URSS

Investigating cGMP Signalling in Plants

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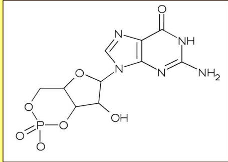
Introduction

- Cyclic guanosine monophospate (cGMP) is a second messenger which is common in animals and has been shown to increase in quantity in plants after salt and drought treatment.



The structure of cGMP[1].

- Microarray gene expression data has identified the genes that are upregulated and downregulated in the model organism *Arabidopsis thaliana* in the presence of cGMP.
- During this project 6 genes were selected based on the microarray data:
  - At4g18290, which was identified as being upregulated by both the microarray data and a reference journal[2].
  - At1g77260, which was the gene most upregulated by cGMP.
  - At3g52040, which was the gene most downregulated by cGMP.
  - At3g19490, identified as being a sodium: hydrogen antiporter that is upregulated by cGMP.
  - At4g35250, the second most upregulated gene by cGMP.
  - At1g69250, the second most downregulated gene by cGMP.
- The main aim of the project was to discover the degree of regulation of these six genes when cGMP is introduced to the plant.
- A secondary investigation was also conducted into how a number of *Arabidopsis thaliana* mutants responded to infection by *Botrytis cinerea*, a fungus that infects many plant species.

Infiltration of the plants with cGMP

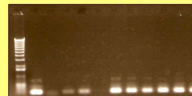
- Wild type *Arabidopsis thaliana* plants were grown for 4 weeks under constant light conditions.
- At the end of the this incubation period, a syringe was used to infiltrate some of the leaves with cGMP.
- This process involved pressing the syringe onto the underside of the leaf, creating a seal. Once the syringe was squeezed, the cGMP entered the leaf through the stoma, which are more abundant underneath the leaf than they are on top of it. Sterilised water was used as a control.

Infiltration being performed[3].

- Half of the infiltrated leaves were removed from the plants after 1 hour, and the other half after 2 hours. This allowed investigation of whether the effects of cGMP alter over time.
- Once removed the leaf samples were immediately placed in liquid nitrogen to cool them to -80˚C. They were stored at this temperature until RNA extraction to prevent freeze-thaw damage to the RNA.

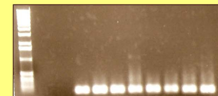
RNA Extraction

- RNA was extracted from the leaves by Phenol-chloroform extraction. This process utilized a phenol - containing TRIzol reagent along with chloroform. After centrifugation a lower organic phase, DNA rich interphase and upper aqueous phase were produced. The RNA was located in the upper phase and precipitated using isopropanol.
- The resultant solution was subsequently tested for the presence of any DNA impurities:



The samples in lanes 7-12 have large amounts of DNA in them. Lane 2 is a positive control.

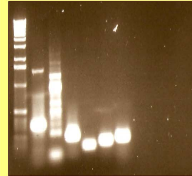
- After DNAse treatment and the further purification of the RNA, cDNA could be produced:



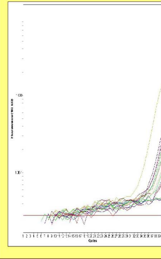
cDNA is present for all samples, although the positive control did not produce a band for an unknown reason.

Real - Time PCR

- Primers were ordered for each of the genes , and gel electrophoresis used to test their functionality:



- Lane 1 of the gel is the 1kb ladder, and lanes 1, 2, 3, 4, 5 and 6 are the results that the primers produced when tested on genomic DNA. As can be seen, the primers in lane 3 (for At1g77260) produced multiple products, and so the annealling temperature of the PCR reaction was raised in the future to correct this.
- The RT - PCR reaction was conducted on a 384 well plate. For each gene to be tested, 5 dilution points were prepared to enable a standard curve to be calculated.
- Actin primers were used to give reference measurements to compare the rest of the results against.



- The amount of DNA produced during the reaction was quantified by a DNA binding dye which binds to double-stranded DNA.
- The relative concentration of DNA was determined by plotting the number of PCR cycles against the fluorescence on a logarithmic scale. The graph appears as a straight line when the DNA replication was exponential. The graph on the left shows the dilution series for the gene At4g18290.
- By comparing the results to the standard curve generated by the serial dilutions, the amount of DNA and thereby activity of the gene in the sample can be calculated. The actin housekeeping gene was normalised against to ensure accuracy.

Botrytis Infection

- Wild-type *Arabidopsis* plants along with 4 mutant strains were infected with the fungus *Botrytis cinerea*.
- The *Botrytis* spores were diluted down to a concentration of 100,000 spores per ml using half-strength grape juice as the dilutant.
- 10µl of the spore solution was then added to the leaves with half-strength grape juice as the control.
- Photos were taken to monitor the fungal growth on the leaves:

CNG13 leaves at 19h30mins after infection

CNG13 leaves at 26h30mins after infection

CNG13 leaves at 44h after infection

CNG13 leaves at 51h after infection

CNG13 leaves at 68h30mins after infection

- The mutant plants used were CNG10, CNG13, CNG19.306 and CNG195. Photos similar to the ones above were taken for all the plants.
- The circumference of the brown fungal infection that appeared on the leaves was then measured to test whether any of the mutant plants had a different degree of susceptibility when compared to the wild-type.

Results

- The results of the RT-PCR indicate that the microarray data was correct.
- For the gene At1g77260, which had previously been determined to be upregulated, the DNA concentration was more than twice as high for those leaves which had been exposed to cGMP for 4 hours when compared to the control plants.
- This is in contrast to At1g69250, which did not increase in activity after exposure to cGMP, and was reduced in the case of cGMP exposure for 2 hrs.
- In the Botrytis infections, none of the mutant plants showed a significant difference from the wild-type in terms of their response to infection.

References

1. Adapted from 'http://content.answers.com/main/content/wp/en/thumb/e/e7/180px-CGMP.png'
2. Maathuis F J M; 2005; cGMP modulates gene transcription and cation transport in Arabidopsis roots; The Plant Journal (2006) 45, 700-711
3. Adapted from 'http://www.nature.com/nprot/journal/v1/n4/images/nprot.2006.286-F3.jpg'