Over expression of the Q-type ZFP StZFP2 in potato increases resistance to potato late blight (Phytophthora infestans) infection

Susan D. Lawrence, Nicole G. Novak, Frances G. Perez and Richard W. Jones

ABSTRACT
Late blight in potato caused by Phytophthora infestans remains a major impediment to potato growers worldwide. Utilization of regulators that affect defence may augment control measures to achieve a basal defence against late blight. This work examines the effect of over-expressing the transcription factor StZFP2 in potato on P. infestans infection. StZFP2 is induced upon infestation by chewing insects such as Manduca sexta and the pathogen P. infestans. StZFP2 was transformed into potato under the control of the CaMV 35S promoter. Three over-expressing lines (OE-StZFP2) resulted in significantly lower lesion size five days after infection (DAI). The increased expression of StZFP2 slowed the spread of infection at 5DAI. Levels of StZFP2 and classic pathogen response marker genes, StPR-1b and StPR-2 were significantly lower in more resistant lines, suggesting that OE-StZFP2 affects this pathogen response.

Introduction
Late blight caused by Phytophthora infestans, responsible for the Irish potato famine remains a major disease threat to potatoes, the fourth most important food crop worldwide. Attacking both tomato and potato, it ranks as the number one plant-pathogenic oomycete (Kamoun et al. 2015). The hemibiotrophic pathogen’s lifecycle starts as a biotroph early upon infection and ultimately becomes a necrotroph, killing the plant tissue as the hyphae spread. During the biotrophic stage the pathogen penetrates the plant and does not cause cell degradation but induces plant genes responsive to salicylic acid (SA). Subsequently, P. infestans ultimately kills plant tissue initiating an interaction similar to necrotrophs resulting in induction of plant genes responsive to jasmonic acid (JA) and ethylene.

Upon infection the plant identifies the pathogen thru pathogen-associated molecular patterns (PAMPs) via PAMP receptors (Jones and Dangl 2006). An additional aspect of defense signaling is detection of damage to plant cells, through damage-associated molecular patterns (DAMP). This can occur in conjunction with insect feeding, pathogen attack or simply through a mechanical non-biotic wound. DAMPs have their own unique DAMP receptors that activate expression of wound-responsive genes (for current reviews Gust et al. 2017; Ranf 2017).

Ultimately defence signaling, whether recognizing DAMPs, PAMPs or if due to herbivores (HAMPs), results in the production of downstream defence genes. During pathogen infection, numerous pathogen response (PR) proteins are induced (van Loon et al. 2006). The infection causes a cascade of defence proteins that have been characterized by purification and molecular identification, placing them into 17 groups to date (PR-1 to PR-17) based on the proteins type. For example, PR-2 proteins are β-1,3-glucanases while PR-3 are chitinases. Glucanases can degrade cell wall components of P. infestans, but since this pathogen does not contain chitin the PR-3 proteins are not considered to be effective against it (Pieterse et al. 1992). Even though these genes are PR genes by definition in reality PR-3 proteins can be JA responsive and inhibit infesting insects (van Loon et al. 2006). One PR protein PR-1, which is frequently used as a molecular marker for the response to SA or biotrophic pathogens, clearly acts as an antimicrobial in vitro as well as when over expressed in transgenic plants (Alexander et al. 1993; Niderman et al. 1995). Recent work shows that both acidic and basic PR-1s bind sterols (Gamir et al. 2017). Since Phytophthora species contain sterols in their membranes, and are sterol auxotrophs, these pathogens require sterols from the plants they infect and are particularly inhibited by PR-1 binding, which depletes sterol pools needed by the pathogen. Another role for PR-1 in tomato is as a cleavage product. Upon wounding PR-1b is cleaved and a peptide named CAPE1 is produced. CAPE1 refers to a conserved cysteine-rich region of the PR-1 family of proteins. CAPE1 acting as a DAMP induces several different defence genes including PR-1b. CAPE1 creates both an antiherbivore as well as a significant antipathogen response in tomato (Chen et al. 2014). Therefore PR-1, the SA marker, functions as both a sterol-binding protein and as a signaling molecule in plant defence.

Much is known about proteins involved in regulating pathogen response, such as WRKY transcription factors and NPR1, which act as positive regulators of the pathogen defence response (Birkenbihl et al. 2017), and induction of the plant’s basal immunity. Many other regulatory proteins positively enhance or negatively depress the expression of the downstream PR genes (Alves et al. 2014). These pathways are modulated by plant hormones with SA inducing defence genes that inhibit biotrophic pathogens, JA and ethylene inducing defence genes inhibiting necrotrophic pathogens and JA and abscisic acid generally inducing defence against...
chewing insects. While SA and JA tend to act antagonistically, other hormones as described above interact synergistically. This also includes synergy between SA and cytokinins in several reported biotic stress responses (Verma et al. 2016). A balance is required to successfully respond to various pathogens or insects that have unique modes of attack and require different hormonal pathways. A balanced response is also needed for the plant to effectively respond to other stresses, such as water loss, an abiotic stress that requires a different mix of hormones. Consequently, constitutively turning on one specific defence pathway also affects growth and successful reproduction for the next generation. Plants that are continuously producing defence genes, while they may prove more resistant to pathogens or pests tend to be stunted and result in lower yield. Therefore, manipulating a balance between defence and growth is a tradeoff that ultimately requires optimization (Hout et al. 2014).

One important family in the plant response to stress are the Q-type C2H2 zinc finger proteins (Q-type ZFPs), which are named for their unique zinc finger domain that is capable of binding two zinc molecules, allowing precise interactions with particular DNA or RNA motifs thus regulating specific genes (Kielbowicz-Matuk 2012). Q-type describes the invariant QALGGH motif found within the 30 amino acid conserved zinc finger domain. C2H2 refers to the two invariant Cys and His residues within this domain. There are, 176 C2H2 zinc finger proteins identified in the Arabidopsis genome (Engelbrecht et al. 2004), but only 18 are two fingered Q-type ZFPs (Ciftci-Yilmaz and Mittler 2008).

Recently several Q-type ZFPs have been described in the Solanaceae; from Capsicum annum, CaZFP1, which enhances tolerance to drought and infection by Pseudomonas syringae pv. tomato when expressed in Arabidopsis (Kim et al. 2004). Two Q type ZFPs have been identified in tobacco NtZFT1 and NtZFP1. They are both responsive to spermine (Mitsuya et al. 2007). The tomato SICZFP1, enhances cold tolerance in Arabidopsis and rice (Zhang et al. 2011). Only two Q-type ZFPs have been described in potato, StZFP1 (Tian et al. 2010) and StZFP2 (Lawrence et al. 2014). OE-StZFP1 confers salt tolerance in tobacco, but the transcript in untransformed potato is also induced by P. infestans infection and SA (Tian et al. 2010). Both StZFP1 and StZFP2 are induced by wounding and infestation by the chewing insect’s tobacco hornworm and Colorado potato beetle (Lawrence et al. 2014).

Given the ability of over-expressed Q-type ZFPs to enhance tolerance, this work constructs and tests over expressing transgenic lines of StZFP2 (OE StZFP2) for increased resistance to P. infestans. Three transgenic lines were selected and tested for classic defence marker genes to determine how OE-StZFP2 affected pathways involved in defence.

**Materials and methods**

**Construction of the StZFP2 over expression vector**

The entry vector pENTR.EV47CTF was constructed by amplifying StZFP2 from pGEM.EV47 (sequence of EV47 EST at NCBI-BJQ11206.1) using primers CTF/NTF.EV47-forward 5’-CACCATGACATCTATGAAAAGAGCGA-GAAGACA-3’ and CTF.EV47-reverse 5’-AAGGGATTT AAAAAAGCATCGCAA-3’ with Advantage 2 polymerase (Clontech) according to manufacturer instructions. TOPO cloning (pENTR/D-TOPO Cloning Kit, Invitrogen) was utilized to ligate the resulting amplification product to create pENTR.EV47CTF. LR recombination was performed between pH7WG2 (Karimi et al. 2002) and pENT.REV47.CTF to result in the OE-StZFP2 construct.

**Plant transformation**

*Solanum tuberosum* var. Kennebec was transformed with the 35S-StZFP2 construct via Agrobacterium tumefaciens-mediated transformation (Banerjee et al. 2006). The transformed plants were stored long term in an incubator with 16 hr light, 8 hr dark at 19°C. New cuttings were made by transferring to fresh media every six months. Successful transformation was confirmed by PCR amplification of transformants using 35Sf promotorseq 5’-tctctgcaagacctctctctat-3’ and 35Sr terminator 5’-cactagcgaacacctataagaacc-3’. The appropriate size fragment (899 bp, data not shown) was confirmed and the twelve OE-StZFP2 transgenic lines were maintained in tissue culture as plantlets.

**P. infestans assay**

Sporangia were harvested from two-week-old cultures of *P. infestans* (race US 11) by flooding plates with 5 ml sterile water and decanting the sporangia into a sterile petri dish. Harvested sporangia were refrigerated for 1 hr at 4°C, followed by incubation at room temperature for 30 min to induce zoospore formation. Leaflets from four-week old greenhouse grown potato plants were detached from the center of the plants and placed onto moistened paper towels in incubation trays. Fifty μl aliquots of the sporangia/zoospore mixture were applied at individual sites on the abaxial side of the leaves. Incubation trays were sealed with plastic wrap and placed in an incubator (18°C). Inoculated leaves were kept in the dark for 24 hr, followed by 14 hr light/10 hr dark lighting cycles. The lesion size and infection rate were scored at 5 and 8DAI. Untransformed Kennebec and all 12 sense transformics were tested. In each assay two leaves with three leaflets were all individually infected. The lesion size was calculated and graphed as averages with error bars representing standard error.

**RNA isolation and qRT-PCR**

RNA was isolated using TRIzol according to the manufacturer’s protocol. Custom Taqman assays were designed for each gene used for qRT-PCR (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized using 1ug RNA and SuperScript III First Strand Synthesis SuperMix for qRT-PCR (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized using 1ug RNA and SuperScript III First Strand Synthesis SuperMix for qRT-PCR (Life Technologies, Carlsbad, CA, USA). The qRT-PCR was performed using the 7500 Real Time PCR System and Taqman assays were completed according to manufacturer’s guidelines using 25 ng cDNA (Life Technologies, Carlsbad, CA, USA). Luciferase positive control RNA (Promega, Madison WI, USA) was added to the reverse transcription master mix. cDNA was made using SuperScript VILO Master Mix (Invitrogen). 2.5ug template RNA and 1 ng luciferase RNA (Promega) was used in a total volume of 20ul. cDNA was diluted to 5 ng/ul and 10ul was used for qRT-PCR. Custom Taqman assay primer/probe sequences are listed in (Table 1). The Comparative Ct method was used to calculate transcript abundance for each gene (Schmittgen and Livak 2008). The results are graphed using the ΔΔCt values as ‘mean transcript
levels’ with St18S as an endogenous control. The relative transcript levels represent three to five P. infestans infected leaflets. The 3DAI leaflets from untransformed Kennebec were compared to the transgenics. The error bars represent standard deviation between the leaflets.

Statistical analysis
The P. infestans assays were analyzed using a Student’s t-test comparing untransformed Kennebec and each individual transgenic line. Significant differences of $P < 0.05$ are represented by (*), and $P < 0.01$ by (**). qRT-PCR was also analyzed with a Student’s t-test to determine significance. $P < 0.05$ is represented by (*) while $P < 0.01$ by (**) and $P < 0.001$ (***)

Results
To confirm that StZFP2 was affected by late blight infection in potato, detached leaflets from Kennebec were inoculated with P. infestans (US11) as described in the material and methods, harvested after 3DAI and snap frozen in liquid nitrogen. RNA was isolated from the leaflets and qRT-PCR was performed using St18S as an endogenous control. Levels of StZFP1 and StZFP2 were compared to control Kennebec and leaflets exposed to P. infestans for three days (Figure 1). StZFP1 is the first Q-type ZFP described in potato and Tian et al. (2010) showed that it was induced by P. infestans infection. Both StZFP1 and StZFP2 were induced at 3DAI.

The open reading frame of StZFP2 was cloned into pH7WG2 between the 35S promoter and terminator. The construct was transformed into S. tuberosum L. var. Kennebec as described in the materials and methods. Twelve transgenic lines were selected and the OE-StZFP2 lines were named S1-S12 (for sense 1, sense 2, etc.). The twelve independent transformed lines were transferred to pots and grown in the greenhouse for one month. P. infestans detached leaf infection assays were performed comparing untransformed Kennebec and the twelve OE-StZFP2 lines as described in the materials and methods. The assays were repeated twice and three of the transgenic lines showed significantly decreased lesion size in both assays at 5DAI (Figure 2). In the first assay, lesion formation was slower than in the second assay with the final lesion size at 8DAI much higher for untransformed Kennebec but not the OE-StZFP2 lines. Consequently, when comparing the OE-StZFP2 lines to untransformed Kennebec, in the first and second assay, S3 was reduced to 10.5% but only 76% in the second assay, S4-10.5% or 63%, S12 0% or 50%. At 8DAI the difference was only significant with S4 in the first assay and S3 in the second assay, suggesting that the increase in StZFP2 slows the initial spread of the infection. Ultimately the P. infestans infection catches up regardless of the over-expression of StZFP2.

Expression of StZFP2 transcript in untransformed and OE-StZFP2 lines was determined before and three days after infection with P. infestans (Figure 3). The non-stressed plants contained the following levels of StZFP2 transcript in comparison to untransformed Kennebec. S3 had greater than 10,000 fold, S4 more than 13,000 and S12 has about 8700 times more StZFP2 transcript than P. infestans infected untransformed Kennebec. S4 with the highest level of StZFP2

Table 1. Primers used for qRT-PCR.

| Target   | F Primer          | R Primer          | Probe               |
|----------|------------------|------------------|---------------------|
| luciferase | AGAGCTGTTCCTTACGATCCCTTCAG | GCGAAGAATGAAATAGGGTTGGT | ACGCACTTTGGAAATTIT |
| SNIPR1   | AGCTGCTACCTCCTGTAATTATT | GGGAAATAGGAGGATAGGAAGA | CTCGCCCAAAATCA |
| SPAL2    | GCTGGGCCCTCAATGGAGTC | TCAATGGATGTCGGCTCAGTGG | CGTATGCACAACTGAA |
| SP1R1    | GTGATCTACGACGAGATGCAAA | GGTTGGATGGTGTTGTTAAGAG | ACGCCGCAATATCTAG |
| SP1R1B   | GGAAGAAGGCAAAACTCAAACATGGT | GTGGGCGAGACTACTGTAGTATAG | CGGCCACCTTGCGC |
| StZFP2   | AGGAGGCGGCAATGCGATTGTGGT | CTGATGAGAAGGTTTGAACAGA | CAAAGGCAGACTAAG |
| St18S    | CTGCTGATCTCAGAATACAGAT | CCCGGGACCAAAAACTTGGATT | ACATCGCCGCCACCT |
| StZFP1   | GGGGATAAAAAGCAGTACGCTATTAA | AGACGTGACGGATGTATCAC | CGTGCAGCCGGAGAGC |

Figure 1. ZFP1 and ZFP2 are induced at 3DAI by P. infestans infection in Kennebec.
transcript was also most notably stunted in comparison to untransformed Kennebec (Figure 4).

Levels of the SA pathway genes StNPR1 and StPR-1b (Figure 5) were tested in the three most resistant lines and compared to Kennebec. StNPR1 (Figure 5(A)) was only significantly reduced in S4, the line with the most severe stunting. StPR-1b (Figure 5(B)) was significantly repressed in all three of the more resistant lines.

StPR-2 encodes a β-1,3-glucanase and was also significantly repressed, but only in S3 and S4 (Figure 6). Neither StZFP1 nor the JA marker genes StPAL a phenylalanine ammonia lyase, and the StPIN2 a proteinase inhibitor were significantly altered in comparison to untransformed Kennebec upon infection with P. infestans (data not shown).

Discussion

OE-StZFP2 lines had increased resistance to initial infection by P. infestans but were ultimately not able to deter infection over time (Figure 2). The OE StZFP2 lines had extremely high levels of StZFP2 from 8700 to 13,000 fold higher than P. infestans infected untransformed Kennebec. S4 had the highest levels of StZFP2 (Figure 3) and contained the lowest amount of any marker gene tested (Figures 5 and 6). Since S3 had the second highest amount of StZFP2 and also contained the second lowest amount of marker genes, this suggests that the higher the levels of StZFP2 the lower the amount of SA pathway marker genes. S4 was a stunted plant (Figure 4), which indicates more than defence against P. infestans infection was repressed in the S4 line. Several groups have shown that infection of potato by P. infestans causes increases in numerous PR transcripts or proteins, such as PR-1b (Hoegen et al. 2002; Wang et al. 2008), with PR-1b, PAL1 and PR-5 induced in Kennebec (Wang et al. 2008). Local infection of Kennebec with US1 or US8 significantly increases StPR-1b expression from 2-5DAI (Wang et al. 2008). Well within the 3DAI time frame tested here. The OE StZFP2 plants have increased resistance to P. infestans but have reduced levels

Figure 2. Transgenic lines S3, S4, S12 had smaller lesions sizes at 5DAI compared to untransformed Kennebec. Only the lesions for S4 (A) in the first assay and S3 (B) in the second assay were significantly smaller at 8DAI. A Student’s t-test was performed comparing each transgenic line to untransformed Kennebec, \( p < 0.05 (*) \), \( p < 0.01 (**) \).
of the classic PR marker genes StPR-1b and generally StPR-2 compared to untransformed Kennebec (Figures 5 and 6). StNPR1 was not repressed except in the stunted S4 transgenic compared to untransformed Kennebec (Figure 5). The StPAL gene used for this work is a JA responsive gene (Gallou et al. 2009) and was not affected by OE StZFP2 (data not shown). Typically, when plants are more resistant to *P. infestans* infection they also have higher amounts of PR-1, which correlates with high levels of SA (Alexander et al. 1993; Niderman et al. 1995; van Loon et al. 2006). However, the OE StZFP2 lines while more resistant to *P. infestans* infection at early stages of infection had lower levels of the SA marker genes StPR-1b and StPR-2.

The effect of SA on *P. infestans* infection and defence response in potato has been tested using a transgenic line containing the NahG gene that degrades SA, depleting the accumulation of SA and results in higher growth of *P. infestans* along with a slower induction of StPR1 (Halim et al. 2007). Therefore, the loss of SA during infection increases *P. infestans* levels in the leaf and slows the induction of the SA marker. Since OE-StZFP2 decreases lesion formation (Figure 3), suggesting lower amounts of *P. infestans* and decreases in StPR-1b expression (Figure 5), it is difficult to simply suggest that levels of SA alone are affected in the OE-StZFP2 plants. Using RNAi knockdowns of three different genes involved in JA synthesis, the loss of JA has no effect on *P. infestans* growth (Halim et al. 2009). OE StZFP2 also had no effect on the JA markers StPAL and StPIN2 (data not shown) however the OE StZFP2 did result in lower lesion size (Figure 3). Consequently, OE StZFP2 does not seem to simply affect JA levels during *P. infestans* infection since lower lesion size suggests that levels of *P. infestans* are also lower.

**StZFP2 effects may be more complicated than simply altering JA or SA function during *P. infestans* infection.** *StZFP2* localizes to the nucleus, is induced by wounding, infestation (Lawrence et al. 2014) and *P. infestans* (Figure 1). It contains an EAR motif (Lawrence et al. 2014), which in other ZFPs has been shown to negatively regulate gene expression (Ohta et al. 2001; Sakamoto et al. 2004). Therefore, *StZFP2* and the OE-StZFP2 lines are expected to repress genes in potato.

Recently *StERF3* has been characterized in potato, which is a nuclear localized TF containing an EAR motif and induced during infection by *P. infestans* (Tian et al. 2015). Over expression and RNAi transgenics were examined during *P. infestans* infection. OE-StERF3 plants actually result in greater susceptibility manifested by larger lesion sizes during infection and lower levels of StNPR1 and StPRI. The authors
Figure 5. 3DAI with *P. infestans* significantly decreased the level of SA defence pathway gene *StPR-1b*. Comparing the level of transcript at 3DAI for the SA pathway markers: A: *StNPR1* decreased significantly in only S4 while B: *StPR-1b* decreased significantly in all three over expression lines. Filled squares below or above each bar represent levels of *StNPR1* or *StPR-1b* prior to infection. Student’s t-test were performed to comparing levels of transcript between Kennebec and the sense transgenics at 3DAI *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***)

Figure 6. Expression of the *StPR-2* gene was significantly lower in S3 and S4. *StPR-2* encodes a β-1,3-glucanase and was also significantly repressed in S3 and S4 but not S12. Filled squares below each bar represent levels of *StPR-2* prior to infection. Student’s t-test were performed to compare levels of 3DAI transcript between Kennebec and the sense transgenics at 3DAI *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***)
conclude that STERF3 is acting as a negative regulator of SA pathway genes and the pathogen response. The RNAi construct of STERF3 had smaller lesions and higher amounts of StPR1, thus confirming that STERF3 functions as a negative regulator of the pathogen response in potato. STERF3 OE and RNAi lines also have the same effect on salt tolerance, so that STERF3 also acts as a negative regulator to an abiotic stress, which generally involves different hormonal pathways. Therefore, the OE-StZFP2 and OE-STERF3 have opposite effects on the SA pathway genes.

The Arabidopsis Q-type ZFP Zat7 increases salt tolerance when over-expressed in Arabidopsis, but the increase in salt tolerance is lost when the EAR motif is mutated, demonstrating that the negative regulator domain critically effects enhanced salt tolerance (Ciftci-Yilmaz et al. 2007). The OE Zat7 line demonstrates that this negative regulator domain can enhance tolerance to salt stress and may simply negatively regulate genes that oppress expression of genes associated with salt tolerance. OE-StZFP2 appears to positively regulate resistance to P. infestans and at the same time negatively regulate StPR-1b expression. The levels of PR-1b generally demonstrate the induction or repression of the SA branch of pathogen defense, so it is difficult to reconcile how transgenic plants with increases in resistance lower levels of StPR-1b.

Further work is underway to identify the expression of additional genes affected by OE-StZFP2. As well as the identification of promoters that StZFP2 binds and what effects the neutralization of the EAR domain plays on resistance to P. infestans and the expression of the SA pathway genes StPR-1b and StPR-2.

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