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A second set of XEGIP-encoding genes resides on chromosome 8 of potato and tomato.

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Abstract

Xyloglucan-specific endoglucanase inhibitor proteins (XEGIP) are present in a wide range of dicots, where they are believed to play a role in defense from pathogens. The XEGIPs are generally present as two or three copies, however, they are reported to be present as a cluster of ten copies in potato and tomato on chromosome 1. We have now identified a second set of XEGIP encoding genes, where two inverted copies are present on chromosome 8 of potato (*Solanum tuberosum*). The same set exists, in the same order, on chromosome 8 of tomato (*Solanum pennellii*). Transcript expression analysis indicates that XEGIP10 is transcribed only in roots, while XEGIP11 transcripts were not detected under normal growth conditions. Transformed potatoes (cvs. Bintje and Kennebec) overexpressing these genes did not show any changes in phenology. Foliar screening of transgenic lines for resistance to the late blight pathogen *Phytophthora infestans* did not demonstrate a large reduction in disease progression or pathogen sporulation.

Keywords: xyloglucan-specific endoglucanase, late blight, potato

Introduction

Plants produce a number of inhibitory proteins that interact with proteases, pectinases, pectin methylesterases, xylanases and endoglucanases (Juge 2006, Pogorelko *et al.* 2013). Production increases during biotic stresses, such as pathogen attack, and during abiotic stress such as temperature extremes. Since the inhibitory proteins can interfere with pathogen enzymes, they

are believed to play a role in host defenses. Closer study indicates that the inhibitory proteins can have a direct effect on the host, independent of any pathogen interactions (Alexandersson *et al.* 2011, Jones and Perez 2014) which may help explain their up-regulation by abiotic stress. One group of inhibitory proteins is the xyloglucanase-specific endoglucanase inhibitor proteins (XEGIPs) found in dicots. These proteins were first discovered due to their interaction with a fungal endoglucanase in Glycosyl Hydrolase family 12 (Qin *et al.* 2003). A parallel inhibitory protein TAX-1 exists in monocots, and inhibits GH11 xylanases (Igawa *et al.* 2004). The *Xegips* are present in a wide range of dicots, found as two or three copies in the genomes (Qin *et al.* 2003, Jones 2012). An exception in copy number exists for the xyloglucan-specific endoglucanase inhibitor protein encoding genes in potato and tomato. Extensive duplication occurred at a pre-speciation time period, resulting in ten copies of *Xegip* within a 90kB region of chromosome 1 from potato and tomato (Jones 2012). During our ongoing analysis of potato XEGIP-encoding genes we identified a second set of *Xegip* genes clustered on another potato chromosome. We report on the sequence, transcription and effects in plant transformant expression.

Materials and methods.

Identification, cloning and expression of *Xegips*.

Genome sequences deposited at NCBI GenBank were periodically scanned using tblastn and sequences from previously identified potato *Xegips* (Jones 2012). Two sequences were identified in GenBank which mapped to potato chromosome 8. Primers spanning the predicted coding regions were used to PCR amplify the gene sequences from potato (cv. Russet Norkotah). The PCR products were cloned (TOPO 2.1, Invitrogen) and sequenced commercially in both

directions (Macrogen, USA). Sequences were analyzed for signal peptides using SignalP 4 (Petersen *et al.* 2011).

Comparisons were made between the sequences found in potato and similar sequences detected in the tomato genome, using Simple Synteny (Veltri *et al.* 2016)

(<http://www.dveltri.com/simplesynten>) with an e-value cutoff threshold of 10.

Transcriptional analysis was performed using total RNA extracts (Zymogen RNA kit) from leaves and from roots. A cDNA template was prepared using ProtoScript (New England Biolabs) and oligo dT in a 20 microliter reaction, according to the manufacturer's protocol. One microliter of cDNA was used in a 40 microliter PCR reaction containing GoTaq (Promega) and one of the following primer sets; X10 f (atgaaattatctcctatgtcaccctcc) and X10 r (ccattcctttaacattttgcctaa), X11 f (atgtctatcatgtccaatatttacacac) and X11 r (cgatttcagcaccagagtagaaagt), Actin f (atgcatatgttgccgatgaagctcaa) and Actin r (agaggacagcttgaatggcgacatac). PCR reactions were run at 96 C for two minutes, followed by 30 cycles of 53 C for 15 sec, 72 C for 40 sec, and 96 C for 30 sec, followed by a final cycle of 5 min at 72 C. Three microliters of each reaction was loaded into a 1% agarose gel and run at 100 V for 30 min. Exposure time was based on absence of saturated pixels under UV illumination. Samples were quantified using BioRad Quantity One software.

Transformation and plant regeneration.

The gene was PCR amplified with primers to the coding region plus the addition of 5'XbaI and 3'SacI restriction sites for X11 or XhoI and BamHI for X10. Amplified product was TOPO cloned (Invitrogen), and plasmid isolated after transformation into *E. coli*. Plasmid was restricted and the insert was gel purified (Zymo Genetics) then ligated with Instant Sticky End Ligase

(New England Biolabs) into an Xba1-Sac1 restricted, gel purified pBI121 or Xho1-BamH1 restricted, gel purified pGD binary vector. Ligated vector-insert combinations were transformed into *E. coli*, and plasmid isolated for further use. *Agrobacterium tumefaciens* cells (LBA4404, Life Technologies) were electroporated with each of the two constructs.

Leaf tissues from tissue culture grown plantlets of potato (*cvs.* Kennebec and Bintje) were transformed as previously described (Jones *et al.* 2004). Rooted regenerants were screened by PCR amplification using 35S promoter and nos terminator primers. Plants were grown in a greenhouse until maturity. The PCR products generated throughout the study were sequenced to confirm sequence integrity. Transcript levels of X10 and X11 in the foliage of transformed plants were tested using the procedures outlined previously for detection of X10 and X11 transcripts in control plants.

Inoculations with *Phytophthora infestans*

Leaflets were removed from the center of two month old greenhouse grown plants and spot inoculated with a sporangial suspension of *Phytophthora infestans* US 11 as previously described (Jones and Perez 2014). Inoculated leaflets (two sets) were maintained in covered plastic boxes and assessed for lesion size and sporulation six days after incubation at 18 C.

Results

Identification, cloning and transcript analysis.

Two new *Xegip* genes were identified and named following the known potato *Xegips* (X-0 to X-9). Sequences were deposited in GenBank as X10 (KF769949) and X11 (KC960886). The two

sequences were found on the NCBI whole genome shotgun sequence (wgs) submission AEW01019573 originating from potato (*Solanum tuberosum*). Similar sequences were also found on a tomato (*Solanum pennellii*) genome sequence HG975447. In both cases the genome sequence was found on chromosome 8. Comparison of the regions was made using the web based program Simple Synteny (Veltri *et al.* 2016). Genes were located within 6kB of each other and transcription would occur in opposite directions (Fig. 1). Comparisons based on amino acid alignment of the two secreted proteins indicated a similar overall pattern, however X10 lacked the second arginine that is generally required to bind to GH 12 enzymes (Fig. 2).

Transcript analysis revealed that X10 transcripts are abundant in potato roots of the two cultivars tested, but they are lacking in the foliage. Transcripts for X11 were not evident in roots or foliage (Fig. 3).

Transformation and regeneration.

Independent lines of overexpressing X10 and X11 were obtained and grown in the greenhouse to maturity. The transgenic X11 lines were readily generated in both Bintje and Kennebec, while the X10 lines were difficult to regenerate, resulting in only four transgenic Bintjes and one transgenic Kennebec. While the regeneration was poor for X10, there were no phenotypic differences in growth or tuber formation in mature plants for either cultivar tested for X10 or X11. Under normal greenhouse growth, transcripts of X10 and X11 are not produced in leaf tissue, thus it is not possible to compare transgene expression levels between transgenics and the control plants. However, all transgenic plants expressed transcripts of the corresponding transgene in the foliage, with a maximum of two-fold difference between the highest and lowest expressing transgenic.

Inoculation with *Phytophthora infestans*.

Individual leaflets of greenhouse grown transformed plants did not show large differences in disease (lesion size) or sporulation. (Table 1). The effect of X10 was slightly greater than X11 in transformants, but at best they contribute to a slight reduction in lesion size and sporulation.

Discussion

The identification of two new XEGIP-encoding genes in potato expands the number of *Xegips* to twelve. The high number of *Xegips* is similar to the high number of GH12 xyloglucan-specific endoglucanase encoding genes reported in *P. infestans* (Costanzo *et al.* 2006), suggesting a pattern of co-evolution. Based upon blastn analysis, the X10 and X11 genes are most closely related to X1, which is located at the 5' end of the *Xegip* gene cluster on chromosome 1. A phylogenetic tree comparing all the previously reported *Xegip* genes from potato has been published (Jones 2012). It can only be speculated if one cluster is the progenitor of the other through a translocation.

An interesting aspect of the new *Xegips* is the transcript presence. We did not find X11 transcripts in control plants under greenhouse conditions, while X10 transcripts were abundant in roots and absent in foliage. Expression of XEGIPs can be very tissue-specific, an example being NEC4, found in the nectar of tobacco (Navqi *et al.* 2005). There are no apparent ESTs for X11 in GenBank, while there is EST confirmation for X10. It has been noted that the late blight resistant potato cultivar Sarpo Mira relies on the presence of roots to exhibit the full defense response (Orlowska *et al.* 2012). It would be of interest to determine if specific XEGIP proteins could be translocated to other parts of the plant. Regions of the legume genome harboring *Xegips* have been associated with resistance to the root attacking nematode *Pratylenchus* (Oldach *et al.* 2014),

thus there may be specific pests and pathogens that are differentially affected by individual XEGIPs. In tobacco, one XEGIP was studied and shown to have an effect on susceptibility to a bacterial pathogen, but no effect on two fungal pathogens (Xie *et al.* 2008).

Introducing the production of two new XEGIPs to the foliage of potato through transformation allowed for functional testing of these XEGIPs in their ability to interfere with *P. infestans* infections. Neither XEGIP is normally produced in potato foliage, however transcripts were readily detected from cDNA of the transgenics. On an individual basis, neither XEGIP resulted in sufficient reduction in lesion size to be considered exclusively as a disease control measure. There was a general reduction in lesion size and sporulation that could be beneficial if added along with other defense genes. The limited effectiveness may be due to the complexities introduced by the large number of GH12 enzymes produced by *P. infestans*, or that interference with GH 12 is insufficient to limit *Phytophthora*. Transformant expression of a TAX-1 xylanase inhibitor in wheat grain spikes (Moscetti *et al.* 2013) delayed *Fusarium* head blight, providing one of the few examples where an inhibitor similar to XEGIP was effective. In this case there is a less complex situation with the limited number of GH 11 xylanases being produced by the pathogen.

The amino acid sequence suggests that X10 may not be a good inhibitor of GH 12 enzymes, although it should be noted that xyloglucan-specific endoglucanase activity exists in GH 5 and GH 74 families also. There are two arginines required for binding to the inner pocket of GH 12 (Yoshizawa *et al.* 2012), and the second one is absent in X10. This does not mean that it could not interact with other enzymes.

XEGIPs can take on many different functions in addition to neutralizing GH 12 enzymatic activities. Studies in tobacco nectar have shown that binding of XEGIP with GH 12 initiates secondary effects that have a direct impact on pathogen success (Harper *et al.* 2010). In hops and apple (Habrylo *et al.* 2013, Bai *et al.* 2015), only some of the XEGIPs interact directly with GH 12, while a role for direct activity on the host is suggested for the other XEGIPs. This was demonstrated with X-0 in potato, where overexpression led to drastically altered plant phenotypes (Jones and Perez 2014). Altered phenotypes were not observed when over-expressing the XEGIPs in this study, demonstrating that each XEGIP can differ in function.

The number of XEGIPs in other dicots may need to be re-evaluated, given the large number that still remain mis-annotated. While some are identified as similar to aspartic proteases, many are identified as basic 7S globulins. These proteins have a similar structure, but lack inhibitory activity (Scarafoni *et al.* 2010, Yoshizawa *et al.* 2011). The true globulins form tetramers at proper pH and have other functions in the plant. Within the globulin family, unique characteristics have been discovered that could allow them to be inhibitory. While normally processed, the lack of processing found in a yeast expression system lead to the discovery of activity against GH11 xylanase (Scarafoni *et al.* 2016).

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Figure Captions

Figure 1. Comparison of locations for X10 (KF769949) and X11 (KC960886) on a region of chromosome 8 of potato (*Solanum tuberosum*) and tomato (*Solanum pennellii*). Diagram generated by SimpleSynteny shows the conservation in transcriptional direction and general distance between the two copies.

Figure 2. Amino acid alignment by ClustalW comparing secreted proteins from X10 (AHG54973), X11 (AGT17598), X0 (AAP84703) and X1 (ADU58603). Arrows indicate conserved arginines that bind with GH 12.

Figure 3. Transcript analysis of control plants. Transcripts are only present for X10 and only in root samples. Lane 1, Bintje leaf X10, lane 2 Bintje root X10, lane 3 Kennebec leaf X10, lane 4 Kennebec root X10, lane 5 Bintje leaf X11, lane 6 Bintje root X11, lane 7 Kennebec leaf X11, lane 8 Kennebec root X11, lane 9 Bintje leaf actin, lane 10 Bintje root actin, lane 11 Kennebec leaf actin, lane 12 Kennebec root actin.

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Table 1. Assessment of foliar inoculation with *Phytophthora infestans* (race US 11) at 6 days.

<u>Transformant</u>	<u>Lesion size (cm)</u>	<u>Sporulation (0-4 scale)</u>
Kennebec control	3.0	3.0
Bintje control	3.0	4.0
Kennebec X11-1	4.0	1.0
Kennebec X11-2	3.0	2.0
Kennebec X11-3	3.5	2.0
Kennebec X11-4	3.5	2.0
Kennebec X11-5	3.5	4.0
Kennebec X11-6	3.75	3.0
Kennebec X11-7	3.0	2.5
Kennebec X11-8	3.25	2.5
BintjeX11-1	3.5	4.0
BintjeX11-2	3.0	3.5
BintjeX11-3	3.5	3.5
BintjeX11-4	3.75	3.5
BintjeX11-5	3.25	2.5
BintjeX11-6	3.5	4.0
BintjeX11-7	3.25	4.0
BintjeX11-8	3.5	4.0
BintjeX11-9	2.75	2.0
Kennebec X10-1	2.75	2.5
BintjeX10-1	1.25	1.0
BintjeX10-2	1.25	0.5
BintjeX10-3	1.0	0.5

BintjeX10-4

1.5

1.0

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