequenced.

Crosses and genetic segregation analysis

The *Leptosphaeria* T-DNA insertional mutant was crossed to isolate D9. Plugs cut from the two isolates were placed on V8 agar mating medium [20% V8, CaCO₃ (0.2%), agar (2%)]. Strains were grown for one week at 22°C with a 12 h photoperiod then overlaid with water agar (1% agar). After 4-6 weeks incubation at 14°C with a 12 h black-light photoperiod, pseudothecia were produced and individual ascospores were isolated. Progeny were screened for resistance to hygromycin, colony morphology and pathogenicity on *B. napus*. In addition, the mating type of each of the progeny was determined by PCR as described previously [7].

Complementation of the *Leptosphaeria* mutant strain

The T-DNA insertion in the *Leptosphaeria* pathogenicity mutant was located in the homolog of a gene encoding a Sit4-associated protein homolog. The mutant was complemented with the wild type allele. The genomic sequence of the *L. maculans sap1* gene (Lema_T071490) from isolate v23.1.3 was obtained from the Joint Genome Institute database. The predicted intron-exon sequence was corrected by mapping RNA-seq reads [21] onto the gene using Integrative Genomics Viewer [33] and from sequencing cDNA clones. The gene information available on the JGI database contained 150 bp of unknown sequence. Covering this region using a combination of Sanger sequencing and next generation sequencing reads revealed that this 150 bp, in addition to 21 bp and 268 bp upstream and downstream, respectively, is not present in isolate D5 (Fig S1). Seven hundred and forty-one bp of sequence upstream of the start codon was taken as the promoter and 819 bp of sequence downstream of the stop codon as the terminator. Three PCR fragments covering the gene were obtained from isolate D5: one corresponding to the promoter of the gene (AU172 5' GCCTCACCGCGGCCCATGGTCATGCCCACCCACTTTTGC 3' and AU138 5' GCCGAAGCGCCAAAACATGG 3'), one corresponding to the coding region of the gene (AU131 5' ATGTTTTGGCGCTTCGGCGG 3' and AU141 5' TTATCGATAGTCCATGTCAGC 3') and a third to the terminator (AU173 5' GCTGACATGGACTATCGATAATCC 3' and AU174 5' GTCATCTTCTGTCTGAATCGGTACCCAGCTATCCCGATCCCTTGCCG 3'). The three fragments of the

gene were then cloned using Gibson assembly (New England Biolabs) into plasmid pPZPNat1, which includes the gene for nourseothricin acetyltransferase for selection [12], linearized with XbaI and XhoI. *Agrobacterium*-mediated transformation was used to transform this complementation construct into the *Leptosphaeria* SAP mutant strain with selection on medium containing nourseothricin (200 µg/ml in the overlay). Two independent transformants, named SAPC1_1 and SAPC9_1, were characterized.

Radial growth and pycnidiospore germination assays

The *in vitro* growth rates of strains were determined by placing 2 µl of pycnidiospores (10^7 spores/ml in H₂O) onto the center of agar plates. A cross was drawn on the bottom of the plate and growth (at 22°C with a 12 hour photoperiod) along each of the four lines was measured at regular intervals from day 4 to day 16. Two different agar media were used: water agar (1% agar in H₂O) and clarified V8 juice medium. The colonies grown on water agar did not show sufficient contrast to the media to be photographed, so the hyphae were stained using lactophenol cotton blue (0.05 g cotton blue, 20 g phenol, 20 ml lactic acid, 40 ml glycerol and 20 ml of distilled H₂O) by flooding the stain over the surface of the media and removing excess stain after several minutes.

Spore germination was assessed *in vitro* with a method adapted from that used by [9]. Spores (200 µl of 1×10^7 spores/ml in distilled H₂O) were mixed with 10 ml of molten 0.8% V8 agar. The mixture was poured into a petri dish and kept at 22°C in a dark incubator. After 48 h, a portion of agar was cut from the center of the plate, stained with lactophenol cotton blue, placed on a microscope slide and then pressed under a coverslip. Approximately 100 spores were counted under a compound light microscope and the proportion of spores that had germinated was recorded. Bright field images of hyphae *in vitro* and *in planta* were taken using a Leica DM2500 compound microscope with a Leica DC 300F digital camera.

Results

Isolation of a mutant of *L. maculans* with reduced pathogenicity on canola

One strain with reduced pathogenicity was identified after screening 150 strains derived from *A. tumefaciens* transformation. The mutant caused markedly reduced disease on cv. Westar, with an average lesion area of 2.1 mm² compared to 19.7 mm² for the wild type (Figs 1A and 2). This difference was statistically significant as determined by a Student's *t*-test. In cotyledons infected with the wild type *L. maculans* isolate, long hyphae

penetrated far into the leaf, whereas in the mutant such hyphae were rarely seen. In cases where a small lesion had formed, the hyphae never extended far from the initial inoculation point and were highly branched (Fig 1B).

Identification of the T-DNA insertion site within a gene (*sap1*) encoding a Sit4-associated protein homolog

One flank of the T-DNA insertion was obtained by inverse PCR. Sequencing and subsequent BLAST of the inverse PCR product indicated that the insert was located on SuperContig 7 of the *L. maculans* genome, into the first exon 307 bp downstream of the start codon of the gene Lema_T071490.1, which is annotated as “similar to Sit4-associated protein

(SAP185)”. A second PCR was used to define the other side of the insertion and revealed a deletion of 22 nucleotides from the genomic DNA of *L. maculans* (Fig 3). Due to ambiguities in the annotation of the gene from the genome sequence project, the coding regions of the *sap1* gene were confirmed by isolating and sequencing cDNA molecules. An annotated version of the DNA sequence and the coding region for the *sap1* gene is presented in Fig S1. As a consequence of the T-DNA insertion, the predicted protein is truncated to just 119 amino acids, of which 102 match to the start of the wild type SAP1 sequence of 964 amino acids.

The growth rate of the SAP mutant is altered

The growth rate of the *Leptosphaeria* SAP mutant strain, as measured by colony diameter, was reduced on both water agar and V8 agar. On water agar the growth rate of the wild type was 2.1 mm/day while the SAP mutant grew at 1.0 mm/day (Fig 4A). On V8 agar the wild type grew at 1.6 mm/day compared to 0.78 mm/day in the SAP mutant (Fig 4B). This difference is attributed to hyphal growth rather than a germination defect, because the spores of the wild type and the *Leptosphaeria* SAP mutant had a similar germination frequency after 48 h in two independent experiments (93% and 74% for wild type spores and 94% and 82% for the SAP mutant spores).

Hyphal and colony morphology are impaired in the SAP mutant

The morphology of the *Leptosphaeria* SAP mutant colonies grown on V8 agar differed from the wild type due to the presence of short hyphal branches, giving the mycelium a slightly ‘fluffy’ appearance (Fig 5). There was considerable difference between the *Leptosphaeria* SAP mutant and the wild type on water agar because the

mutant did not show the reduction in branching displayed by the wild type (Fig 5). The wild type colony was sparse, consisting of long trailing hyphae extending into the medium, whereas the *Leptosphaeria* SAP mutant formed a smaller, denser colony of highly branched hyphae.

Genetic segregation and complementation experiments confirm that mutation of the *sap1* gene is responsible for the mutant phenotypes

Twenty-four progeny were obtained from a cross between the *Leptosphaeria* SAP mutant and isolate D9: 14 were hygromycin-sensitive and 10 were hygromycin-resistant. This ratio is consistent with a single T-DNA insertion event into the genome. Thirteen out of the 14 hygromycin sensitive progeny were able to cause disease to a similar extent to that of a wild type isolate and all ten of the hygromycin-resistant progeny showed reduced pathogenicity. Of the hygromycin-sensitive progeny, 10 showed a wild type phenotype *in vitro* (fast growth with a smooth colony edge), three showed a reduced growth rate but normal hyphal morphology, and one showed an unusual hyphal morphology somewhat similar to the phenotype of the *Leptosphaeria* SAP mutant. Of the hygromycin-resistant progeny, seven showed a branching phenotype similar to the *Leptosphaeria* SAP mutant, two showed an intermediate phenotype in which the hyphae appeared only slightly more branched and one isolate showed little growth. The mating types of the progeny were assessed by PCR, indicating that the progeny set includes recombinant classes. Of the hygromycin sensitive isolates six were MAT1-2 and eight were MAT1-1. Of the resistant progeny six were MAT1-1 and four were MAT1-2.

A wild type copy of the *sap1* gene was transformed into the *Leptosphaeria* SAP mutant.

Complementation with the wild type copy of the gene restored the pathogenicity of the mutant back to the wild type level in two independently complemented strains (average lesion areas of 18.2 mm² in strain SAPC9_1 and 21.7 mm² in strain SAPC1_1, Figs 1A and 2). These values were not statistically significantly different from the wild type as determined by a Student's *t*-test. Additionally the complemented isolates had the equivalent growth rates as wild type (Fig 4), and they had a smooth colony edge and lacked the branching hyphal protrusions present in the mutant (Fig 5).

Discussion

Of 150 insertional mutants of *L. maculans* screened on *B. napus* cotyledons, one transformant was identified as having reduced pathogenicity. In a cross with this transformant, the T-DNA segregated with the reduction of pathogenicity. The mutant phenotype was rescued by complementation with a wild-type copy of

the SAP gene. Together, these data indicate that the T-DNA insertion is responsible for the reduction of pathogenicity. Bourras *et al.* showed that T-DNA insertion is biased towards regulatory regions of genes, including promoters, and that this T-DNA integration pattern can be explained by the existence of microhomologies between promoter sequences and the T-DNA left border flanking sequence [5]. Interestingly, microhomologies to the T-DNA left and right borders were also detected at the T-DNA insertion site of our transformant which might explain why the T-DNA has inserted into the coding region of the *sap1* gene.

Although the *Leptosphaeria* SAP mutant grew more slowly than the wild type isolate, this does not solely account for the reduction of pathogenicity phenotype because wild type *L. maculans* isolates show a wide range of growth rates. A more likely contributing factor to the reduction in pathogenicity is the increased branching phenotype seen in the hyphae of the *Leptosphaeria* SAP mutant. This phenotype was most obvious on the low nutrient water agar where hyphae of the wild type and complemented strains were markedly less branched than that of the mutant. This *in vitro* phenotype is reflected *in vivo* during infection. When inoculated onto plants the wild type produces long penetrating hyphae that extend into the leaf, while the *Leptosphaeria* SAP mutant was impaired in this process..

The disrupted gene in the *Leptosphaeria* SAP mutant is a homolog of a component in the target of rapamycin (TOR) signaling pathway. TOR signaling plays an important regulatory role in eukaryotic cells. In the model fungus *S. cerevisiae* TOR signaling couples cellular metabolism to nutrient availability [27]. TOR promotes Tap42 to form complexes with type 2A and type 2A-like phosphatases [36]. One such type 2A-like phosphatase is Sit4. TOR is active when nutrients are available, which results in the association of Sit4 and Tap42 to form an inactive complex. On the other hand, when nutrients are limited, TOR is inactive and the TAP42-Sit4 complex dissociates leaving the Sit4 phosphatase active [2]. One of the roles of the active Sit4 protein in *S. cerevisiae* is to dephosphorylate the transcription factor Gln3, which results in untethering from the cytoplasmic protein Ure2 and subsequent localization into the nucleus leading to transcription of genes needed for adaptation to nutrient stress [2]. In addition to Tap42, in both *S. cerevisiae* and *F. graminearum* Sit4 also interacts with a number of other proteins including Sit4-associated proteins (SAPs) [22, 36]. *S. cerevisiae* contains four SAP homologs - *SAP185*, *SAP190*, *SAP155* and *SAP4*. This contrasts to *L. maculans* in which there is only a single SAP homolog in the genome, as assessed by BLAST.

Deletion of Sit4 results in serious growth defects or loss of pathogenicity in a number of fungi including *S. cerevisiae* [22], the human pathogens *Aspergillus fumigatus* [4] and *Candida albicans* [19] and the filamentous plant pathogen *Fusarium graminearum* [36]. SAP loss-of-function mutants have only been reported

for *S. cerevisiae* [17, 22, 27]. Deletion of either *SAP185* or *SAP190* alone did not cause a significant decrease in growth rate. However, in the *sap185 sap190* double mutant, growth rate was significantly reduced. A quadruple mutant of all four *SAP* genes had a severe decrease in growth rate of an equivalent magnitude to a *sit4* knockout. The fact that the quadruple *SAP* knockout in *S. cerevisiae* shows the same phenotype as a *sit4* knockout indicates that the *SAP* genes function in the same pathway as Sit4.

In wild type *L. maculans* the degree of hyphal branching is influenced by nutrient availability in that growth when nutrients are limiting leads to reduced branching, as seen by the pattern on the low nutrient water agar. The *Leptosphaeria* SAP mutant showed a higher degree of branching on water agar than the wild type or complemented strains, indicating that SAP plays a role in regulating branching in filamentous fungi. As a hemibiotrophic pathogen, this fungus initially grows in the apoplast of the plant, which is not a nutrient-rich environment. This may explain the unusual branching phenotype observed during cotyledon infection.

TOR type 2A phosphatases have not previously been implicated in hyphal branching. However, another TOR target, the kinase FgSch9, regulates hyphal branching in *F. graminearum* [13]. Interestingly, the way in which Sch9 regulates branching differs between species: in *F. graminearum* the *sch9* knockout has reduced branching, whereas in *Candida albicans* a *sch9* knockout shows an increase in branching [32].

In conclusion, in this study we have identified a new pathogenicity factor in *L. maculans*. Future research should explore the signaling system through which this homolog of a Sit4-associated protein integrates environmental nutrient levels to impact the ability of this fungus to cause disease on canola.

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Figure legends

Fig. 1 Isolation of a T-DNA insertional mutant with reduced pathogenicity. (A) Representative lesions 14 days post inoculation on *B. napus* cv. Westar cotyledons of the wild type isolate, the *Leptosphaeria* SAP T-DNA

insertional mutant, and two complemented strains (SAPC1_1 and SAPC9_1). (B) Infected cotyledon tissue stained with trypan blue. The hyphae of the wild-type infection appeared less branched and penetrated further into the cotyledon tissue. Scale bar = 0.1 mm.

Fig. 2 Distributions of lesion sizes of four *L. maculans* strains 14 days post inoculation on *B. napus* cv. Westar. The lesions generated by the *Leptosphaeria* SAP mutants were in all cases only very small or not visible. A reversal back to the wild-type phenotype occurred in the two complemented strains (SAPC1_1 and SAPC9_1).

Fig. 3 Location of the T-DNA insertion within the first exon of Lema_T071490.1, 307 bp downstream of the start codon in the *Leptosphaeria* SAP mutant. (A) The genomic sequence at the insertion site is shown for the wild type and SAP mutant. The T-DNA replaces between 22-27 nt, with this ambiguity being due to the regions of microhomology (underlined) between the *L. maculans* genomic DNA and the T-DNA sequence. (B) Diagram of the position of the T-DNA insertion (bar) into the *sap1* gene. Black boxes represent exons and white boxes represent introns. The positions and orientation of the primers used to amplify fragments to make the complementation construct are presented as arrows.

Fig. 4 The *in vitro* growth rate of the *Leptosphaeria* SAP mutant compared to the wild type and two complemented strains (SAPC1_1 and SAPC9_1) on (A) water agar and (B) V8 juice agar media. Growth assays are from seven replicates on V8 media and three replicates on water agar. Error bars represent ± 2 times the standard deviation.

Fig. 5 *In vitro* growth of *L. maculans* strains after 10 days. The top panel of four images are colony morphologies on clarified V8 juice agar. The middle and bottom panels are the colony edge morphologies of strains growing on clarified V8 juice or water agar. The hyphae growing on water agar were stained using lactophenol cotton blue to increase contrast. Scale bars represent 1 cm for the whole colonies and 750 μ m for colony edges.

A **B**

A

Wild type GGCCCGCTCCCTGTCCACCAGCAAGTCCGATGCGGGCGACAATGAGGAGAACAAGGAGGAGC
Mutant GGCCCGCTCCCTGTCCACCA-----T-DNA-----TGAGGAGAACAAGGAGGAGC

B

A

B

[illegible]

ATTTCCTCAAGGCCATCATCACCATTATCTGGGAATGCCACACCAAGACCAAGCTCTGTGATGGCC
D F L K A I C I T I S A N T T Q D Q S V I G
AACAGCAATGACGGCCAGCTGTCTCTCGAGACCTGGCTGAAGCAATGTAGGACTTCAGTGGT
N E L T R Q L V S E T C L K Q L I D F M L R
TGGGAACCCGTTGACTGTAGGCGTGGTATCATTATCGAAGTCATCCGGA AAAACAATTACAGACTA
G N P L T V G V G I I E I V I R K N N S D Y
ACCTGGGAATCAAATCGGACCGCTCCCAAAGAATTCTGATCCCAATTACCTAGGCACATTACTAC
D L E N Q I G F V P F K N S D P I Y L G T L L
GAGTTTGC AAAACCATACCTCAGTTCTATGGAAGCTGGTCAACAGCTCAAGCCAAATCATCAAGCAA
E F A N H I P Q F M E L V N S S Q I I T Q
CGATGGCAGCAGCAGCAGCAAGAAGCCGAGCTCAAGACGGCCTTTGGCGAAAAGATTGAGCCGCT
D G S T T T T K K R E L K T A F G E K I E F L
CGATTTCAGCAATTCAGACCTTGTGAATCATGGCCGGAATTGTCTACATCGACCAAGCTCATGGCCCTAC
G F D R F K T C E L M A E L L H C S N M A L
AACAGCACTCGGGATGAGGCCAGGTGAGGAGGCCGGATGCCAGGCCAGACGCTCTCAAGGCACAG
N E R G S E A E V R R R D A E R E R L K A E
CAAGCTCATCTCTGCTGGTGGCAACCTTGGGGAATTTCTCCAAACGCTCGATAGCCAGGCTTCCA
K L T S A G G N L G F E F S N S V D S Q G F H
ATGCAAGTGCACCATCATGGACTCGGCTCGGGAGATCAAGCCATTGCACGTACAGAAATGATGGGG
H A R A P S M D G S P A E I K F L H V Q N S A
GACGACTTTGAAAAGGTCATTGCTCCCGAAGTGGCCATCCAGTGCGCCCAACGATAGTTCGCGGGAG
D D F E K V I A F E V A I T S G S T D S F E
GGGACCAATCGCGGAGCCATAGAAAGTTCAACCAAAAGTCATGCCCAAGGAATCCCTCGCGGAGAA
E Q I G E F P L E R S P K V M P K E S P A E K
CGGCTCAAGCGAAGGCGAATTCTGCTGTGAGCCGCTGACCCCAAGCATACATCGGCACGA
P A S S E G G E F V V E I L T P P K D T S A T
GAGTGCACAGCAGCATCTCCCAAGCGCGAGATGATGCGGAGTCACCTACGTGGCTGGACTGTCT
E S T S S S T T F K P A D D A E S F T S A G L S
CAAGTCTCGCGGCTTGTGATCTGGACAAGATACAGTGTATGAACGATGTCGAAGACGCAACGGAGCC
K V G G L D L D N D T V M N D R Q D A T E P
CAAAAATAGAGGCTGCAACCAAGACCTTCCTCTCTCTTGAACGCAACAACTTAGOCAATCTTCCA
T K I E A A T K D L F S L L T Q L S Q S S
ACCGAGCATCTTCTGAACCACTATCAACCATGAGGATGATAGCGTCGCGCCACTCTTCGGCGAT
T D D L F E F L S P H E D D K F A P L F A N
GAGGCCGGAAGCAATACCGAGGCTCTCGCGCGGCTGACACGTGCGGATTTACCGCACTCGCAT
I S R E S N T E A P A V D T S R F T A P A S

[illegible]

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