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Investigating the Structure and Function of Ribosome-Inactivating Proteins in Barley (*Hordeum vulgare*) and *Nicotiana benthamiana*

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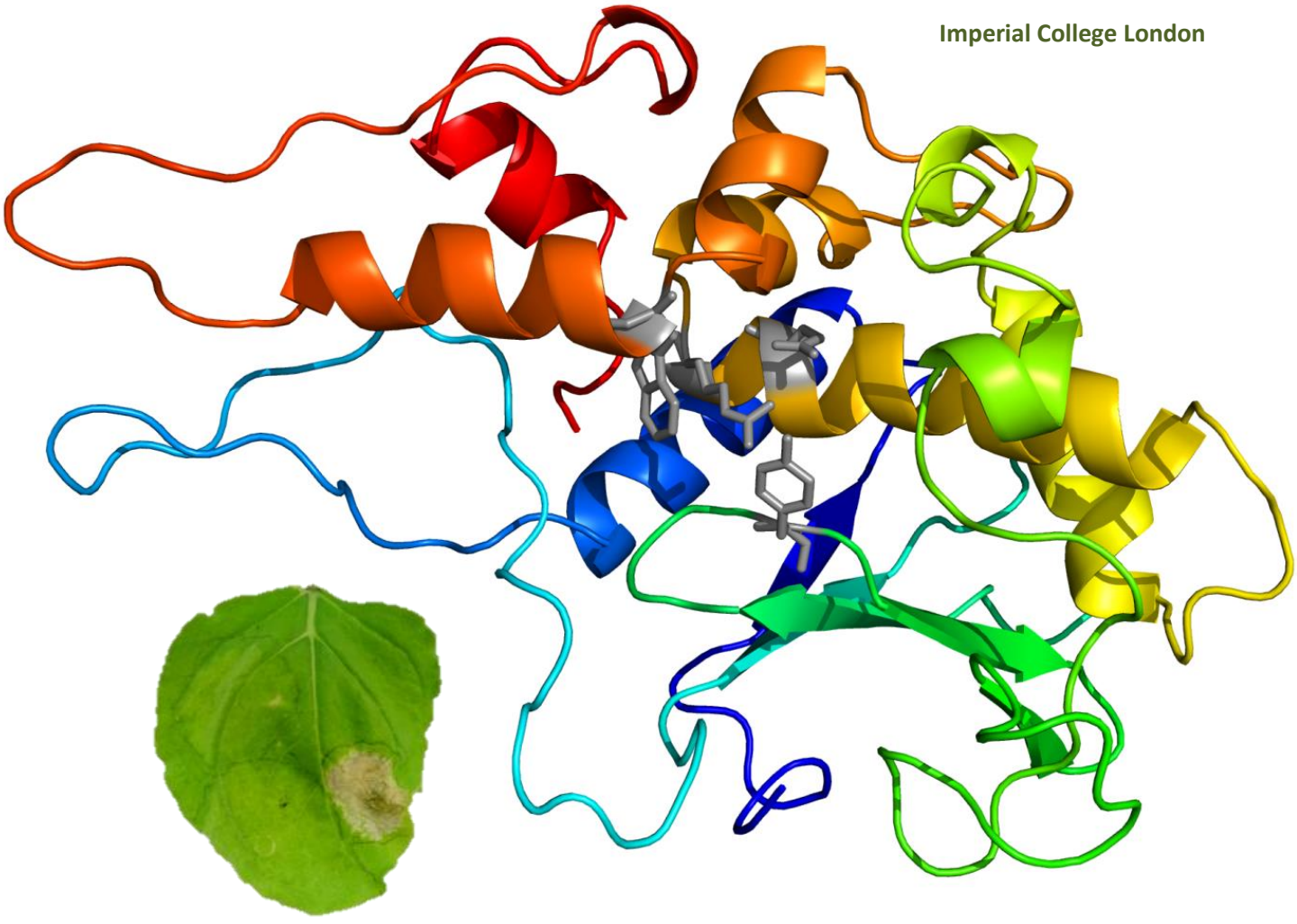

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Abstract

Ribosome-inactivating proteins (RIPs) form a large and widely-taxonomically-distributed class of RNases, particularly in the plant kingdom. Acting as repressors of translation, they inhibit ribosomal function – a role performed by a RIP domain which they all share. This protein domain has a largely-poorly-conserved amino acid sequence, with only a few well-conserved amino acid sites. Using gene cloning,site-directed mutagenesis on specific sites of interest, and transient expression in *Nicotiana benthamiana* via *Agro*-infiltration, the interaction between the structure and function of this domain in the barley RIP jasmonate-induced protein 60 was investigated. In a related experiment, the recently-sequenced genome of *N. benthamiana* itself was searched for putative RIPs, and the plant was investigated for RIP expression in leaves. This project reveals new insights into the function and structure of the RIP domain in plants, with implications on the evolution of RIP genes.

(139 words)

Introduction

Discovering plant defence-related proteins and characterising their functions form an essential element of plant pathology. Ribonucleic acid degrading enzymes, or RNases, form a large and diverse class of proteins, for example many of which target messenger RNAs and are involved in gene silencing (e.g. as reviewed by 22). In contrast, one subclass of RNases – ribosome-inactivating proteins – target ribosomal RNA, and are involved in repression of the translation, which in the context of the organism can lead to a variety of effects – in some cases cell death [32], which could be important as a stress-mediated defence response in plant-pathogen interactions. A proposed example of one such plant defence system is the role of ribosome-inactivating protein JIP60 (jasmonate-induced protein 60) in barley, a plant affected by the important biotrophic pathogen barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) [8]. However, evidence of this link is yet to be determined, and the interaction of the structure and function of JIP60 (both in a mechanistic context and in the context of the whole organism) is not well understood.

Ribosome-Inactivating Proteins

The biochemical function of ribosome-inactivating proteins (RIPs) is inhibition of translation and therefore prevention of protein synthesis. This occurs by modification of ribosomes by N-glycosidase activity [14], via depurination of an adenine residue, position 4324, on the eukaryotic 28S rRNA alpha-sarcin/ricin loop (SRL) [15; 21; 32; 49]. This prevents the binding of elongation factor 2 (eEF2) to the SRL and therefore translation at the translocation step [33; 55]. Whereas the mechanism and biochemical function of RIPs is well characterised, the biological roles of these proteins, in the context of the whole organism, are varied, and in some cases not fully understood [28]. Antiviral roles for some RIPs have been proposed, e.g. shiga toxins [44] and pokeweed antiviral protein [9]; whilst ricin is thought to act as a deterrent to herbivores, given its high toxicity and action on heterospecific ribosomes (Chaudhry *et al.* 1994); and *Mirabilis expansa* RIPs expressed in roots have been shown to have antifungal and antibacterial properties [53].

The ribosome-inactivating activity of RIPs is conferred by their shared domain, termed the RIP domain, or A-chain [27; 28], which typically has a molecular weight of around 30kDa (Chaudhry *et al.* 1994). At a size of roughly 185 amino acids, the primary sequence of this domain is highly variable and mostly poorly conserved, with only a handful of well-conserved residues, some of which have been predicted to consistently form the active site across different RIPs [27; 28]. This domain hence forms the basis of functional studies into specific RIPs, and its distribution among taxa poses questions on the origins and evolution of the domain. For example, an intensive bioinformatic search for RIP genes across a wide phylogenetic range revealed evidence for RIPs not only in plants, where they are particularly preponderant, but in animals and fungi [28], which led to the hypothesis of the origin of the RIP domain prior to the evolution of the three domains of life. Lapadula *et al.* 2013 argued that in evolution, RIP genes have been vulnerable to loss, as well as multiple duplications in the plant lineage, being the cause of the great diversity of RIPs in the plant kingdom. This is a more parsimonious hypothesis than that of horizontal gene transfer from plants to eubacteria, given also the presence of RIPs in gram-positive and gram-negative bacteria [28; 38].

As well as the depurination of adenine on the SRL, another feature of ribosome-inactivating function of some RIPs was investigated by Lapadula *et al.* (2012). The interaction of some RIPs with the highly acidic conserved motif on the C-terminal ends of ribosomal P proteins, which form a structure known as the ribosomal stalk, is necessary for full ribosome-inactivating function [27]. Interestingly, the C-terminal acidic motif of P proteins is essential for the function of the ribosome in relation to eEF2 (eukaryotic elongation factor 2), without which peptidyl tRNA cannot be translocated from the A to the P site in the ribosome [29]. However, interaction with P proteins is not essential for the function of all RIPs [27].

Based on protein structure, ribosome-inactivating proteins are divided into three classes [28]. All three classes share the RIP domain in their protein structure. Type I RIPs, or A RIPs, have no additional domain; whereas type II (AB) RIPs have a 30kDa C-terminal lectin domain (B-chain) which is involved in cell entry via the binding of galactose sugars on cell surfaces [51]. A third type of RIPs, or AC RIPs, describes those which possess a C-terminal domain with unknown function (C-chain) [39; 50].

Jasmonate-Induced Protein 60

Jasmonate-induced protein 60 (JIP60), a type III RIP, is a potent translational inhibitor in barley (*Hordeum vulgare*) (3; Chaudhry *et al.* 1994). JIP60 has a C-terminal domain with a sequence similar to eukaryotic initiation factor 4E (eIF4E) [43] – an essential factor in the initiation of translation, forming part of the closed loop (eIF4E) complex which selectively recruits mRNAs for translation by binding their 5' cap [5]. The eIF4E-like domain contains signature sequence S19, which is similar to the eIF4E binding site [43]. (The full amino acid sequence of the JIP60 protein is presented in appendix B, with regions and sites of interest on the RIP domain as described by Lapadula *et al.* (2012, 2013), and Rustgi *et al.* (2014).)

Two models of mechanism of JIP60 translation inhibition have been proposed, subject to post-translation processing to remove the C-terminal domain [43] as well as a part of the RIP domain known as the internal processing site (43, see appendix B). In the first, described by Chaudhry *et al.* (1994), processed JIP60 depurinates 28S rRNA as described earlier, causing irreversible inhibition of translation. The second proposed mechanism, described by Reinbothe *et al.* (1994) results in reversible translation inhibition, via ribosome disassociation caused by JIP60 possessing both domains. Rustgi *et al.* (2014) provided evidence that JIP60 is processed in methyl jasmonate-treated and senescent barley plants, suggesting that a "molecular switch" from reversible to irreversible protein synthesis inhibition, hence a JIP60-mediated reprogramming of translation, could be a stress response in barley. *JIP60* has been shown to be expressed in powdery mildew-infected barley cytoplasm in its processed form [37], and could be important in a barley defence response to the biotrophic pathogen, mediated by stress. This project relates to the first mechanism, involving processed JIP60 and the N-glycosidase ribosome-inactivating function.

JIP60 is a RIPs which has been observed to cause cell death, although the molecular pathway between inhibition of translation and cell death is unknown [32]. It is an issue of interest, since plant RIPs have been noted for antiviral and antitumour properties and hence can be the basis for cancer treatments and other medical applications [41]. Recently biomedical research into RIPs as has also focused on increasing the specificity of inhibition, reducing antigenicity, and understanding and enhancing cell entry [41]. For example, ricin, a RIP from the castor bean, has been used to treat Hodgkin's lymphoma patients after it was shown to be highly toxic to cancer cells [45]. RIPs could also potentially be used in crop plant defence-related agricultural applications in the future, given

the antifungal and antibacterial properties of some, such as *M. expansa* RIPs [53]. Additionally, maize ribosome-inactivating protein has been shown to cause significant decreases in feeding of cigarette beetle (*Lasioderma serricorne*) and corn earworm (*Helicoverpa zea*) larvae when over-expressed in transformed tobacco [10].

Agro-Infiltration and Nicotiana benthamiana

Agrobacterium tumefaciens-mediated transformation via infiltration of leaves, or *Agro*-infiltration, is a well-established method for genetically transforming plants [36]. Transformation of *Nicotiana benthamiana* in this way is commonly used to investigate plant-fungal pathogen interactions, for example experimental studies on plant resistance and fungal avirulence proteins; and *N. benthamiana* is also widely investigated in its own right as a model host for plant pathogens [18]. Known naturally as the cause of crown gall disease in many plant species, *A. tumefaciens* forces the integration of oncogenes from its own DNA into the genomic DNA of its plant host cell, resulting in their expression and the growth of tumours. Such genes constitute "Transfer-DNA" (T-DNA) and are housed on a Ti (tumour-inducing) plasmid. Experimentally, plasmid vectors can be constructed to experimentally exploit this system, with genes of interest placed on synthetic Ti plasmids, between 25-28 base pair T-DNA "left" and "right" border regions – these being the only essential *cis*-acting elements for plant transformation by *Agrobacterium* [36]. The tomato bushy stunt virus protein P19 can also be transgenically introduced to aid expression of genes of interest, acting as a repressor of gene silencing in the host [54]. Tobacco (*Nicotiana tabacum*), a close relative of *N. benthamiana*, has been shown to exhibit functional JIP60 when transiently expressing the *JIP60* gene – with inhibition of translation via N-glycosidase activity [11; 12] and an increased ratio of polysomes to monosomes [19]. Processed JIP60 (RIP domain-encoding gene sequence only) in *N. benthamiana* has been shown to cause cell death on leaves when transiently-expressed via *Agro*-infiltration [37].

In this project, the interaction between the primary sequence and function of the RIP domain of JIP60 (processed JIP60) was experimentally tested by the creation of single-site mutant forms of *JIP60*, selectively cloned using plasmid vectors, with the aim of assessing the importance of well-conserved RIP domain amino acids regarding function compared to the wild type. This was tested using *Agro*-infiltration of *N. benthamiana* leaves, and the functionality was assessed by observing cell death. This formed one of the experiments during this project, referred to as the "JIP60 experiment". The model species *N. benthamiana* was used in different ways in two separate experiments. Additionally, the genome of *N. benthamiana* was searched for RIP genes, and efforts were made to find functional RIPs in the plant's expressed proteome. This has been named the "*Nicotiana benthamiana* experiment". The two experiments forming this project are linked by their focus on *N. benthamiana* and the amino acid sequence of the widespread and variable plant RIP domain.

Materials and Methods

The JIP60 and *Nicotiana benthamiana* experiments both involved bioinformatics initially, as the foundation of both was the amino acid sequence of the RIP domain. Whereas the JIP60 experiment involved the use of gene cloning, site-directed mutagenesis, and *Agrobacterium*-mediated transformation to assess the role and importance of amino acids in the RIP domain of JIP60 on the function of the protein; the *N. benthamiana* experiment involved using existing knowledge of the RIP domain in other plants for efforts to detect the expression of a similar protein in a plant species with a recently-sequenced genome.

BIOINFORMATICS

Prior to starting laboratory-based methods for the *N. benthamiana* and JIP60 experiments, the use of bioinformatics was necessary to investigate existing knowledge of the RIPs and the RIP domain in particular and hence determine specific experimental approaches. This involved assembling an alignment of ten plant RIP domains (figure 1) which was the starting point for both the JIP60 experiment and the *N. benthamiana* experiment.

Searching for RIPs in the N. benthamiana genome

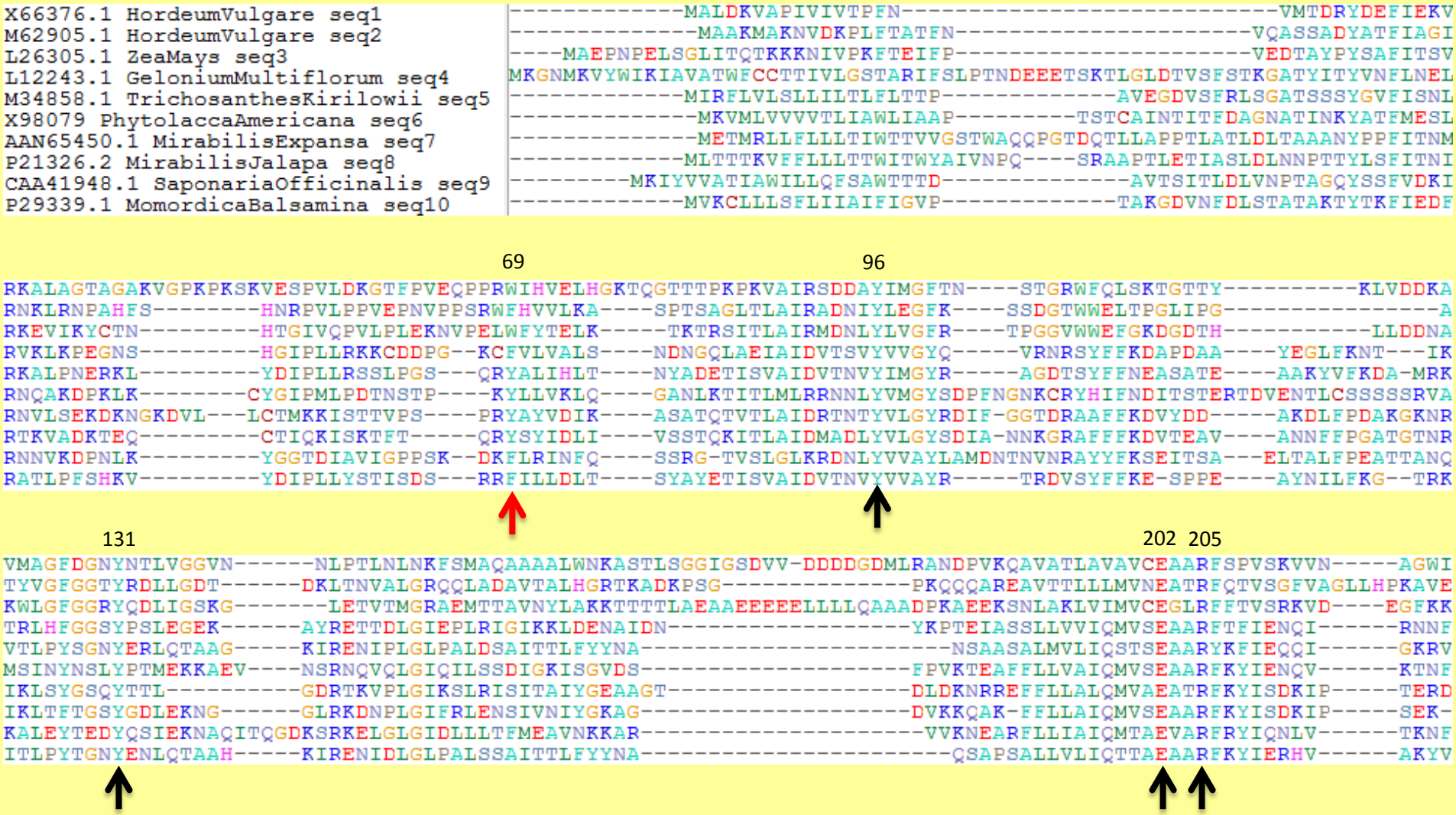
Given that the genome of *N. benthamiana* had been sequenced [48] and was accessible online https://solgenomics.net/organism/Nicotiana_benthamiana/genome, BLAST searches were carried out in attempts to find similar sequences to the RIP domain in JIP60. Different flavours of BLAST were used, but BLASTn, tBLASTn, and tBLASTx searches all failed to produce significant matches. The reason for this is, as explained earlier, the RIP domain amino acid sequence amongst known RIPs is poorly conserved, with only a handful of characteristic well-conserved positional residues [28]; and therefore BLAST was too superficial a searching technique to be reliable for finding RIPs.

Therefore a search method which considered and utilised the relatively weak but significant alignment of known RIP domains was required, namely one involving probabilistic inference based on multiple amino acid sequences. An example of such a search tool is HMMER [13; 16] The basis of this was a hidden Markov model (HMM) algorithm [13] assembled using the programs WinSCP and PuTTY, via which an alignment of ten known plant RIP domains [2; 3; 17; 25; 30; 34; 35; 40; 46; 52] (see table 1), deliberately chosen from a wide phylogenetic distribution, was used to search the predicted proteome of *N. benthamiana*. Shown in figure 1, this alignment of amino acid sequences was made using the CLUSTAL algorithm.

The HMM predicted proteome search yielded a single result, a shorter amino acid sequence than expected for a RIP domain – and subsequently the respective gene or DNA sequence was named *Short Candidate Ribosome-Inactivating Protein* (*SCRIPt*).

Table 1: Ten Plant RIPs. A Clustal alignment of the amino acid sequences of the RIP domains of these proteins was used partly as the basis for selecting mutagenesis sites for *JIP60,* as well as an input for a hidden Markov model search for RIPs in the predicted proteome of *Nicotiana benthamiana.*

Species	**Name**	**RIP Type**	**NCBI Accession No.**	**Reference**
Barley (*Hordeum vulgare*)	Jasmonate-Induced Protein 60 (JIP60)	Type III	X66376.1	Becker & Apel 1992
Barley (*Hordeum vulgare*)	cRIP30	Type I	M62905.1	Leah *et al.* 1991
Maize (*Zea mays*)	RIP-2	Type I	L26305.1	Bass *et al.* 1995
False lime (*Gelonium multiflorum*)	Gelonin	Type I	L12243.1	Nolan *et al.* 1993
Chinese cucumber (*Trichosanthes kirilowii*)	Trichosanthin	Type I	M34858.1	Shaw *et al.* 1991
American pokeweed (*Phytolacca Americana*)	Pokeweed Antiviral Protein (PAP)	Type I	X98079	Poyet & Hoeveler 1997
Mauka (*Mirabilis expansa*)	ME1	Type I	AAN65450.1	Vepachedu *et al.* (unpublished)
Four o'clock flower (*Mirabilis jalapa*)	Antiviral protein	Type I	P21326.2	Kataoka *et al.* 1991
Common soapwort (*Saponaria officinalis*)	Saporin	Type I	CAA41948.1	Fordham-Skelton *et al.* 1991
Balsam pear (*Momordica balsamina*)	Momordin	Type I	P29339.1	Ortigao & Better 1992


X66376.1 HordeumVulgare seq1
M62905.1 HordeumVulgare seq2
L26305.1 ZeaMays seq3
L12243.1 GeloniumMultiflorum seq4
M34858.1 TrichosanthesKirilowii seq5
X98079 PhytolaccaAmericana seq6
AAN65450.1 MirabilisExpansa seq7
P21326.2 MirabilisJalapa seq8
CAA41948.1 SaponariaOfficinalis seq9
P29339.1 MomordicaBalsamina seq10
69
96
131
202
205

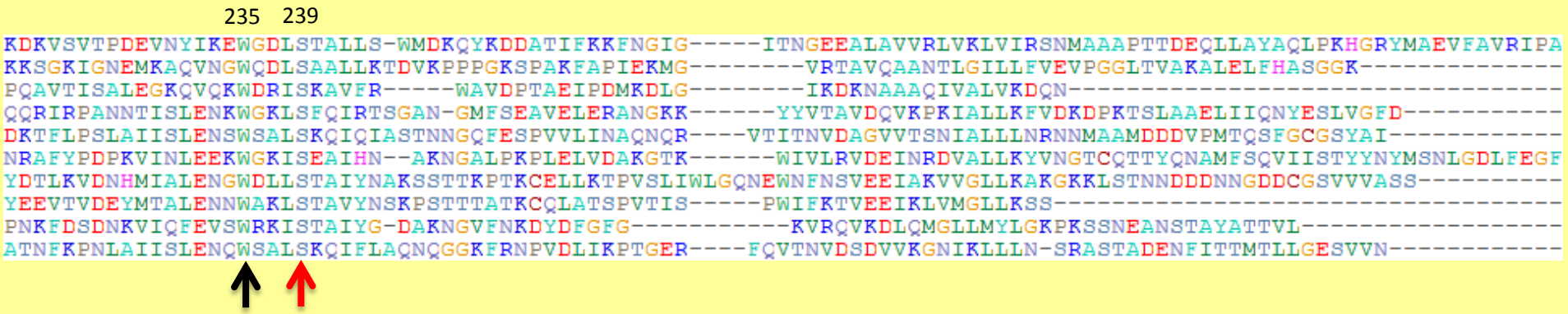


Figure 1: Clustal Alignment of Ten RIP Domain Amino Acid Sequences. This alignment was the starting point of both experiments – forming the HMM input for searching the *N. benthamiana* genome for RIPs; and part of the basis for selecting sites to target in site-directed mutagenesis in the JIP60 experiment. Red arrows indicate conserved amino acids at the start and end of the conserved domain as described by Lapadula *et al.* (2012, 2013); black arrows indicate conserved amino acids predicted to form the active site [27; 28]. Numbers above amino acids correspond to their position in the primary sequence of the JIP60 RIP domain [3] encoded by the construct used in the JIP60 experiment. Details of the ten RIPs are described in table 1.

Short Candidate Ribosome-Inactivating Protein (SCRIPt) – Gene Annotation

As described in the predicted proteome, the amino acid sequence of SCRIPt was 61 residues in length (see appendix A), but a manual annotation of the corresponding nucleotide sequence was carried out in order to gain clarity on the positions of start and stop codons as well as introns, and make a prediction on the exact size of the translated protein in *N. benthamiana*, as the predicted amino acid sequence was clearly not correct (see appendix A). From the manual annotation (shown in figure 2), SCRIPt was predicted to be a type I RIP (consisting only of a RIP domain) of 111 amino acids, with the genomic nucleotide sequence being of 438 bases, including three introns. This sequence reads in reverse from position 24921 on scaffold 893 of the assembled genome "Ni_ben" [48] available on the Sol Genomics website (https://solgenomics.net/jbrowse_solgenomics/?data=data%2Fjson%2FNiben1.0.1&loc=Niben101Scf00893).

Analysing the RIP Domain of JIP60 and Selecting Sites for Mutagenesis

Being a type III RIP [6], JIP60 has an N-terminal RIP domain which is separated from the adjoining C-terminal domain after translation [43]. Hence a good starting point in characterising the importance of the structure on the function of this protein is by assessing the importance of key amino acids in the RIP domain. Four amino acids in the RIP domain were chosen for mutagenesis, with the aim of creating four different *JIP60* mutants – each with a mis-sense mutation resulting in the replacement of the given amino acid residue with alanine, a small and comparatively inert amino acid. The four residues chosen were tyrosine – position 96, glutamic acid – position 202, arginine – position 205, and tryptophan – position 235. The reason for these choices was that the amino acids in question were both (i) conserved in all ten RIP sequences from the alignment used earlier, as shown in figure 1, and (ii) predicted to be involved in active site function by Lapadula *et al.* (2012; 2013), which was supported by the predicted protein structures of JIP60 as assembled using Phyre2 Protein Fold Recognition Software [26], as shown in Figure 3. For convenience these JIP60 mutants will be referred to as Y96A, E202A, R205A and W235A. Interestingly, in the case of E202A, the same corresponding site in maize RIP (E207) had been tested in a previous published study [31]. The authors found that maize RIP mutant E207A had a reduced ribosome-inactivating function of around 556-fold compared to the wild type when tested on rabbit reticulocyte lysate [31]. The central hypothesis was that each of these amino acid sites were essential for the N-glycosidase activity of JIP60, and therefore that the mutants would lack the ribosome-inactivating function of wild type JIP60, shown by lack of cell death in *Agro*-infiltrated *N. benthamiana* leaf tissues expressing the mutants; and that in contrast, expression of wild type JIP60 would result in cell death. In the case of the E202A mutant, this would support the study by Mak *et al.* (2007) in suggesting the importance of the conserved glutamic acid residue in the role of the active site of plant RIPs in general.

ATG|CTT|TTG|ATT|CGG|ACG|TTA|CTC|ACC|TTT|ACC|GTC|TCG|CCG|ATA|CAC|TTC|ATA|TCA|
M L L I R T L L T F T V S P I H F I S

TTC|ACG|CCG|TCT|CCA|GTT|AGA|TTC|ACA|TCC|TTA|GTT|TCC|
F T P S P V R F T S L V S

GTTTGAATAACCGATTGCTCGTAATAATTGTTAATCTAG|
Intron

AGC|TCC|ATT|TAT|TCT|AAT|TTT|GTT|TCA|GGT|CTG|AGA|AAT|GAG|ACT|ACA|CAG|GCA|CTC|
S S I Y S N F V S G L R N E T T Q A L

ATG|GAG|AAG|CTC|GTT|GTC|GCA|GAT|TTA|GAG|GTT|GCT|CTG|ATC|
M E K L V V A D L E V A L I

AGTATTTCTCTCTCTTTTTCGGTTACTTTAAGAATTAG|
Intron

GCT|ATG|ATT|AAT|AAT|TTT|GCG|TTA|TGT|ATT|ATC|AGA|CGT|TAT|GTT|AAT|GTA|TTA|AAA|
A M I N N F A L C I I R R Y V N V L K

TCT|GCA|TAT|ACA|GTG|TAT|TAT|CAA|AAA|AAC|AAA|ATT|ACT|ACT|
S A Y T V Y Y Q K N K I T T

CAGTTTGATTTTTAATTGGATTAAG|
Intron

ATT|TGT|ACT|AAT|AAC|ACC|CTG|GCC|TAT|GTT|AAA|TTT|ATC|TAA
I C T N N T L A Y V K F I STOP

Figure 2: Manual Annotation of *SCRIPt* from the *Nicotiana benthamiana* Genomic Sequence. The predicted amino acid sequence of the SCRIPt protein is highlighted in green, beneath the genomic sequence. Non-coding nucleotides are shown in red.

JIP60 EXPERIMENT

Experimental JIP60

Before describing the methods for gene cloning and use of plasmid vectors in the JIP60 experiment, it is important to highlight the differences between the version of JIP60 used experimentally – i.e. the sequence of the *JIP60* insert – and the JIP60 sequence as described by Becker & Apel (1992) and used for the alignment shown in figure 1. Firstly, the *JIP60* insert only included the RIP domain-encoding part of the gene sequence, as this experiment investigates the irreversible translation inactivation (N-glycosidase and depurination activity) of the JIP60 protein, proposed to be performed by processed JIP60 [6; 43]. A stop codon, however, has not been added to the *JIP60* insert, due to the use of a *eGFP* tag as an expression reporter, detailed below, via the production of a JIP60-eGFP protein in the transgenic host plant *N. benthamiana*. Finally, the sequence of the *JIP60* insert has been modified to mimic the internal activation process which takes place *in vivo* after translation. As shown in appendix B, this involves the removal of a region of the JIP60 RIP domain as identified by Rustgi *et al.* (2014) as the "internal putative processing site (P1)". Removal of this region and the placement of an ML (methionine-leucine) linker (see appendix B, figure B(i)) results in the production of an active ribosome-inactivating JIP60 product, causing cell death when transiently-expressed in *N. benthamiana* [37]. Internal activation is a property which JIP60 shares with maize b-32, another type III RIP, on which the same method has been successfully used [31]. This is why the amino acid sequence of experimental JIP60 was shorter (267 amino acids) than that of the JIP60 RIP domain described by Becker & Apel (1992) (283 amino acids).

Gene Cloning

Gateway Cloning® technology (Invitrogen) was the method used to clone *JIP60*. Based upon site-specific recombination naturally performed by phage λ, DNA inserts can be transferred between plasmid vectors using BP Clonase® and LR Clonase® enzymes which target *att* sites [20; 24]. The *JIP60* insert described above, cloned into commercially-available plasmid pDONR201 (Invitrogen), was the starting point for *JIP60* mutagenesis. This is a reliable entry vector plasmid for use in gene cloning [57]. The *JIP60* insert was 864 nucleotides in size, and the entire plasmid had a size of 3059 nucleotides. This was the template for the site-directed mutagenesis reactions which generated *JIP60* mutants on the plasmid vector. Once successful mutagenesis had been confirmed by Sanger sequencing, *JIP60* was transferred to commercially-available plasmid pK7FWG2 (Invitrogen), an *Agrobacterium*-suitable vector [4]. (See appendix C for the sequences all plasmid vectors and *JIP60*-plasmid constructs used in this experiment.) The resulting construct in each case was used in the *Agro*-infiltration of *N. benthamiana* leaves to test the importance of the given amino acid site on the ribosome-inactivating function JIP60.

Using Plasmid Vectors pDONR201 and pK7FWG2

The empty plasmid vector pDONR201 (see figure 5a) has a size of 4470 nucleotides, containing an insert region housed between *attP1* and *attP2* sites – which are converted to *attL1* and *attL2* sites when a gene of interest is inserted, via the BP Clonase® reaction. Crucially, within the insert region of the empty vector lies *ccdB*, a suicide gene [56] and outside the region lies a Kanamycin resistance gene, with the former acting as selectable marker against the empty vector in *E. coli* and the latter as a selectable marker for the presence of the vector. Prior to the start of the project, a former member of the Spanu research group [37] had assembled the *JIP60*-pDONR201 construct, with a size

of 3059 nucleotides, for safekeeping and future use. PDONR201 was a useful vector due to its relatively small size, and was rapidly amplified using *E. coli* by heat-shock transformation, agar plating and overnight broths using selective media. Plasmid DNA was isolated after overnights using the QIAprep® Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. Also due to its size, this construct was suitable for site-directed mutagenesis reactions.

In each case, once mutant (or wild type)-*JIP60*-pDONR201 constructs (see figure 5b) had been confirmed, by Sanger sequencing, and amplified, the presence of *attL* sites on the vector allowed transfer of the gene of interest to plasmid pK7FWG2 using the Gateway® LR Clonase® II Enzyme Mix (Invitrogen) according to manufacturer's instructions.

Following the LR Clonase® reaction, in each case the mutant (or wild type)-*JIP60*-pK7FWG2 construct, of size 11021 nucleotides, (see figure 5d) was amplified using *E. coli*, miniprepped and confirmed by Sanger sequencing.

The empty plasmid vector pK7FWG2 (see figure 5c) has a size of 11880 nucleotides, and like pDONR201, contains the suicide gene *ccdB* within its insert region, which lies between *attR1* and *attR2* sites – which are converted to *attB1* and *attB2* sites when a gene of interest is inserted, via the BP Clonase® reaction. The antibiotic resistance marker in the case of pK7FWG2 is for spectinomycin. Importantly, pK7FWG2 is a Ti (tumour-inducing) plasmid, with a T-DNA region encompassing the insert region (i.e. the insert region lies between left and right T-DNA border regions), meaning that it is an *Agrobacterium*-suitable plasmid, could be used for *Agro*-infiltration and hence this is how JIP60 was transiently expressed in *N. benthamiana*. Furthermore, the plasmid contained an *eGFP* - (*Enhanced Green Fluorescent Protein*) gene sequence which resulted in a eGFP C-terminal tag on the expressed JIP60 RIP domain protein – on the condition that the sequence used for JIP60 lacked a stop codon, which was the case. Hence eGFP acted as an expression (translational) reporter for JIP60 [24]. (The protein structure of eGFP is shown in figure 4.) This marker enabled confirmation of the expression of the gene of interest in *N. benthamiana* following *Agro*-infiltration.

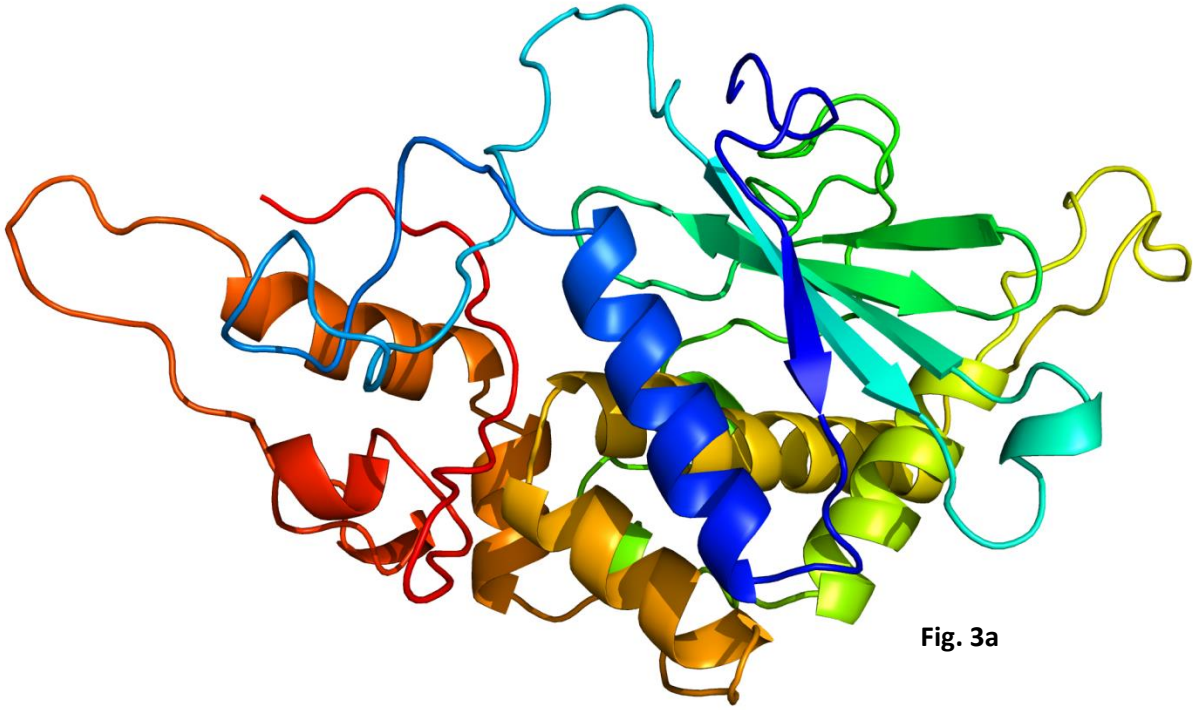


Fig. 3a

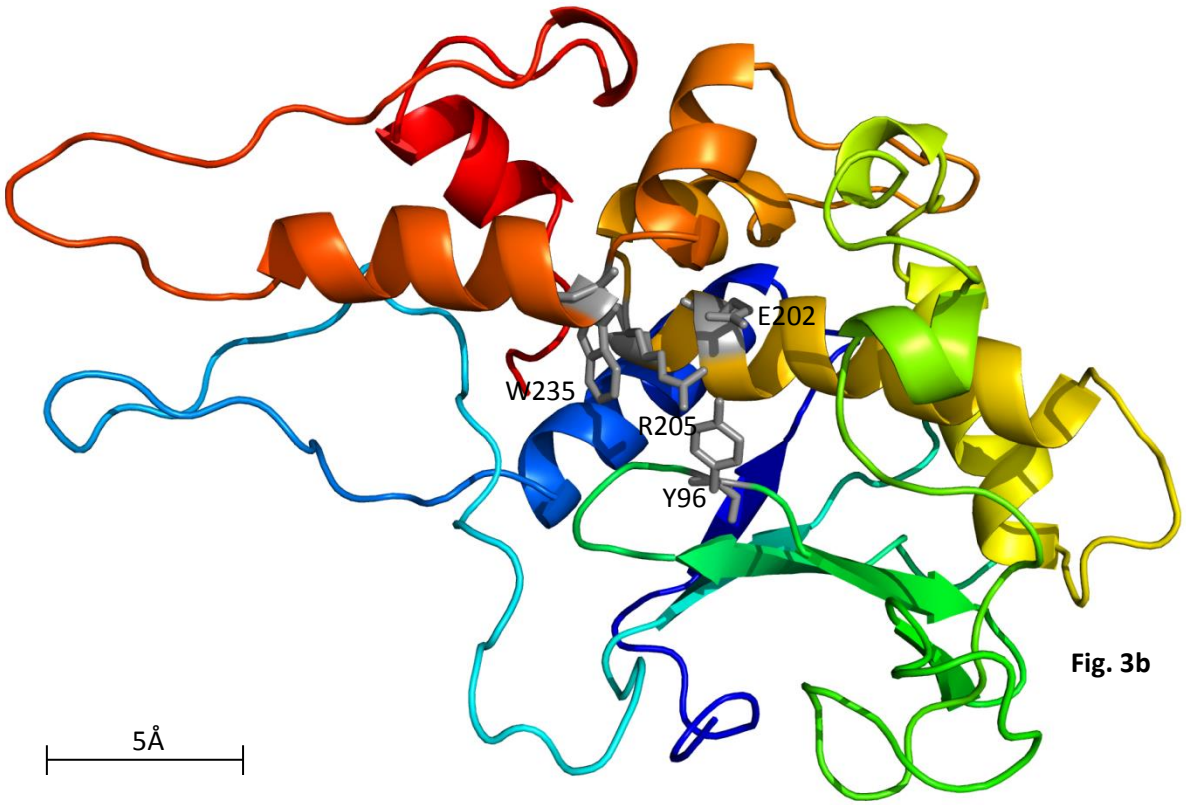


Fig. 3b

Figure 3: JIP60 RIP Domain Protein Structures. These cartoons display the structures of JIP60 RIP domains as predicted by the Phyre2 Protein Fold Recognition Server [26] and are coloured by a spectrum with in both cases the N-terminus in blue and C-terminus in red. Figure 3a: JIP60 RIP domain, according to the sequence described by Becker & Apel (1992); figure 3b: JIP60 RIP domain encoded by the sequence of the *JIP60* insert used in the JIP60 experiment, with the residues of interest (Y96, E202, R205, and W235) targeted for mutagenesis shown (skeletal structures superimposed) in grey.

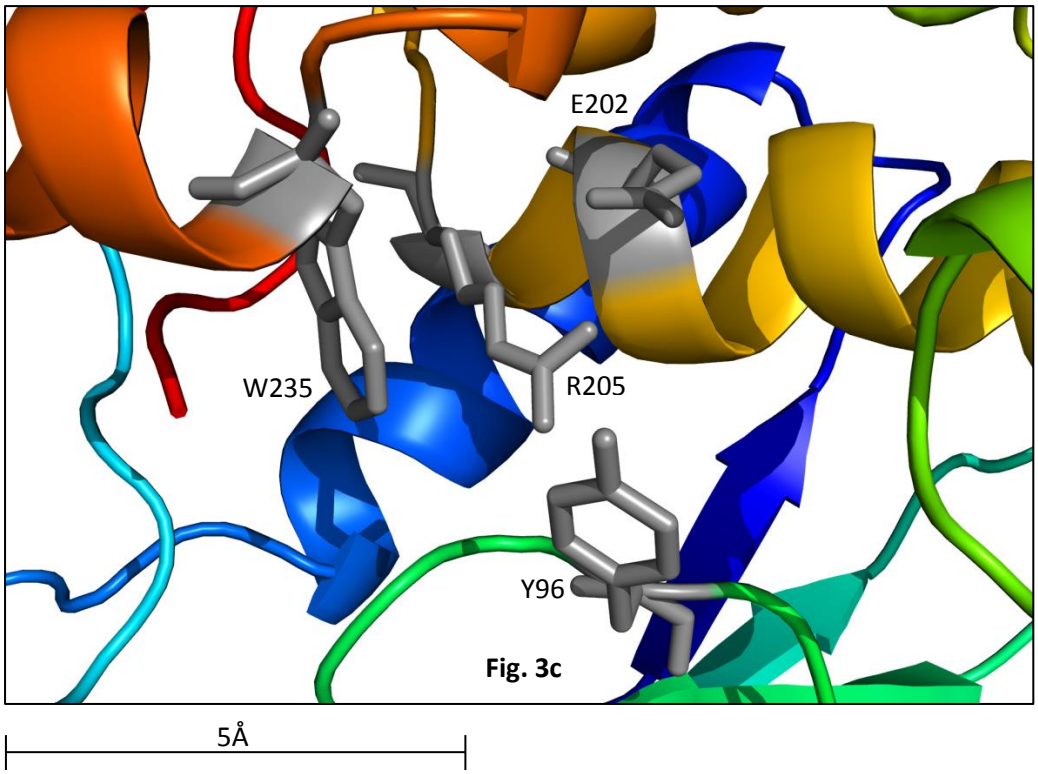


Figure 3 (continued). Figure 3c: a closer view of the proposed active site [27; 28] of the JIP60 RIP domain encoded by the sequence of the *JIP60* insert used in the JIP60 experiment, with the residues of interest (Y96, E202, R205, and W235) targeted for mutagenesis shown (skeletal structures superimposed) in grey.

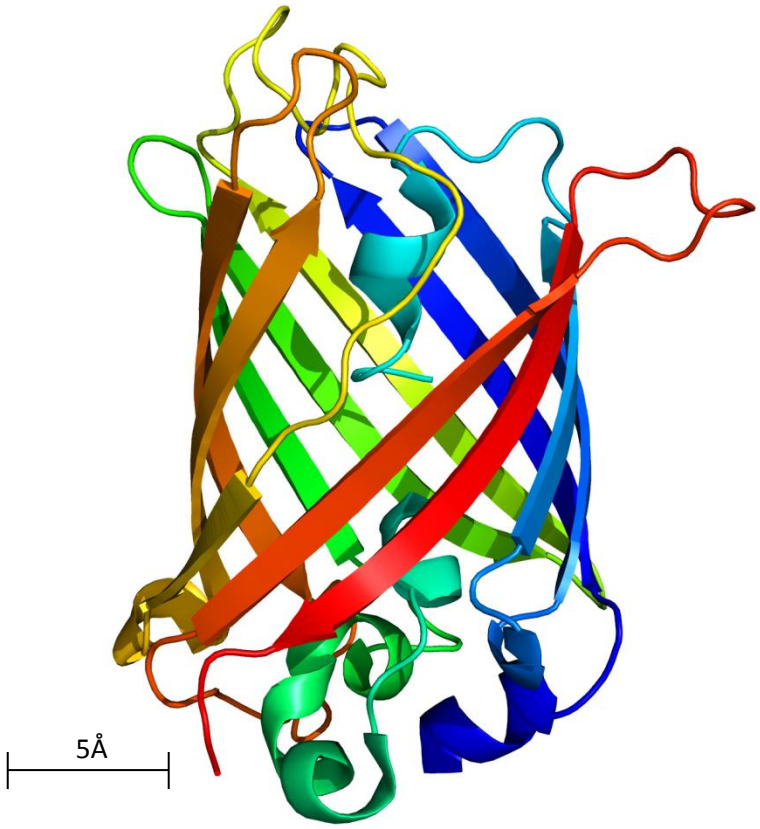


Figure 4: Enhanced Green Fluorescent (eGFP) Protein Structure. This cartoon displays the structure of eGFP as predicted by the Phyre2 Protein Fold Recognition Server [26], and is coloured by a spectrum with the N-terminus in blue and C-terminus in red. Together with the JIP60 RIP domain shown in figures 3b and 3c, this forms the expressed product in the epidermal cells of *N. benthamiana* leaf tissues *Agro*-infiltrated with *JIP60*-pK7FWG2-transformed *Agrobacterium* in the JIP60 experiment.

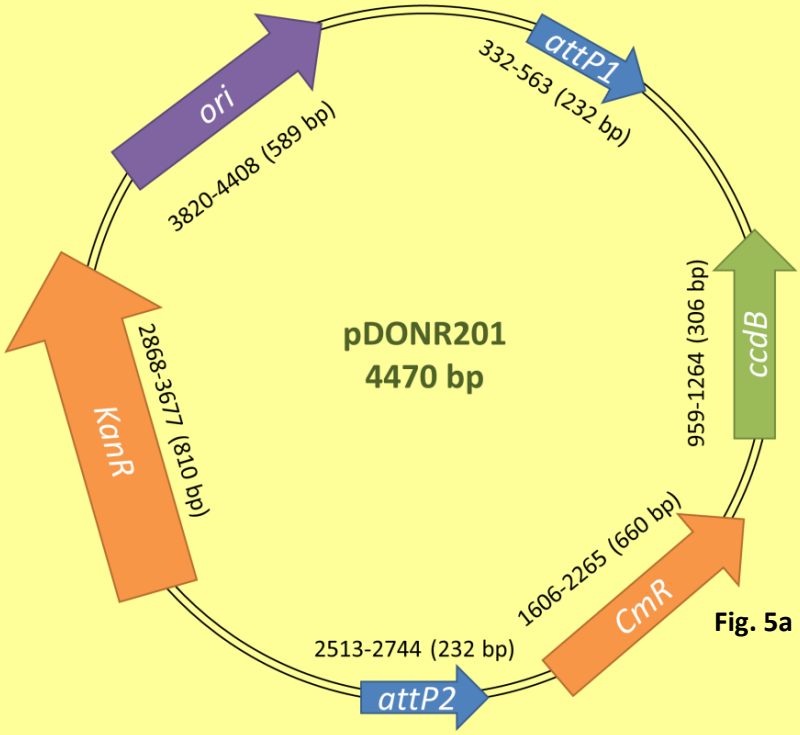


Fig. 5a

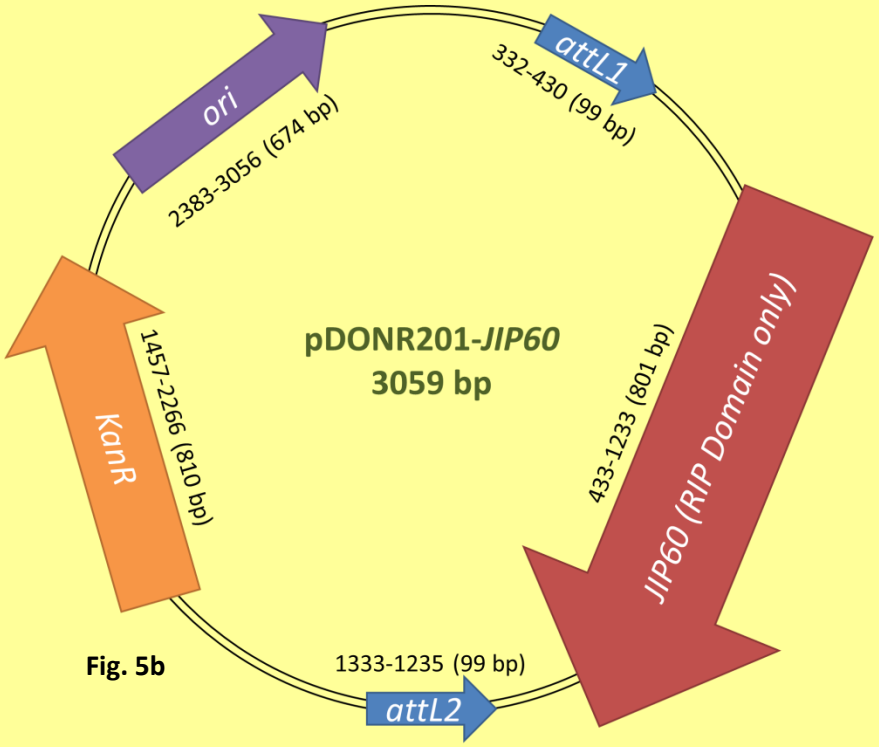


Fig. 5b

Figure 5: Plasmid Maps Relevant to the JIP60 Experiment, Featuring Sequences of Interest. Figure 5a: empty plasmid pDONR201 (Invitrogen); figure 5b: plasmid vector pDONR201 with *JIP60*. *Ori*: origin of replication [23]; *KanR*: kanamycin resistance gene; *CmR*: chloramphenicol resistance gene; *ccdB*: a suicide gene.

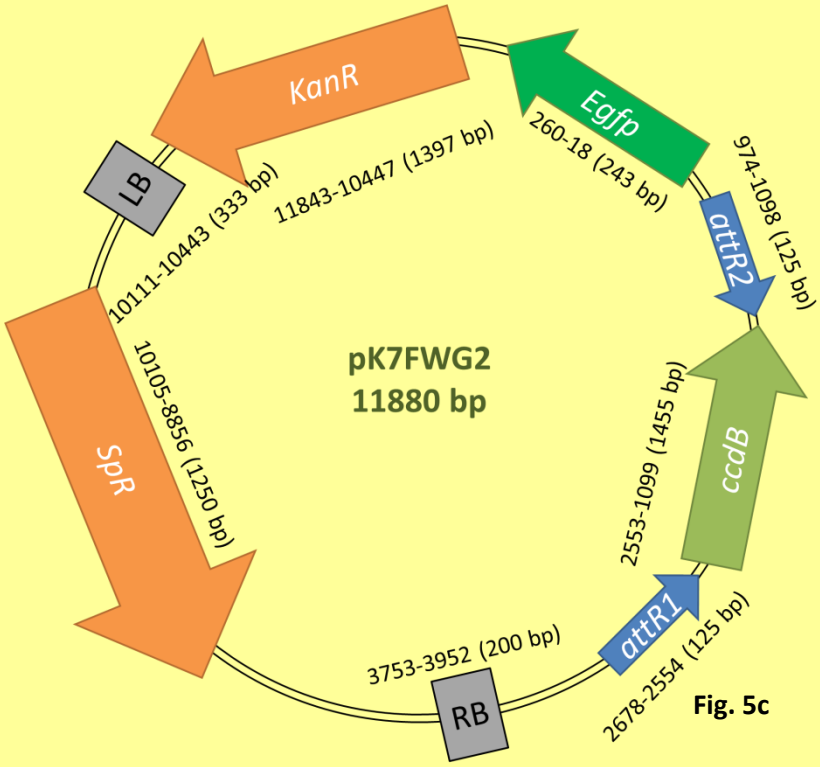


Fig. 5c

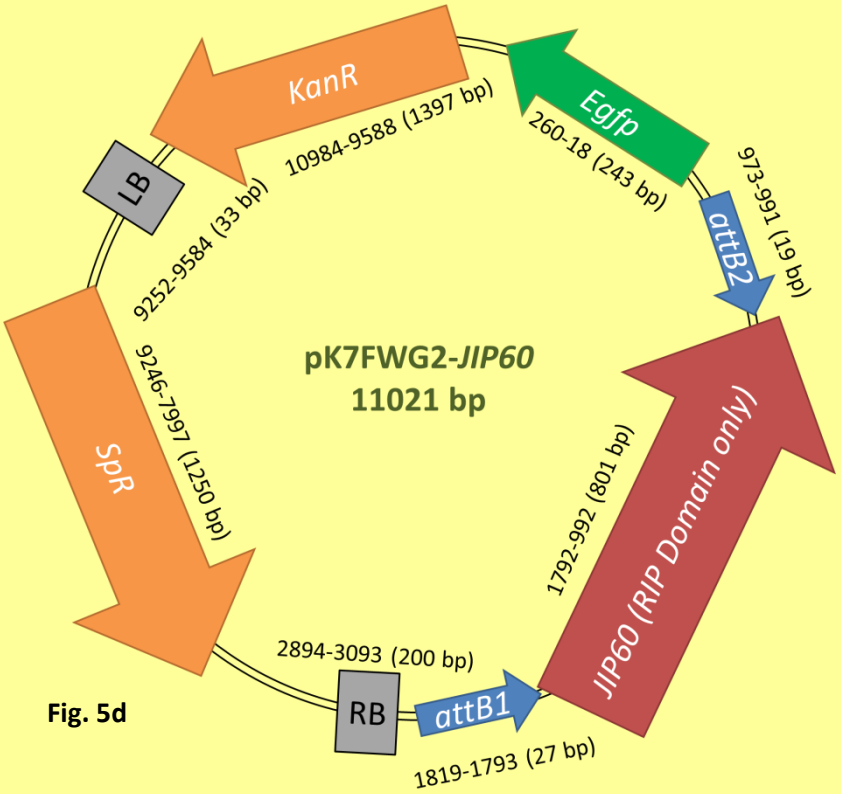


Fig. 5d

Figure 5 (continued). Figure 5c: empty *Agrobacterium*-suitable plasmid pK7FWG2 (Invitrogen); figure 5d: plasmid vector pK7FWG2 with *JIP60*. *Egfp*: enhanced green fluorescent protein; *KanR*: kanamycin resistance gene; *SpR*: spectinomycin resistance gene; *ccdB*: a suicide gene.

Site-Directed Mutagenesis

The QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies) was used for site-directed mutagenesis. At first, in the case of the Y96A mutant, the QuikChange® I reaction method was performed, but subsequently, in the case of the E202, R205, and W235 mutants, the QuikChange® II reaction method was used due to a greater efficiency and lower cost. The principles behind the two reactions are similar, but whereas QuikChange® I requires primers which do not overlap at all but meet at their 5' ends – with the forward primer containing the amino acid change (mutation site) in the middle of its sequence – QuikChange® II requires completely complementary and overlapping primers, both of which contain the mutation site. Unlike QuikChange® I, QuikChange® II produces a circular product, and so does not require primers which are phosphorylated at the 5' end or a subsequent ligase reaction. The primers used for generating each of the four mutants are described in table 2. Reaction mixtures, of total volume 25 µl, contained 1 µl of template at a concentration of 1 ng/µl, 0.5 µl of each primer, 0.5 µl Pfu Ultra High-Fidelity® DNA polymerase, 10 times Pfu reaction buffer, and 0.5 µl dNTP mix (10mM). The reaction conditions used were 95°C for 120 seconds, 20 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 210 seconds, then 72°C for 600 seconds. The products were then treated with *Dpn* I restriction enzyme and left overnight at 37°C (and the Y96A QuikChange® I linear product ligated using DNA ligase) before amplification in *E. coli*.

Table 2: Primers Used in *JIP60* Site-Directed Mutagenesis. Amino acid changes are in bold.

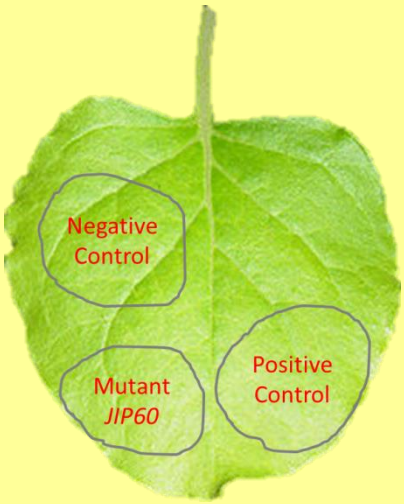
Mutant	Primers (5'-3') Forward; Reverse	Melting Points	Mutagenesis Method
JIP60 (Y96A)	~~**TAC**~~ CGACGACGCC**GCA**ATCATGGG; CTTCGGATGGCCACCTTGGGATTAGG	 61.3°C 61.3°C	QuikChange® I
JIP60 (E202A)	~~**GAG**~~ GCCTTCTGC**GCG**GCCGCGAGATTCATCC; ~~**CTC**~~ GGATGAATCTCGCGGC**CGC**GCAGAAGGC	 70.3°C 70.3°C	QuikChange® II
JIP60 (R205A)	~~**AGA**~~ GCGAGGCCGCG**GCA**TTCATCCCTGTCTCC; ~~**TCT**~~ GGAGACAGGGATGAA**TGC**CGCGGCCTCGC	 69.8°C 69.8°C	QuikChange® II
JIP60 (W235A)	~~**TGG**~~ GGTCAACTACATCAGGGAG**GCG**GGTGACTTGTCCACCGC ~~**CCA**~~ GCGGTGGACAAGTCACC**CGC**CTCCCTGATGTAGTTGACC	 70.7°C 70.7°C	QuikChange® II

Agro-Infiltration of N. benthamiana

JIP60-pK7FWG2 constructs were introduced into *Agrobacterium tumefaciens* strain GV3101. The bacteria were transformed with 200ng of the plasmid by electroporation with a 2.2kV pulse for 5.9 milliseconds, before one hour-long incubation in LB broth and then plating on selective media (spectinomycin agar), and were left to grow for three days at 28°C.

Subsequently individual *Agrobacterium* colonies were screened for the plasmid using colony PCR, using the primers: 5' AGGTGGCATCGCCCTCGCC 3' (forward) and 5' CTCTATATAAGGAAGTTCATTTCATTTGGA 3' (reverse), (see appendix C) which amplified a region with a size of 1105 nucleotides (and in the case of the empty vector, the size would be 1964 bp). The reaction conditions for this were 95°C for 120 seconds followed by 30 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 60 seconds, then 72°C for 300 seconds; with a 10 µl reaction mixture containing 5 µl template, 0.2 µl of each primer, 0.2 µl dNTP mix, 0.1 µl Taq polymerase and 5 times reaction buffer. PCR templates were prepared by mixing colony bacteria with water and heating at 95°C for ten minutes.

Positive colonies were re-streaked on selective media and incubated for 24 hours prior to *Agro*-infiltration. Leaves of four week-old *N. benthamiana* plants, kept under summer conditions, were infiltrated using syringes without needles and by applying moderate pressure to the abaxial surface. Infiltration mix consisted of bacteria suspended in MMA solution (10mM MES and 10mM $MgCl_2$ at pH 5.3), measured at a standardised absorbance (0.1) on a spectrophotometer. Leaves were infiltrated with *JIP60* (wild type)-pK7FWG2-*Agrobacterium*, *JIP60* (mutant)-pK7FWG2-*Agrobacterium*, and empty (just *eGFP*)-pK7FWG2-*Agrobacterium* in separate patches, as shown in figure 6. These acted as the positive control, treatment, and negative control respectively. In each case, *Agrobacterium* at standard absorbance (0.1) transformed with *P19*-pK7FWG2 was also added. P19 is a tomato bushy stunt virus protein which can be used in *Agro*-infiltration to repress gene silencing in the host plant in order to increase the efficacy of transient expression [54]. Eleven replicates were performed, using third and fourth leaves. Data was collected by photography of leaves at intervals measured in days post infiltration, depending on visible changes in leaves.



Negative control = *Agrobacterium* transformed with empty pK7FWG2 + *Agrobacterium* transformed with P19

Positive control = *Agrobacterium* transformed with *JIP60* (wild type)-pK7FWG2 construct + *Agrobacterium* transformed with P19

Mutant *JIP60* = *Agrobacterium* transformed with mutant-*JIP60*-pK7FWG2 + *Agrobacterium* transformed with P19

(Adaxial surface shown)

Figure 6: *Nicotiana benthamiana* Leaf *Agro*-Infiltration Arrangement.

Testing JIP60 Expression in N. benthamiana and Fluorescence Microscopy

To confirm the expression of experimental JIP60, or to be more specific, JIP60-eGFP, in *N. benthamiana* epidermal cells of *Agro*-infiltrated leaf tissues, fluorescence micrographs were taken using a low magnification light microscope, of the abaxial surfaces. Microscopy settings used were 510 ms exposure, 1.25 x colour saturation, 1.0 x gain, with the sample exposed to ultraviolet light corresponding to maximal excitation of eGFP.

NICOTIANA BENTHAMIANA EXPERIMENT

Several approaches and many PCRs were carried out in attempts to detect the expression of, and successfully isolate, amplify and clone *SCRIPt* from both untreated/unstressed and methyl jasmonate-treated *N. benthamiana* leaves. RNA was extracted and converted into cDNA by reverse transcription, the reason for this being a deliberate effort isolate the expressed gene, i.e. from mRNA in *N. benthamiana* leaves, and not from genomic DNA.

RNA Extraction and Reverse Transcription

RNA was extracted from *N. benthamiana* leaves using RNeasy® Mini Kit (Qiagen) according to manufacturer's instructions. Reverse transcription was carried out with Superscript® III Reverse Transcriptase Kit (Invitrogen) according to manufacturer's instructions, and using oligo(dT) primers (not random primers) in order to target mRNAs.

Methyl Jasmonate Treatment of Leaves

Moderately-sized (approximately 5cm from petiole to tip) leaves from four week-old *N. benthamiana* plants were selected for RNA extraction, including those which were first treated with exposure to methyl jasmonate. This involved immersing the leaves in 100ml water with 1.2µl methyl jasmonate dissolved in 100µl dimethyl sulphoxide, for four days.

Gene Cloning

As in the case of the JIP60 experiment, efforts were made to clone *SCRIPt* into a plasmid vector to enable sending for Sanger sequencing and with the goal of confirming the mRNA sequence of the expressed gene in *N. benthamiana* leaves, as well as confirming the genomic sequence of the gene as described the single whole genome sequence of *N. benthamiana* [48]. Commercially-available plasmid vector pCR8 (Invitrogen) (which has a size of 2817 nucleotides) was used for this (see figure 7 and appendix C), with the pCR8/GW/TOPO® Cloning Kit (Invitrogen), according to manufacturer's instructions. This process relies on the additional adenine base produced on the 3' ends of PCR products [47]. The PCR product (in this case *SCRIPt*), is positioned at two thymine bases in insertion site, also known as the *TOPO* site, of the plasmid vector (see figure 7 and appendix C) [47]. This plasmid vector was chosen due to potential applications as an entry vector in Gateway Cloning® given the presence of *attL* sites [20; 24]. Following transformation of *E. coli* with the TOPO® reaction product, colony PCRs were performed, under the same conditions as the *Agrobacterium* colony PCRs performed in the JIP60 experiment, to detect the presence of *SCRIPt*-pCR8 constructs (as opposed to empty pCR8) in individual *E. coli* colonies. This was important because the pCR8 plasmid lacks a suitable insertion site negative marker (unlike the presence of the *ccdB* suicide genes in pDONR201 and pK7FWG2). These colony PCRs used primers corresponding to the M13 region on the pCR8

plasmid (see figure 7 and appendix C), and these were 5’ GTCACGACGTTGTAAAACGACGGCCAG 3’ (forward) and 5’ CAGAGCTGCCAGGAAACAGCTATGAC 3’ (reverse).

The initial planned method for cloning was to first amplify *SCRIPt* from cDNA using primers which contained *attB* site sequences at 5’ ends, with the intention of then conducting a BP Clonase® reaction and hence transferring *SCRIPt* into a pDONR201 plasmid vector (which when empty contains *attP* sites – see figure 5a and appendix C). These primers were 5’ GGGGACAAGTTTGTACAAAAAAGCAGGCTTC—ATGCTTTTGATTCGGACGTTACTC 3’ (forward) and 5’ GGGGACCACTTTGTACAAGAAAGCTGGGTC—TTAGATAAATTTAACATAGGCCAGGGTG 3’ (reverse); with the first 31 and 30 bases respectively corresponding to the construction of *attB* sites, as recommended in the manufacturer’s instructions. When this proved unsuccessful, the pCR8 cloning plan described above was implemented. The primers designed for this, lacking the *attB*-site overhangs and amplifying the *SCRIPt* gene alone, were 5’ ATGCTTTTGATTCGGACGTTACTC 3’ (forward) and 5’ TTAGATAAATTTAACATAGGCCAGGGTG 3’ (reverse).

Throughout both experiments, all agarose gels for electrophoresis were prepared using TBE (Tris/Borate/EDTA) buffer and 10 times SYBR Safe® DNA Gel Stain (Invitrogen).

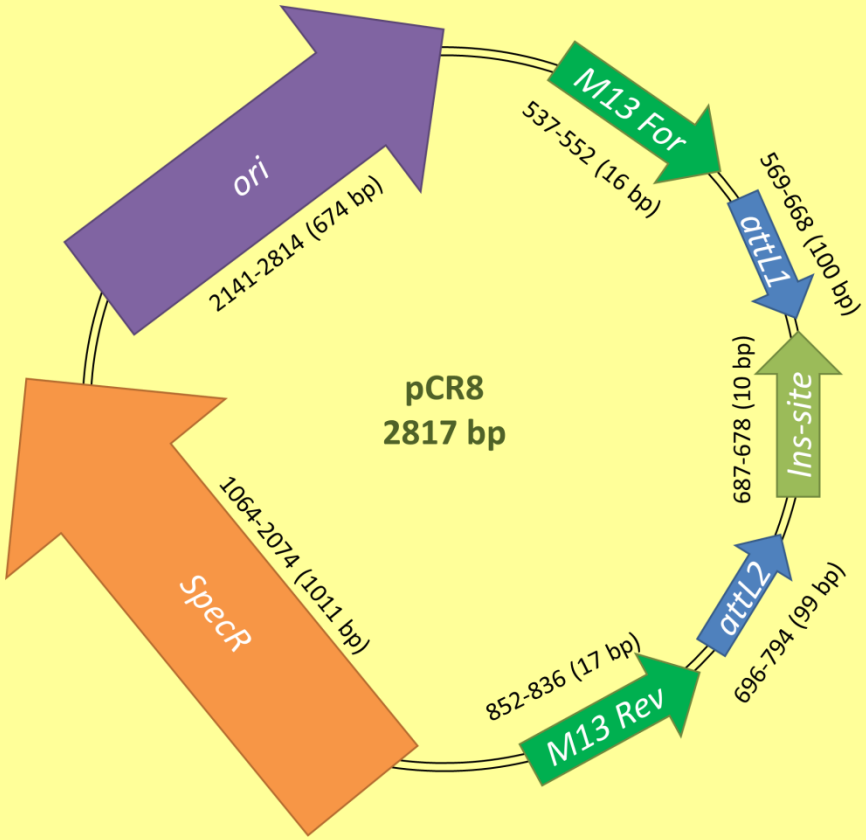


Figure 7: Map of the Empty pCR8 Plasmid Vector (Invitrogen), used for Cloning in the *N. benthamiana* Experiment. *SpecR*: spectinomycin resistance gene; *ori:* origin of replication; *Ins-site:* insertion site; *M13 For* and *M13 Rev* represent the regions on the plasmid corresponding to the M13 primers used in colony PCRs.

Results

The JIP60 and *N. benthamiana* experiments yielded results suggesting insights into the nature and importance of RIPs and the structure of the RIP domain, in barley and *N. benthamiana*.

JIP60 EXPERIMENT

Successful Cloning of JIP60 Mutants

All four mutants of *JIP60* – Y96A, E202A, R205A, and W235A – as well as the wild type, were successfully generated by QuikChange® reactions on pDONR201 plasmid vectors, and all except for R205A were eventually cloned into pK7FWG2 vectors, as shown by figures 10 (wild type), 11 (E202A), and appendix D. The high efficiency of both site-directed mutagenesis and the LR Clonase® reaction was however unfortunately offset by difficulties in the reliability of *Agrobacterium* transformation – specifically due to the tendency of the emergence of *Agrobacterium* false colonies (not carrying the desired plasmid), even on selective media. One mutant of JIP60 was successfully tested in *Agro*-infiltration: *JIP60* (E202A), following the detection of positive *Agrobacterium* colonies via colony PCR (see figure 9). Figure 8 shows the detection of *Agrobacterium* colonies positive for *JIP60* (wild type)-pK7FWG2.

Agro-Infiltration Results: JIP60 E202A Mutant

The photographic results of the *JIP60* E202A pK7FWG2 *Agro*-infiltration of *N. benthamiana* leaves are shown in figures 12 and 13. Almost exclusively, cell death is only seen in the positive control (wild type *JIP60*) infiltration patches, with the exception of three leaves where spill-over has occurred into the *JIP60* E202A patch, and one additional leaf, perhaps due to accidental contact with the leaf mid vein. Yellowing of the affected patches is visible from five days post infiltration, and darkening with cell death appears from eleven days post infiltration. These results strongly suggest that this mutant, *JIP60* (E202A), lacks the ribosome-inactivating function of the wild-type form.

Expression of JIP60 in Nicotiana Benthamiana

The fluorescence micrographs (figures 14 and 15) conclusively show that JIP60 was expressed by epidermal cells in *Agro*-infiltrated *N. benthamiana*. It is clear from figures 14a and 14b that wild type and mutant forms of JIP60 respectively have been expressed, by the visibility of the eGFP protein which is tagged to the RIP domain; whilst figure 14c shows that eGFP from the empty pK7FWG2 vector is expressed in the absence of JIP60 as expected. The green colour in figures 14 and 15 is not due to autofluorescence of epidermal cells, as shown by the comparative darkness of non-infiltrated tissue (figure 15).

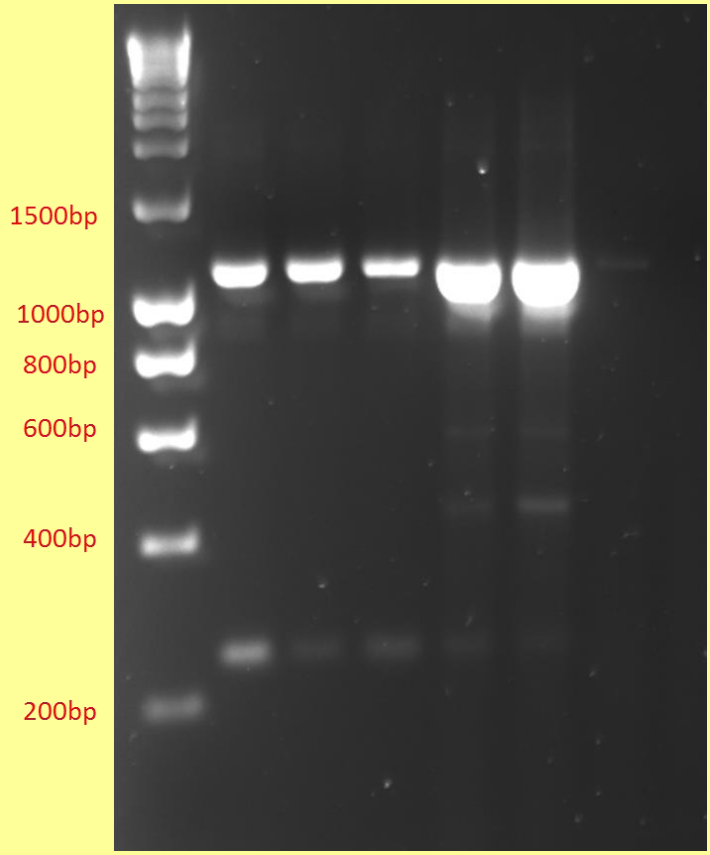


Figure 8: Detection of *JIP60* (wild type)-pK7FWG2 Positive *Agrobacterium* Colonies. Electrophoresis was run at 90 volts for 60 minutes, using a 1.5% agarose TBE gel. Lane 1: DNA ladder with size markers as labelled. Lanes 2-5: positive detection of *JIP60* (wild type)-pK7FWG2 in *Agrobacterium* colonies following colony PCR. The colony corresponding to lane 5 was used in *Agro*-infiltration. The band sizes in lanes 2-5 are between 1000 and 1500 base pairs, which is expected for *JIP60*-pK7FWG2 (amplified region 1105 bp). The expected band size for the empty pK7FWG2 vector is 1964 bp.

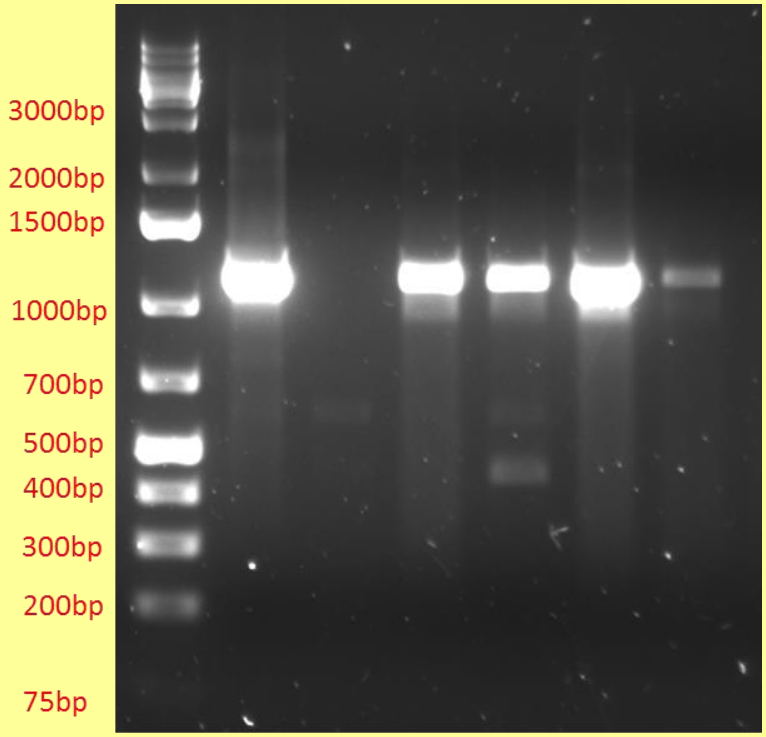


Figure 9: Detection of *JIP60* (E202A)-pK7FWG2 Positive *Agrobacterium* Colonies. Electrophoresis was run at 90 volts for 60 minutes, using a 1.5% agarose TBE gel. Lane 1: DNA ladder with size markers as labelled. Lanes 2, 4, 5, and 6: positive detection of *JIP60* (E202A)-pK7FWG2 in *Agrobacterium* colonies following colony PCR. The colony corresponding to lane 2 was used in *Agro*-infiltration. The band sizes in lanes 2, 4, 5, and 6 are between 1000 and 1500 base pairs, which is expected for *JIP60*-pK7FWG2 (amplified region 1105 bp). The expected band size for the empty pK7FWG2 vector is 1964 bp.

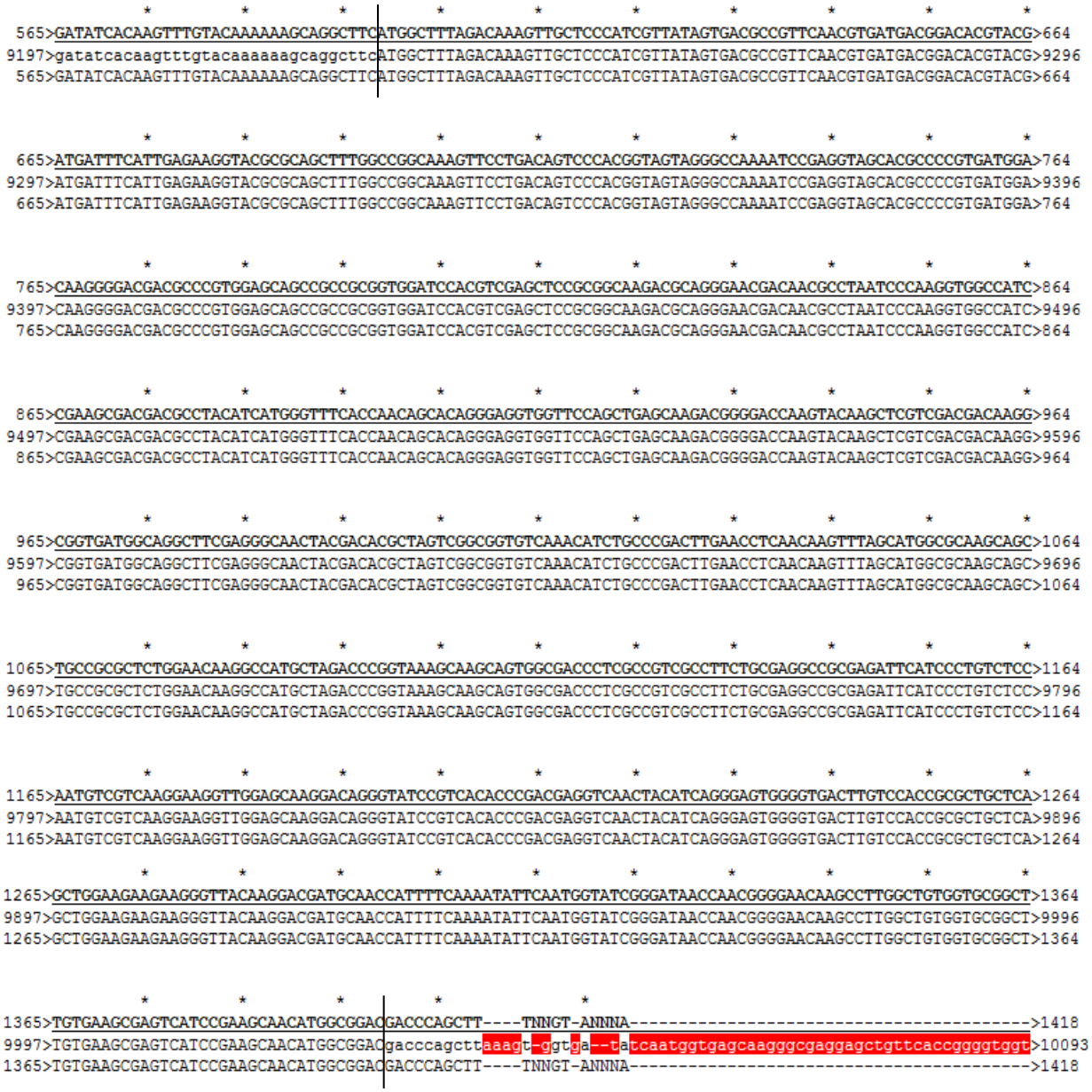


Figure 10: Sequence Data Confirming the *JIP60* (wild type)-pK7FWG2 Plasmid. Top row: alignment; middle row: expected sequence for pK7FWG2 *JIP60* (wild type) (with non-matches in red); bottom row: sequence of pK7FWG2 *JIP60* (wild type) from the sample. The start and end of the *JIP60* insert are indicated by solid vertical lines.

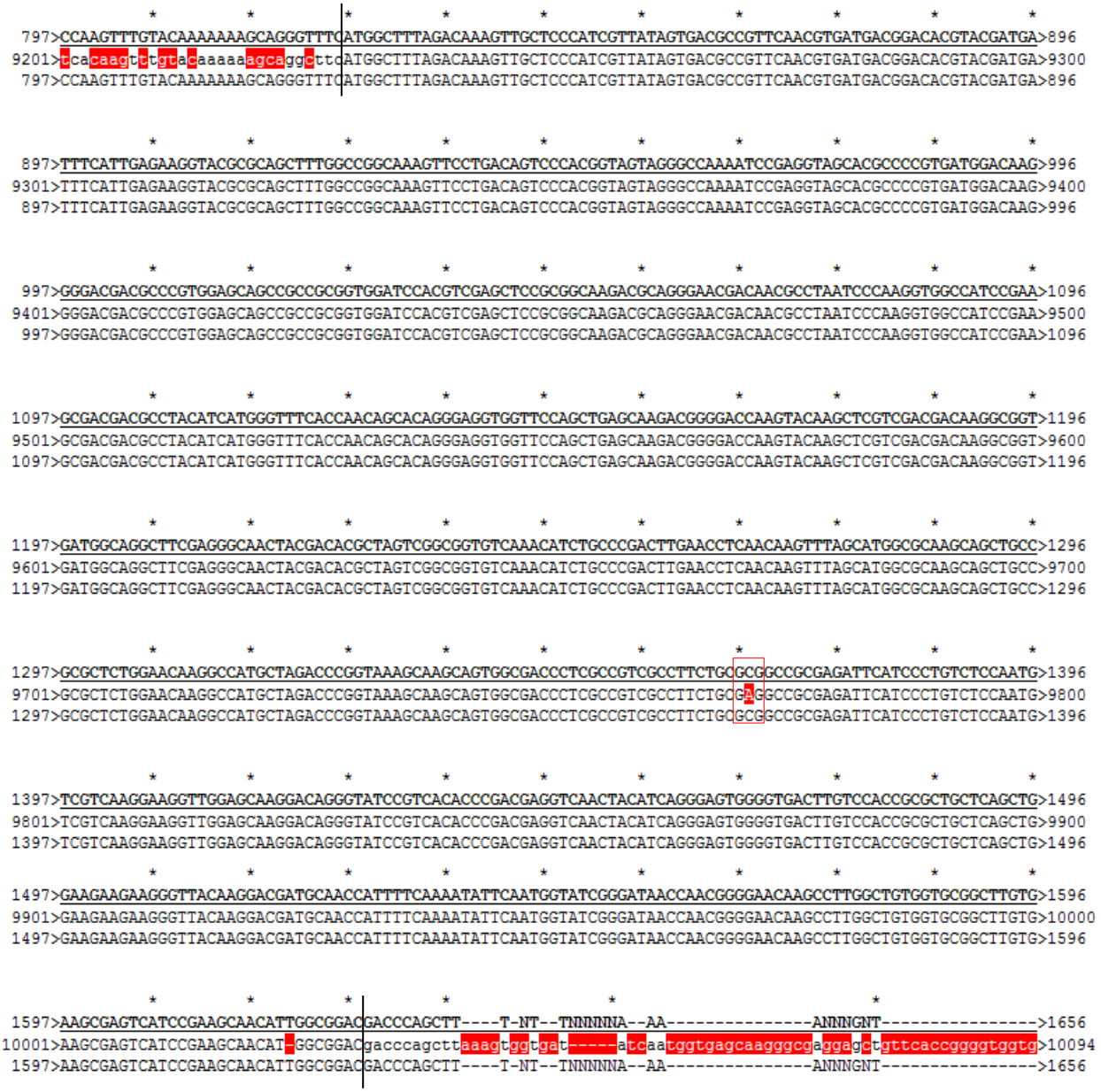


Figure 11: Sequence Data Confirming the *JIP60* (E202A)-pK7FWG2 Plasmid. Top row: alignment; middle row: expected sequence for pK7FWG2 *JIP60* (wild type) (with non-matches in red); bottom row: sequence of pK7FWG2 *JIP60* (E202A) from the sample. The start and end of the *JIP60* insert are indicated by solid vertical lines; the mutation site is indicated by a red box.

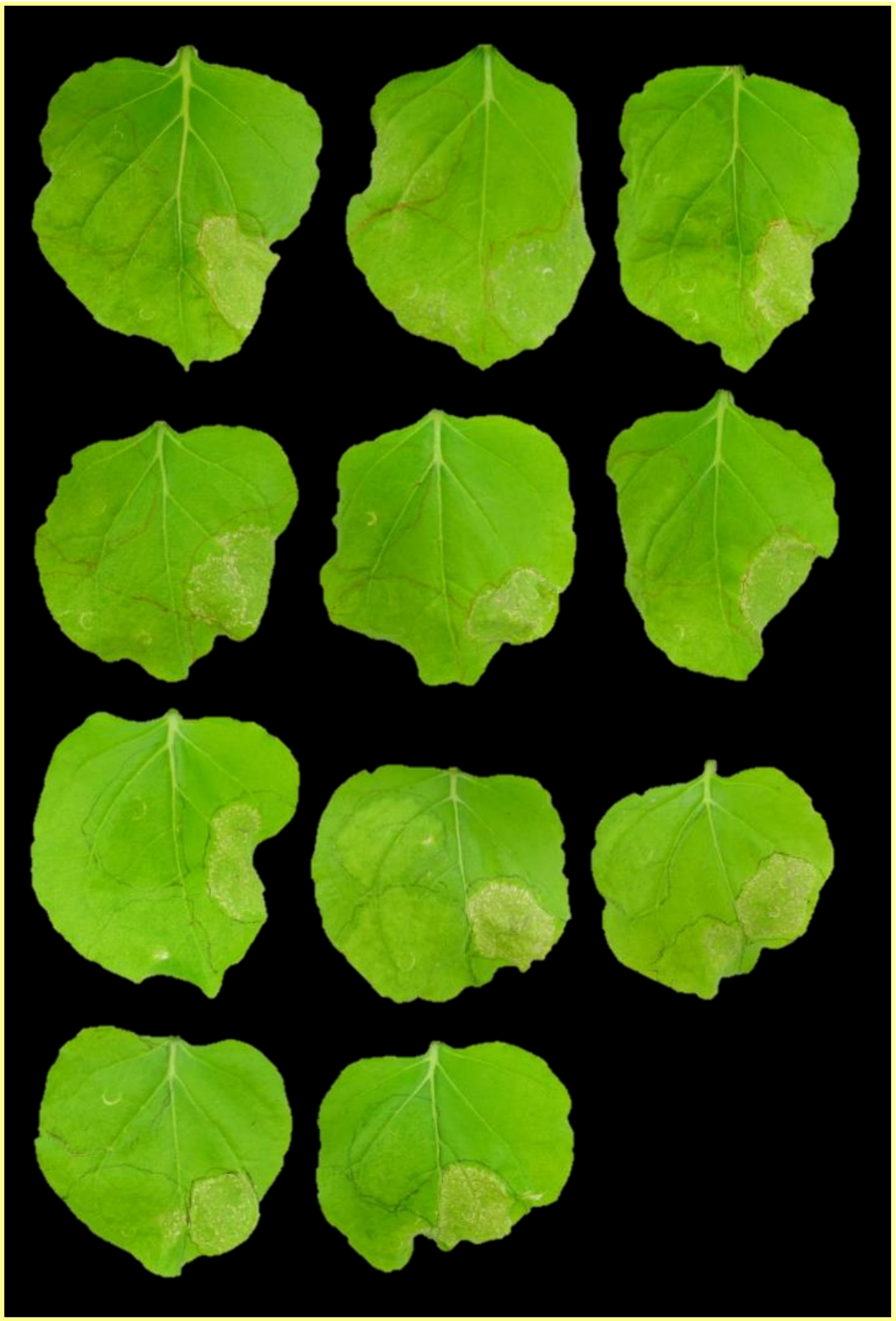


Figure 12: Testing the JIP60 E202A Mutant – *Agro*-infiltrated *N. benthamiana* Photographed for Early Symptoms of Cell Death. Photographs were taken between five and seven days post infiltration, with the adaxial sides of leaves shown. Agro-infiltration patches are outlined in black, and the arrangement of patches is shown in figure 6.

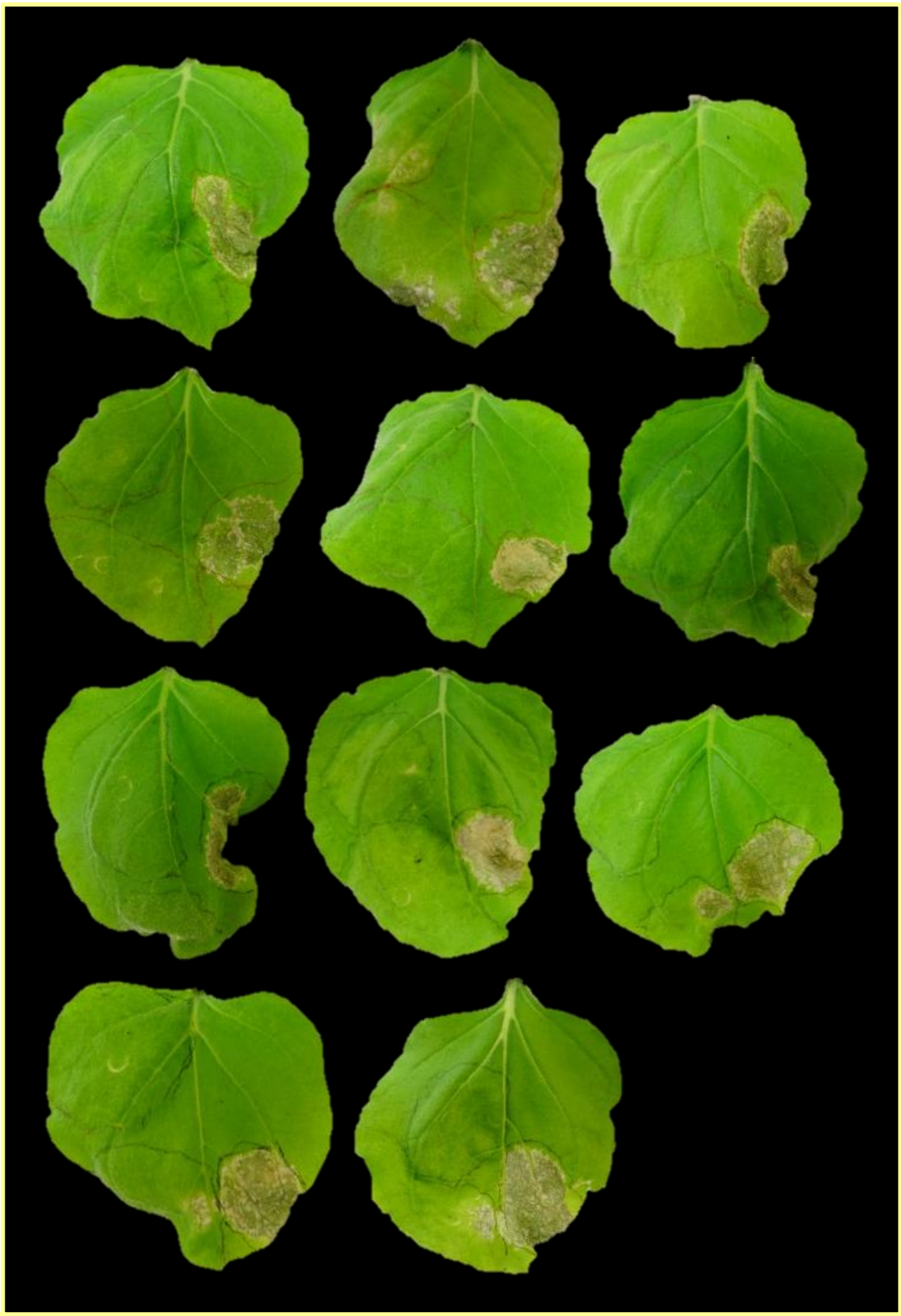
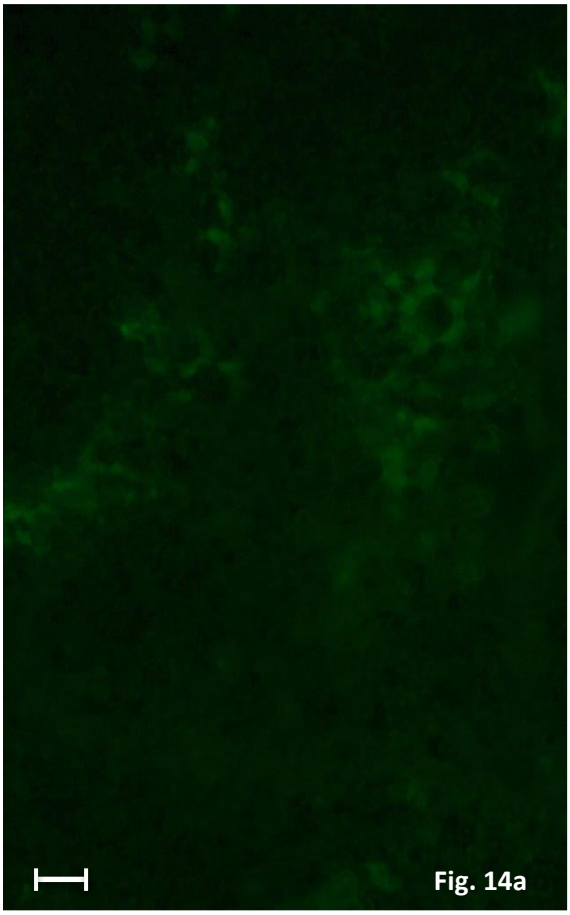
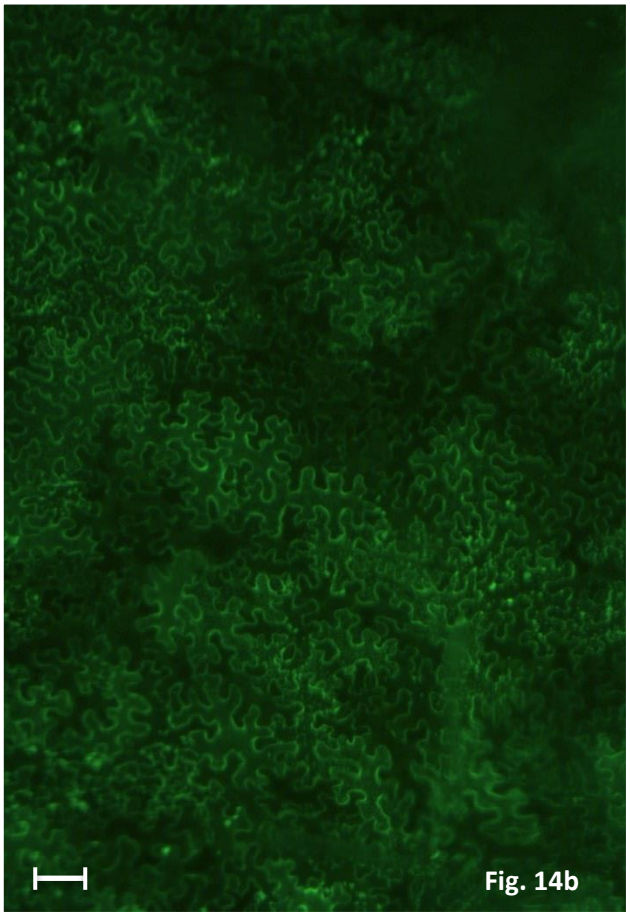


Figure 13: Testing the JIP60 E202A Mutant – *Agro*-infiltrated *N. benthamiana* Photographed for Later Symptoms of Cell Death. Photographs were taken between eleven and twelve days post infiltration, with the adaxial sides of leaves shown. Agro-infiltration patches are outlined in black, and the arrangement of patches is shown in figure 6.





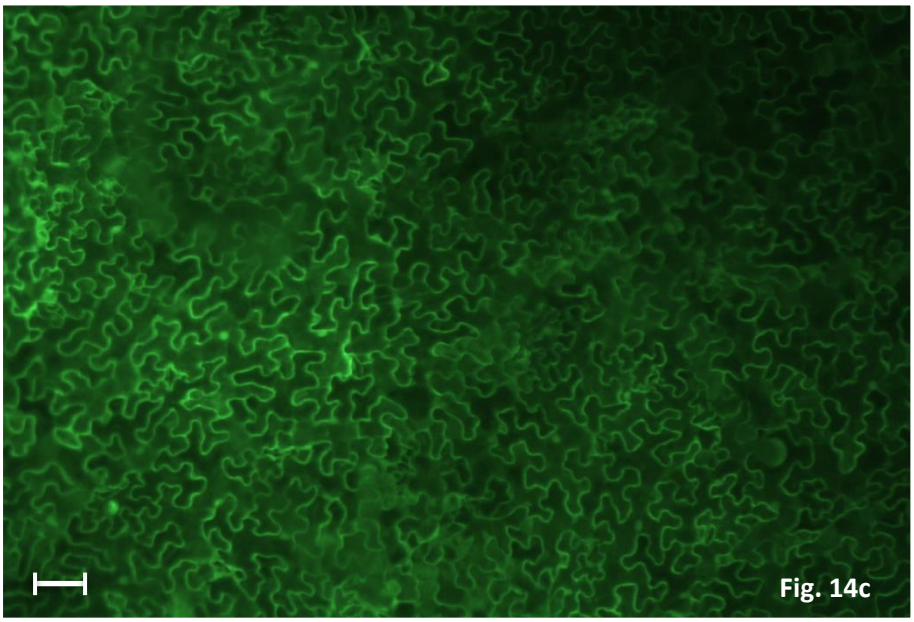


Figure 14: Fluorescence Micrographs of *Agro*-infiltrated *N. benthamiana* Leaves. All images were taken three days post infiltration. The abaxial side is shown, with the bar in each case representing 500 µm. Microscope settings: 510 ms exposure, 1.25 x colour saturation, 1.0 x gain, with ultraviolet light corresponding to maximal excitation of eGFP. Figure 14a: *JIP60* (wild type)-pK7FWG2 + P19 (positive control); figure 14b: *JIP60* (E202A mutant)-pK7FWG2 + P19; figure 14c: empty vector (just *eGFP*)-pK7FWG2 + P19 (negative control).

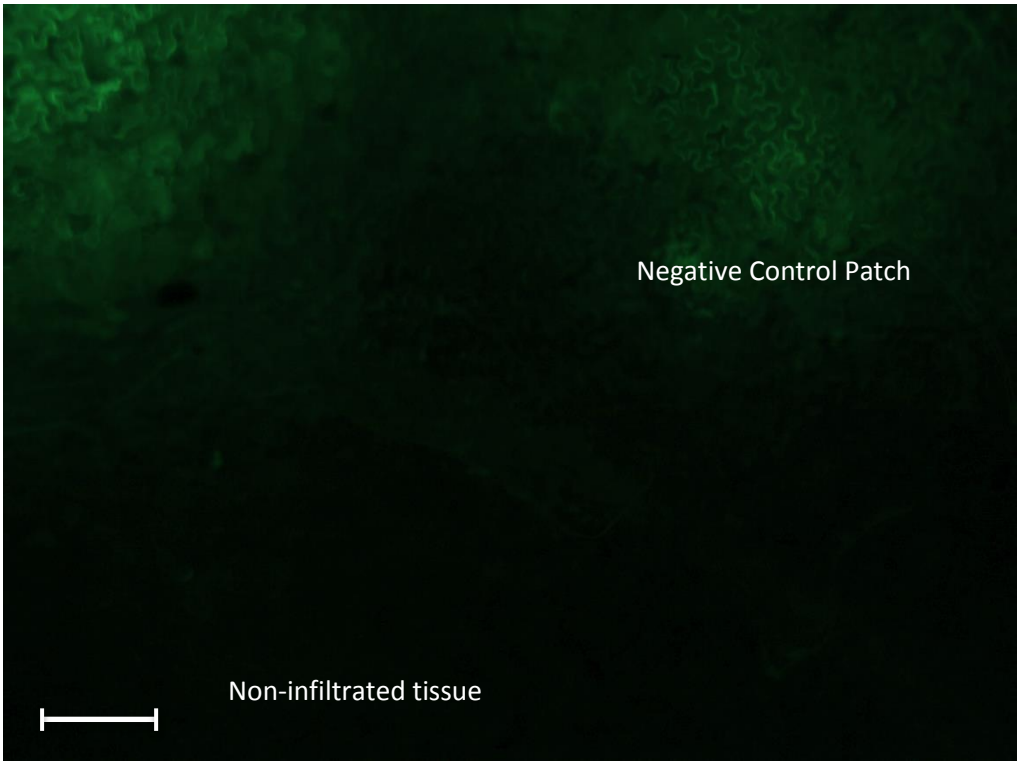


Figure 15: Fluorescence Micrograph of an *Agro*-infiltrated *N. benthamiana* Leaf, Negative Control Patch Boundary. This was taken three days post infiltration. The abaxial side is shown, with the bar representing 1000 µm. Microscope settings: 510 ms exposure, 1.25 x colour saturation, 1.0 x gain, with ultraviolet light corresponding to maximal excitation of eGFP. The boundary of empty vector (just *eGFP*)-pK7FWG2 + P19 (negative control) infiltration patch is shown, with either side as labelled on the image.

NICOTIANA BENTHAMIANA EXPERIMENT

Amplification and Cloning of Genomic SCRIPt from Untreated N. benthamiana

The RNA extracted from an untreated four-week old *N. benthamiana* leaf was at a concentration of 69.0 ng/µl and A206/A208 value of 2.08, indicating a high purity of RNA. The following reverse transcription yielded cDNA of concentration 695 ng/µl and A260/A280 value 1.80, indicating a high purity of DNA. All PCR attempts using the *attB* site overhang primers were unsuccessful, producing no bands in gel electrophoresis. Conditions used consisted of 95°C for 120 seconds, followed by 35 cycles of 95°C for 30 seconds, various annealing temperatures (60°C, 55°C, 50°C, 45°C) for 30 seconds, and 72°C for 105 seconds, then finally 72°C for 600 seconds. Using the primers without the *attB* site overhangs (i.e. for pCR8 cloning), a band was present in the attempt under PCR conditions the same as above, with an annealing temperature of 50°C. This is shown in figure 16: where a band of a size between 400 and 500 bp indicates that genomic *SCRIPt* (438 bp) has been amplified, due to contamination of the cDNA template by genomic DNA. Following cloning of the product into pCR8, transformation into *E. coli*, and finding a positive colony via colony PCR (shown in figure 17, gel lane 9) after many negative bands, an overnight broth and miniprep, Sanger sequencing (see figure 18) confirmed the genomic sequence of *SCRIPt* as suspected. (The plasmid map for pCR8-*SCRIPt* (genomic DNA) is shown in figure 19, and the plasmid sequence detailed in appendix C.) No amplification of expressed *SCRIPt* (i.e. from cDNA produced via reverse transcription of mRNA) was achieved from untreated *N. benthamiana* leaves.

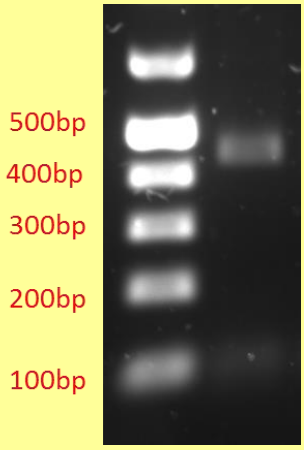


Figure 16: Genomic *SCRIPt* Detected in *N. benthamiana* DNA.
Lane 1: DNA ladder with size markers as labelled. Lane 2: Product of a PCR using non-*attB* site overhang primers for *SCRIPt*, showing a band between the sizes of 400 and 500 bp, which is consistent with the size of the genomic sequence of *SCRIPt* (438 bp). Electrophoresis was run at 90 volts for 60 minutes, using a 1.5% agarose TBE gel.

Amplification of Genomic SCRIPt from Methyl Jasmonate-Treated N. benthamiana

The RNA extracted from methyl jasmonate-treated, four-week old *N. benthamiana* leaf material was also of high quality, at a concentration of 82.8 ng/µl and A206/A208 value of 2.07; and the cDNA product from reverse transcription was of concentration 724 ng/µl and A260/A280 value 1.89. Despite multiple PCR efforts made using the non-*att* site overhang primers, no bands appeared in subsequent gels, other than of a size between 400 and 500 base pairs. Therefore no amplification of expressed *SCRIPt* was achieved from untreated *N. benthamiana* leaves.

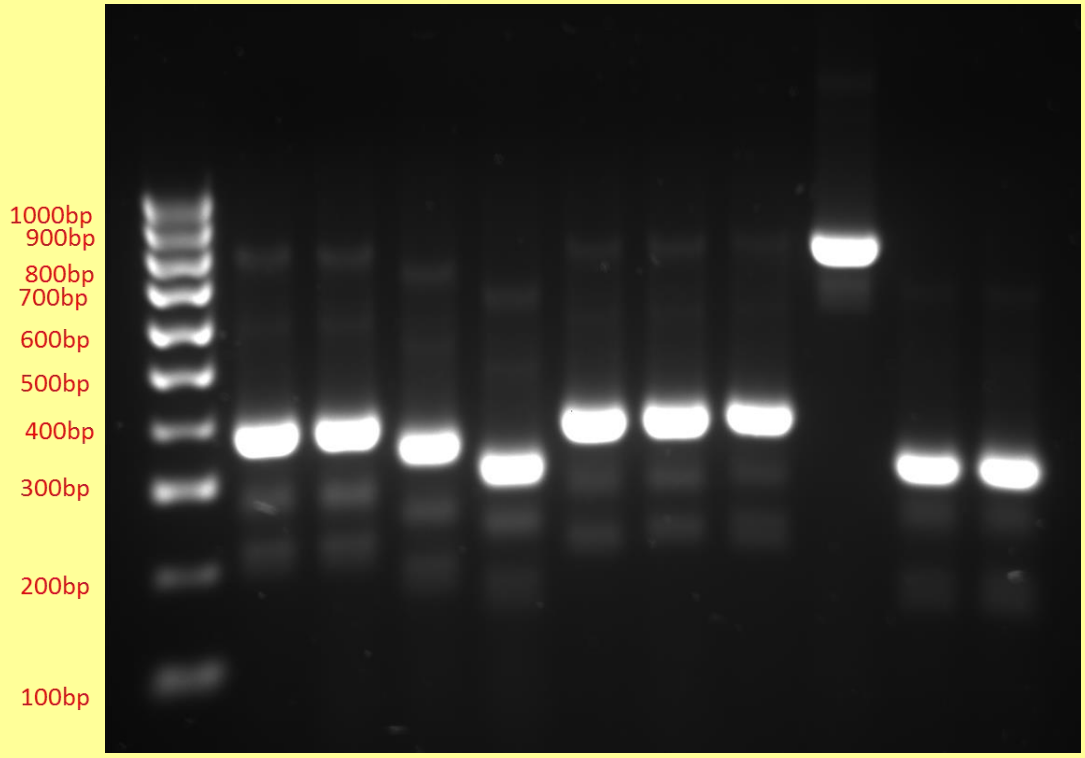


Figure 17: Detection of pCR8-*SCRIPt* (Genomic) Positive *E. coli* Colonies. Electrophoresis was run at 90 volts for 60 minutes, using a 1.5% agarose TBE gel. Lane 1: DNA ladder with size markers as labelled. Lanes 2-8, 10 and 11: colonies negative for the plasmid. Lane 9: positive detection of pCR8-*SCRIPt* (genomic) in *E. coli* colonies following colony PCR. The band in this lane is of a size greater than 700 bp, which is what one would expect to see for pCR8-*SCRIPt* (genomic) (754 bp). For the empty pCR8 plasmid, a band of 316 bp can be expected (see appendix C).

Figure 18: Sequence Data Confirming the pCR8-*SCRIPt* (genomic) Plasmid. Top row: alignment; middle row: expected sequence for pCR8-*SCRIPt* (genomic) (with non-matches in red); bottom row: sequence of pCR8-*SCRIPt* (genomic) from the sample. The start and end of the *SCRIPt* insert are indicated by solid vertical lines.

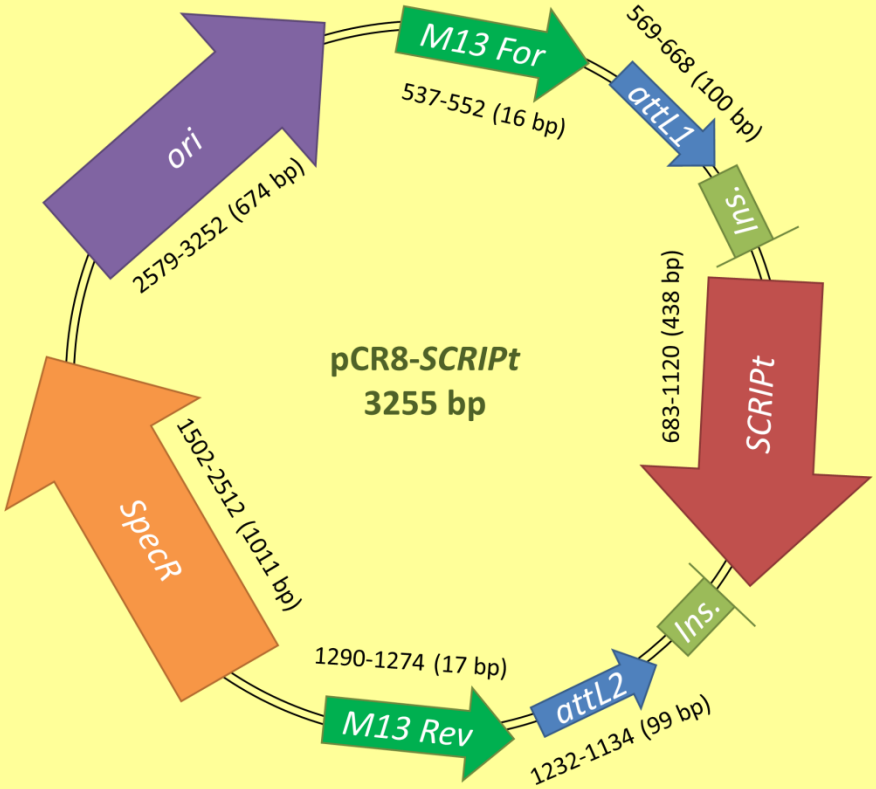


Figure 19: Plasmid Map of pCR8-*SCRIPt* (Genomic), Produced in the *N. benthamiana* Experiment. *SpecR*: spectinomycin resistance gene; *ori:* origin of replication; *Ins:* two halves of the split insertion site, each 5 bp; *M13 For* and *M13 Rev* represent the regions on the plasmid corresponding to the M13 primers used in colony PCRs; *SCRIPt*: genomic *SCRIPt* cloned into the plasmid vector.

Discussion

Both experiments in this project have involved investigating the structure of the characteristic but poorly-conserved ribosome-inactivating protein domain. The JIP60 experiment has produced evidence of an essential amino acid residue in the active site for ribosome-inactivating function; whilst attempts to amplify a predicted RIP gene in the genome of *Nicotiana benthamiana* have suggested the existence of a defunct RIP gene, or pseudogene.

The Function of Experimental JIP60

The *Agro*-infiltrated leaves depicted in figures 12 and 13 show that cell death occurs in wild type *JIP60*-pK7FWG2 treatment patches, and this is clearly apparent in the micrograph figure 14a. As stated earlier, cell death in *Agro*-infiltrated *N. benthamiana* leaves is considered here to be demonstration JIP60 N-glycosidase function [37], with the proposed mechanism for the action of processed JIP60 described by Chaudhry *et al.* (1994) causing irreversible inhibition of translation. The function of wild type JIP60 supports the findings of Pennington (2016, unpublished) regarding similarities in type III RIP post-translation processing shared with maize b-32 [31], as well as the position of the putative internal processing site described by Rustgi *et al.* (2014). This is because the sequence of the *JIP60* insert used in the JIP60 experiment encodes a JIP60 RIP domain similar to the proposed internally-processed *in vivo* JIP60 [43], complete with an ML (methionine leucine) linker placed within the internal processing site [37; 43]. Importantly, in only one case was cell death observed in an empty vector (negative control) patch on *Agro*-infiltrated *N. benthamiana* leaves, strongly showing that the cell death was not simply a response of the *N. benthamiana* to *Agrobacterium* infection. The exception may be due to accidental contact with the leaf vein during infiltration.

The E202A Mutation of JIP60

Figures 12 and 13 also show that cell death does not occur in *Agro*-infiltrated *N. benthamiana* expressing experimental E202A mutant JIP60, and this is also clearly shown by the intact epidermal cells in the micrograph figure 14b. Given that the ribosome-inactivating function of JIP60 by irreversible translation inhibition does not occur in the E202A mutant, this suggests that the glutamic acid residue at position 202 in the amino acid sequence of the JIP60 RIP domain is essential for the function and therefore forms part of the N-glycosidase-performing active site, as proposed by Lapadula *et al.* (2012; 2013) (figure 1) and indicated by the protein structure of JIP60 predicted by Phyre2 [26]. There is however a possibility that the E202A caused changes in the folding of the JIP60 protein, being the reason for the loss of function – hence the structure of JIP60 and its mutant forms of interest need to be experimentally determined, for example by X-ray crystallography [1]. Either way, this is an important finding because it indicates the importance of the conserved glutamic acid residue, position 202, in the function of the RIP domain of JIP60. When tested in maize ribosome-inactivating protein, the same corresponding glutamic acid residue, position 207, was found to be key to ribosome-inactivating function [31]. Crucially, both JIP60 mutant E202A and maize RIP mutant E207A [31] were found to be of reduced functionality compared to their wild type forms, suggesting that this conserved amino acid site is key to the ribosome-inactivating function of plant RIPs in general.

Due to the sequence data shown in figures 10 and 11, and colony PCR results shown in figures 8 and 9, there is no doubt that the correct plasmids were present in transformed *Agrobacterium* used in different treatment groups. Crucially, the mutant and wild type treatment groups were identical in every aspect excluding leaf patch position (and of course the mutagenesis). This included the use of the same plasmids (pK7FWG2, with C-terminal *eGFP* tag), and the same ages and strains of *Agrobacterium* used, making for a robust integrity of findings.

Experimental JIP60 Expression

In all three *Agro*-infiltration patches, the respective insert of pK7FWG2 plasmids was expressed in *N. benthamiana* epidermal cells, as shown by the detection of eGFP in figures 14a (JIP60 (wild type)-eGFP), 14b (JIP60 (E202A)-eGFP), and 14c (eGFP); and it is certain that the green colour shown in these micrographs is not due to autofluorescence of the epidermal cells, as shown by figure 15 – i.e. the darkness of non-infiltrated leaf tissue. Enhanced green fluorescent protein is a stable and reliable expression reporter [24] and this is important as it proves that the failure of E202 mutant JIP60 to perform ribosome-inactivating function was not due to lack of expression.

Future JIP60 Work

One mutant of JIP60 was successfully tested for functionality. A completion of this work on the three other mutants of interest – Y96A, R205A, and W235A, would be interesting in order to observe and shed more light on the importance and conservation of amino acids in the RIP domain. There are also other amino acids in the JIP60 which are well conserved among plant RIP domains, such as F100 and Y131 (see figure 1 and appendix B). It would also be useful to construct mutants by replacing amino acids in the RIP domain of JIP60 which are not well represented in the alignment shown in figure 1, with the hypothesis that these mutations would not result in a loss of JIP60 function. This would start to address the question of divergent evolution of the RIP domain between different RIP genes in general – do poorly conserved amino acids in the RIP domain represent selection towards varying specific functions of RIPs, or are they merely a result of genetic drift?

Given the structure of the C-terminal domain of JIP60, and its similarity to eIF4E [43], it would be interesting to investigate how mutants of the S19 eIF4E binding site region of JIP60 affect the proposed unprocessed function of JIP60 [42], and indeed assess the importance of JIP60 RIP domain mutants on this function, and the RIP domain as a whole.

SCRIPt is Probably a Pseudogene

Attempts to amplify and clone *SCRIPt* in the form of cDNA, i.e. providing evidence of its presence as mRNA, from both methyl jasmonate-treated and untreated *Nicotiana benthamiana* leaves were unsuccessful. *SCRIPt* was only amplified from genomic DNA, and cloning of the PCR product into the pCR8 plasmid and subsequent sequencing confirmed the genomic nucleotide sequence of *SCRIPt* as recorded in the *N. benthamiana* full genome sequence (see figures 18 and 19). This could explain why manual annotation could only predict a protein sequence of 111 amino acids, which is significantly shorter than a typical RIP domain (for example JIP60, 283 amino acids or 267 amino acids after post-translational processing [43]). The results of the *N. benthamiana* suggest that *SCRIPt* is a RIP pseudogene, being a former gene which has lost its function and subsequently accumulated mutations via genetic drift through evolutionary time due to an alleviation of selection pressures [7]. In other words *SCRIPt* may be an evolutionary RIP remnant, which could demonstrate the point made by Lapadula *et al.* (2013) that RIP genes are vulnerable to loss in evolution, explaining their

wide, but sparse, phylogenetic presence in the plant kingdom. However, this is by no means a firm conclusion, as the results of the *N. benthamiana* experiment only show the lack of expression in four week-old *N. benthamiana* leaf cells subjected to normal conditions and a form of jasmonate-induced stress. Perhaps the circumstances required for the expression and ribosome-inactivating function of *SCRIPt* have not yet been met experimentally.

Conclusion

Better understanding the structure and functionality of the RIP domain in JIP60 and other ribosome-inactivating proteins is an issue of some importance and interest, not only because it can provide insights into the evolution of plant-biotrophic pathogen interactions, and potentially give rise to agricultural applications in the future; but also more imminently applications of RIPs as antiviral and antitumour agents in medicine [e.g. 45]. Whilst on one hand, using gene cloning and mutagenesis of the JIP60 RIP domain, the JIP60 experiment has begun to reveal insights into strong selection pressures in the evolution of this poorly conserved but characteristic and well-expressed protein domain – i.e. the conservation of amino acid residue 202, glutamic acid; the *N. benthamiana* experiment has possibly illustrated an example of gene loss by genetic drift by isolating a RIP pseudogene in the genome of *Nicotiana benthamiana*, although a more thorough investigation into possible circumstances of its expression needs to be made. Why some species of plant possess RIPs in their gene expression regulatory repertoire and others, such as *Nicotiana benthamiana*, it seems, do not, also remains a mystery. Further studies on the structure of JIP60 and other RIPs, regarding their molecular structures, functionality and conservation of amino acids, and the extent of their effects on organismal and evolutionary levels are required to expand understanding of this widely taxonomically-distributed and ancient class of RNases.

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INTERNET LINKS

NCBI website, including biological sequence databases: https://www.ncbi.nlm.nih.gov/

Nicotiana benthamiana full genome sequene, Ni_ben v1.0.1.and predicted proteome: released by the Boyce Thompson Institute for Plant Science: http://bti.cornell.edu/our-research/enabling-technologies/nicotiana-benthamiana/; also accessible on Sol Genomics: https://solgenomics.net/organism/Nicotiana_benthamiana/genome; https://solgenomics.net/jbrowse_solgenomics/?data=data%2Fjson%2FNiben1.0.1&loc=Niben101Scf00005%3A1..274325&tracks=DNA&highlight= as well as

Phyre2 Protein Fold Recognition Server: http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index

Appendices

APPENDIX A: *SCRIPt* – SEQUENCES AND MANUAL ANNOTATION

Hidden Markov Model Output:

```
Query:       NbS00012043g0010.1  [L=61]
Scores for complete sequence (score includes all domains):
   --- full sequence ---   --- best 1 domain ---    -#dom-
    E-value  score  bias    E-value  score  bias    exp  N  Model    Description
    ------- ------ -----    ------- ------ -----   ---- --  -------- -----------
    2.7e-06   12.7   0.0    2.8e-06   12.6   0.0    1.0  1  RIPS


Domain annotation for each model (and alignments):
>> RIPS
   #    score  bias  c-Evalue  i-Evalue hmmfrom  hmm to    alifrom  ali to    envfrom  env to     acc
 ---   ------ ----- --------- --------- ------- -------    ------- -------    ------- -------    ----
   1 !   12.6   0.0   2.8e-06   2.8e-06      13      53 ..       7      45 ..       1      58 [. 0.68

  Alignments for each domain:
  == domain 1  score: 12.6 bits;  conditional E-value: 2.8e-06
             RIPS 13 laalptvapitvivfdlvnatadrYdeFiekvRkaladta.k 53
                  l++  tv pi  i+f ++   ++ Y++F++ +R+++  ++ +
  NbS00012043g0010.1  7 LLTF-TVSPIHFISFTPSP--SSIYSNFVSGLRNEIVYETtQ 45
                  2233.3689******7777..899**********9887722 PP
```

Predicted amino acid sequence for SCRIPt, as described in the predicted proteome:

MLLIRTLLTFTVSPIHFISFTPSPSSIYSNFVSGLRNEIVYETTQALMEKLVVADLEVALI

Amino acid sequence for SCRIPt based on the manual annotation (see figure 2):

MLLIRTLLTFTVSPIHFISFTPSPVRFTSLVSSSIYSNFVSGLRNETTQALMEKLVVADLEVALIAMINNFALCIIRRYVN
VLKSAYTVYYQKNKITTICTNNTLAYVKFI

The DNA sequence in genomic context is available on Sol Genomics, at:

https://solgenomics.net/jbrowse_solgenomics/?data=data%2Fjson%2FNiben1.0.1&loc=Niben101Scf00893 reading in reverse.

APPENDIX B: JIP60 SEQUENCES

Amino acid sequence of JIP60, with previously described motifs and sites of interest

Available on NCBI, accession no. X66376.1: https://www.ncbi.nlm.nih.gov/nuccore/19010, first described by Becker & Apel (1992)

RIP domain:

1 MALDKVAPIVIVTPFNVMTDR

22 YDEFIEKVRKALAGTAGAKVGPKPKSKVESPVLDKGTFPVEQPPRWIHVELHGKTQGTTTPKPKVAIRSDD

93 AYIMGF Ribonucleoprotein recognition motif 2 (RNP2) [43]

99 TNSTGRWFQLSKTGTTYKLVDDKAVMAGFDGNYNTLVGGV

139 NNLPTLNL RNP1 [43]

147 NKFSMAQAAAALWN

161 KASTLSGGIGSDVVDDDDGDMLRA Internal putative processing site (P1) [43]

185 NDPVKQAVATL

196 AVAVCEAARFSPVSKVV Shiga-toxin signature-like region [43]

213 NAGWIKDKVSVTPDEVNYIKEWGDLSTALLSWMDKQYKDDATIFKKFNGIGITNGEEALAVVRLVKLVIRS

eIF4E domain [43]:

NMAAAPTTDEQLLAYAQLPKHGRYMAEVFAVRIPATAGGDRPAAPSLCTAATAAATSFTARKKNTPRSKPAATAR
VTWSSLGHRLATSAYGPIVFNLDLHDGNCGQADEEEDEKNTGRIVCDAIGGDFSNYNKAISETVLTRCGPAEVIYAV
LSNGVQGRVDVKLAGLQSRDEVVLVGRIVARSKLFDFGCVLFYNEAAGVRVRPGELVPLARHALAVPLHMPLTIEL
DIRHGGSGDEIVRGELEFKTAIDGLHTGRLVGVNDAEFEVTILWSEYPW

Key:

Conserved in all 10 RIPs used for the HMM search

Conserved in 9 RIPs including JIP60

Conserved in 8 RIPs including JIP60

Amino acid involved in the active site [27; 28]

Experimental JIP60

JIP60 Insert Used in the JIP60 Experiment:

ATGGCTTTAGACAAAGTTGCTCCCATCGTTATAGTGACGCCGTTCAACGTGATGACGGACACGTACGATGATT
TCATTGAGAAGGTACGCGCAGCTTTGGCCGGCAAAGTTCCTGACAGTCCCACGGTAGTAGGGCCAAAATCCG
AGGTAGCACGCCCCGTGATGGACAAGGGGACGACGCCCGTGGAGCAGCCGCCGCGGTGGATCCACGTCGAG
CTCCGCGGCAAGACGCAGGGAACGACAACGCCTAATCCCAAGGTGGCCATCCGAAGCGACGACGCCTACATC
ATGGGTTTCACCAACAGCACAGGGAGGTGGTTCCAGCTGAGCAAGACGGGGACCAAGTACAAGCTCGTCGA
CGACAAGGCGGTGATGGCAGGCTTCGAGGGCAACTACGACACGCTAGTCGGCGGTGTCAAACATCTGCCCG
ACTTGAACCTCAACAAGTTTAGCATGGCGCAAGCAGCTGCCGCGCTCTGGAACAAGGCCATGCTAGACCCGG
TAAAGCAAGCAGTGGCGACCCTCGCCGTCGCCTTCTGCGAGGGCCGCGAAGATTCATCCCTGTCTCCAATGTCGT
CAAGGAAGGTTGGAGCAAGGACAGGGTATCCGTCACACCCGACGAGGTCAACTACATCAGGGAGTGGGGTG
ACTTGTCCACCGCGCTGCTCAGCTGGAAGAAGAAGGGTTACAAGGACGATGCAACCATTTTCAAAATATTCAA
TGGTATCGGGATAACCAACGGGGAACAAGCCTTGGCTGTGGTGCGGCTTGTGAAGCGAGTCATCCGAAGCAA
CATGGCGGAC

Translation:

MALDKVAPIVIVTPFNVMTDTYDDFIEKVRAALAGKVPDSPTVVGPKSEVARPVMDKGTTPVEQPPRWIHVELRG
KTQGTTTPNPKVAIRSDDAYIMGFTNSTGRWFQLSKTGTKYKLVDDKAVMAGFEGNYDTLVGGVKHLPDLNLNK
FSMAQAAAALWNKAMLDPVKQAVATLAVAFCEAARFIPVSNVVKEGWSKDRVSVTPDEVNYIREWGDLSTALLS
WKKKGYKDDATIFKIFNGIGITNGEQALAVVRLVKRVIRSNMAD

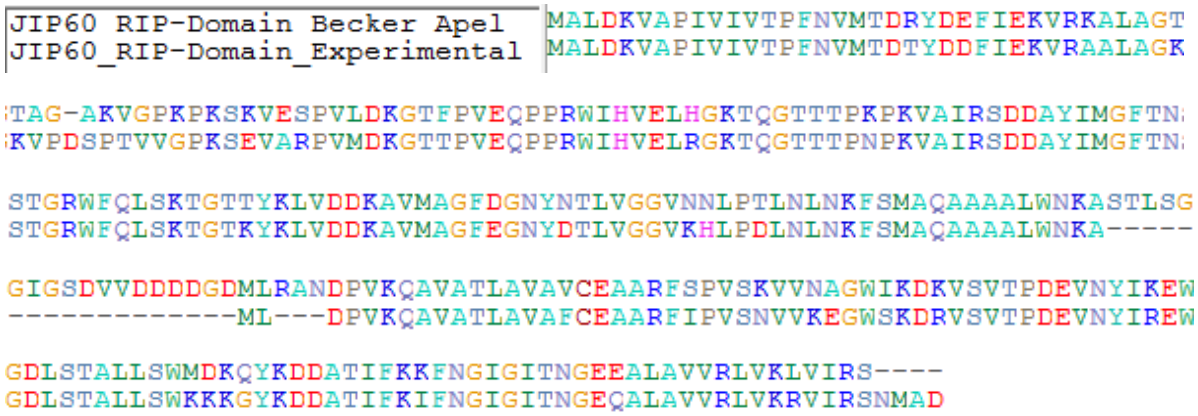
Key:

Conserved in all 10 RIPs used for the HMM search

Conserved in 9 RIPs including JIP60

Conserved in 8 RIPs including JIP60

Amino acid involved in the active site [27; 28]



Appendix Figure B(i): Alignment of the Amino Acid Sequences of the JIP60 RIP Domain [3] and JIP60 RIP Domain Used in the JIP60 Experiment. The ML (methionine-leucine) linker [37] is clearly shown in experimental JIP60, corresponding with the internal processing site [43] of the JIP60 RIP domain, which is missing in experimental JIP60 in order to produce an active translated product in the transgenic host *Nicotiana benthamiana*.

APPENDIX C: PLASMID SEQUENCES

pK7FWG2 (11880 bp) (as shown in figure 5c)

CGACGTCGCATGCCTGCAGGTCACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATTTTACAAATACAAATACATACTAAGGGTTTCTTA
TATGCTCAACACATGAGCGAAACCCTATAAGAACCCTAATTCCCTTATCTGGGAACTACTCACACATTATTCTGGAGAAAAATAGAGAGAGATAGATTTGT
AGAGAGAGACTGGTGATTTTTGCGGACTCTAGCATGGCCGCGGTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAG
CAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGAT
GGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGG
CCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCA
CCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGC
GGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGG
GCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTC
GCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATTGATATCACCACTTTGTACAAGAAAGCTGAACGAGAAA
CGTAAAATGATATAAATATCAATATATTAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAAACACAACATATCCAGTCACTATGGTCGACCT
GCAGACTGGCTGTGTATAAGGGAGCCTGACATTTATATTCCCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCTGAGATCAGCCAC
TTCTTCCCCGATAACGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGCACCACCGGGTAAAGTTCACGGGAGA
CTTTATCTGACAGCAGACGTGCACTGGCCAGGGGGATCACCATCCGTCGCCCGGGCGTGTCAATAATATCACTCTGTACATCCACAAACAGACGATAACG
GCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCATTTCACCAGTCCCTGTTCTCGTCAGCAAAAGAGCCGTTCATTTCAATAAACCGGGCGACCTCAGCCAT
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TTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAGACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAAT
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ACATATTCTCAATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCCGGAAATCGTCGTGG
TATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCC
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AATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTAT
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TACTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCG
CCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCC
AGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGG
AATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCT
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GTTGGCTGGCTGGTGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTAACGCC
GAATTGAATTATCAGCTTGCATGCCGGTCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGC
GTATTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACAGA
AATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAACGATCTGCTTGACTCTAGCTAGAGTCCGAA
CCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTC
AGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCACAGTCGATGAATCCAG
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CAGCCACGATAGCCGCGCTGCCTCGTCTTGGAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCG
GAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCT
TGTTCAATCATGCCTCGATCGAGTTGAGAGTGAATATGAGACTCTAATTGGATACCGAGGGGAATTTATGGAACGTCAGTGGAGCATTTTTGACAAGAAA
TATTTGCTAGCTGATAGTGACCTTAGGCGACTTTTGAACGCGCAATAATGGTTTCTGACGTATGTGCTTAGCTCATTAAACTCCAGAAACCCGCGGCTGAG
TGGCTCCTTCAACGTTGCGGTTCTGTCAGTTCCAAACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGGTCATAACGTGACTCCCTTAATTCTCATGTATG
ATAATTCGAGGGTACCCGGGGATCCTCTAGAGGGCC

Key: *eGFP*; *attR* sites; *ccdB*; left and right T-DNA borders; spectinomycin resistance gene; kanamycin resistance gene; colony PCR primer binding sites.

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pK7FWG2 with JIP60 (wild type) (as shown in figure 5d)

CGACGTCGCATGCCTGCAGGTCACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATTTTACAAATACAAATACATACTAAGGGTTTCTTA
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CAATCCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACCACTGTCGGCAGAGGCATC
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TCCGAGGAGGTTTCCCGATATTACCCTTTGTTGAAAAGTCTCAATAGCCCTTTGGTCTTCTGAGACTGTATCTTTGATATTCTTGGAGTAGACGAGAGTGTC
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CCATCGCAACCCATCTAGCCCGCGCCCTGCAACTCGCCGGGGCCGATGTTCTGTTAGTCGATTCCGATCCCCAGGGCAGTGCCCGCGATTGGGCGGCCGT
GCGGGAAGATCAACCGCTAACCGTTGTCGGCATCGACCGCCCGACGATTGACCGCGACGTGAAGGCCATCGGCCGGCGCGACTTCGTAGTGATCGACGG
AGCGCCCCAGGCGGCGGACTTGGCTGTGTCCGCGATCAAGGCAGCCGACTTCGTGCTGATTCCGGTGCAGCCAAGCCCTTACGACATATGGGCCACCGCC
GACCTGGTGGAGCTGGTTAAGCAGCGCATTGAGGTCACGGATGGAAGGCTACAAGCGGCCTTTGTCGTGTCGCGGGCGATCAAAGGCACGCGCATCGG
CGGTGAGGTTGCCGAGGCGCTGGCCGGGTACGAGCTGCCCATTCTTGAGTCCCGTATCACGCAGCGCGTGAGCTACCCAGGCACTGCCGCCGCCGGCAC
AACCGTTCTTGAATCAGAACCCGAGGGCGACGCTGCCCGCGAGGTCCAGGCGCTGGCCGCTGAAATTAAATCAAAACTCATTTGAGTTAATGAGGTAAAG
AGAAAATGAGCAAAAGCACAAACACGCTAAGTGCCGGCCGTCCGAGCGCACGCAGCAGCAAGGCTGCAACGTTGGCCAGCCTGGCAGACACGCCAGCC
ATGAAGCGGGTCAACTTTCAGTTGCCGGCGGAGGATCACACCAAGCTGAAGATGTACGCGGTACGCCAAGGCAAGACCATTACCGAGCTGCTATCTGAA
TACATCGCGCAGCTACCAGAGTAAATGAGCAAATGAATAAATGAGTAGATGAATTTTAGCGGCTAAAGGAGGCGGCATGGAAAATCAAGAACAACCAGG
CACCGACGCCGTGGAATGCCCCATGTGTGGAGGAACGGGCGGTTGGCCAGGCGTAAGCGGCTGGGTTGTCTGCCGGCCCTGCAATGGCACTGGAACCCC
CAAGCCCGAGGAATCGGCGTGACGGTCGCAAACCATCCGGCCCGGTACAAATCGGCGCGGCGCTGGGTGATGACCTGGTGGAGAAGTTGAAGGCCGCG
CAGGCCGCCCAGCGGCAACGCATCGAGGCAGAAGCACGCCCCGGTGAATCGTGGCAAGCGGCCGCTGATCGAATCCGCAAAGAATCCCGGCAACCGCC
GGCAGCCGGTGCGCCGTCGATTAGGAAGCCGCCCAAGGGCGACGAGCAACCAGATTTTTTCGTTCCGATGCTCTATGACGTGGGCACCCGCGATAGTCG
CAGCATCATGGACGTGGCCGTTTTCCGTCTGTCGAAGCGTGACCGACGAGCTGGCGAGGTGATCCGCTACGAGCTTCCAGACGGGCACGTAGAGGTTTCC
GCAGGGCCGGCCGGCATGGCCAGTGTGTGGGATTACGACCTGGTACTGATGGCGGTTTCCCATCTAACCGAATCCATGAACCGATACCGGGAAGGGAAG
GGAGACAAGCCCGGCCGCGTGTTCCGTCCACACGTTGCGGACGTACTCAAGTTCTGCCGGCGAGCCGATGGCGGAAAGCAGAAAGACGACCTGGTAGA
AACCTGCATTCGGTTAAACACCACGCACGTTGCCATGCAGCGTACGAAGAAGGCCAAGAACGGCCGCCTGGTGACGGTATCCGAGGGTGAAGCCTTGAT
TAGCCGCTACAAGATCGTAAAGAGCGAAACCGGGCGGCCGGAGTACATCGAGATCGAGCTAGCTGATTGGATGTACCGCGAGATCACAGAAGGCAAGA
ACCCGGACGTGCTGACGGTTCACCCCGATTACTTTTTGATCGATCCCGGCATCGGCCGTTTTCTCTACCGCCTGGCACGCCGCGCCGCAGGCAAGGCAGAA
GCCAGATGGTTGTTCAAGACGATCTACGAACGCAGTGGCAGCGCCGGAGAGTTCAAGAAGTTCTGTTTCACCGTGCGCAAGCTGATCGGGTCAAATGAC
CTGCCGGAGTACGATTTGAAGGAGGAGGCGGGGCAGGCTGGCCCGATCCTAGTCATGCGCTACCGCAACCTGATCGAGGGCGAAGCATCCGCCGGTTCC

TAATGTACGGAGCAGATGCTAGGGCAAATTGCCCTAGCAGGGGAAAAAGGTCGAAAAGGTCTCTTTCCTGTGGATAGCACGTACATTGGGAACCCAAAG
CCGTACATTGGGAACCGGAACCCGTACATTGGGAACCCAAAGCCGTACATTGGGAACCGGTCACACATGTAAGTGACTGATATAAAAGAGAAAAAAGGC
GATTTTTCCGCCTAAAACTCTTTAAAACTTATTAAAACTCTTAAAACCCGCCTGGCCTGTGCATAACTGTCTGGCCAGCGCACAGCCGAAGAGCTGCAAAAA
GCGCCTACCCTTCGGTCGCTGCGCTCCCTACGCCCCGCCGCTTCGCGTCGGCCTATCGCGGCCGCTGGCCGCTCAAAAATGGCTGGCCTACGGCCAGGCA
ATCTACCAGGGCGCGGACAAGCCGCGCCGTCGCCACTCGACCGCCGGCGCCCACATCAAGGCACCCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAAC
CTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGG
GTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCAT
ATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCT
GCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAA
AGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCG
AAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTT
TCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCC
CCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGG
ATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGC
TGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTAC
GCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGCAT
GATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGC
ATCTAATCGCTTGAGTTAACGCCGGCGAAGCGGCGTCGGCTTGAACGAATTTCTAGCTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTA
GTGGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGCTGATACTGGG
CCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTT
CGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTC
CGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAA
TGTCATTGCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGG
AGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAA
ATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACT
ACCTCTGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCCCATGATGTTTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTC
CATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACCCCAAAAAAACATGTCATAACAAGAAGCCATG
AAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGACGGCAGTTACGCTACTTGCATTACAGCTTACGAACCGAA
CGAGGCTTATGTCCACTGGGTTCGTGCCCGAATTGATCACAGGCAGCAACGCTCTGTCATCGTTACAATCAACATGCTACCCTCCGCGAGATCATCCGTGT
TTCAAACCCGGCAGCTTAGTTGCCGTTCTTCCGAATAGCATCGGTAACATGAGCAAAGTCTGCCGCCTTACAACGGCTCTCCCGCTGACGCCGTCCCGGAC
TGATGGGCTGCCTGTATCGAGTGGTGATTTTGTGCCGAGCTGCCGGTCGGGGAGCTGTTGGCTGGCTGGTGGCAGGATATATTGTGGTGTAAACAAATT
GACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTAACGCCGAATTGAATTATCAGCTTGCATGCCGGTCGATCTAGTAACATA
GATGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCATC
TCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATC
TTAAGAAACTTTATTGCCAAATGTTTGAACGATCTGCTTGACTCTAGCTAGAGTCCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAG
AAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCC
AACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCAT
CGCCGTGGGTCACGACGAGATCCTCGCCGTCGGGCATCCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATC
ATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGC
AGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGT
CCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTTGGAGTTCATTCAG
GGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCA
GTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCCTCGATCGAGTTGAGAGTGAATATGAGACT
CTAATTGGATACCGAGGGGAATTTATGGAACGTCAGTGGAGCATTTTTGACAAGAAATATTTGCTAGCTGATAGTGACCTTAGGCGACTTTTGAACGCGC
AATAATGGTTTCTGACGTATGTGCTTAGCTCATTAAACTCCAGAAACCCGCGGCTGAGTGGCTCCTTCAACGTTGCGGTTCTGTCAGTTCCAAACGTAAAAC
GGCTTGTCCCGCGTCATCGGCGGGGGTCATAACGTGACTCCCTTAATTCTCATGTATGATAATTCGAGGGTACCCGGGGATCCTCTAGAGGGCC

Key: *eGFP*; *attB* sites; *JIP60*; left and right T-DNA borders; spectinomycin resistance gene; kanamycin resistance gene; colony PCR primer binding sites.

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pDONR201 (4470 bp) as shown in figure 5a

CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCTAGCCAGGAAGAGTTTGTAGAAACGCAAAAAGGCCATCCGTCAGGATGGCCTTCTG
CTTAGTTTGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACT
CAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTACTCTCGCG
TTAACGCTAGCATGGATCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGC
CAAGTTTGTACAAAAAAGCAGAACGAGAAACGTAAAATGATATAAATATCAATATATTAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAA
ACACAACATATCCAGTCACTATGAATCAACTACTTAGATGGTATTAGTGACCTGTAGTCGACCGACAGCCTTCCAAATGTTCTTCGGGTGATGCTGCCAACT
TAGTCGACCGACAGCCTTCCAAATGTTCTTCTCAAACGGAATCGTCGTATCCAGCCTACTCGCTATTGTCCTCAATGCCGTATTAAATCATAAAAAGAAATA
AGAAAAAGAGGTGCGAGCCTCTTTTTTGTGTGACAAAATAAAAACATCTACCTATTCATATACGCTAGTGTCATAGTCCTGAAAATCATCTGCATCAAGAA
CAATTTCACAACTCTTATACTTTTCTCTTACAAGTCGTTCGGCTTCATCTGGATTTTCAGCCTCTATACTTACTAAACGTGATAAAGTTTCTGTAATTTCTACTG
TATCGACCTGCAGACTGGCTGTGTATAAGGGAGCCTGACATTTATATTCCCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCTGAG
ATCAGCCACTTCTTCCCCGATAACGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGCACCACCGGGTAAAGTT
CACGGGAGACTTTATCTGACAGCAGACGTGCACTGGCCAGGGGGATCACCATCCGTCGCCCGGGCGTGTCAATAATATCACTCTGTACATCCACAAACAG
ACGATAACGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCATTTCACCAGTCCCTGTTCTCGTCAGCAAAAGAGCCGTTCATTTCAATAAACCGGGCGA
CCTCAGCCATCCCTTCCTGATTTTCCGCTTTCCAGCGTTCGGCACGCAGACGACGGGCTTCATTCTGCATGGTTGTGCTTACCAGACCGGAGATATTGACAT
CATATATGCCTTGAGCAACTGATAGCTGTCGCTGTCAACTGTCACTGTAATACGCTGCTTCATAGCACACCTCTTTTTGACATACTTCGGGTATACATATCAG
TATATATTCTTATACCGCAAAAATCAGCGCGCAAATACGCATACTGTTATCTGGCTTTTAGTAAGCCGGATCCACGCGATTACGCCCCGCCCTGCCACTCAT
CGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAGACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCT
TGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTG
AGACGAAAAACATATTCTCAATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCCGGAAA
TCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTC
TTTCATTGCCATACGGAATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAA
AAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAA
CGGTGGTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTTATTTCATTATG
GTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCGCCAAAAGTTGGCCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATT
CTGCGAAGTGATCTTCCGTCACAGGTATTTATTCGGCGCAAAGTGCGTCGGGTGATGCTGCCAACTTAGTCGACTACAGGTCACTAATACCATCTAAGTAG
TTGATTCATAGTGACTGGATATGTTGTGTTTTACAGTATTATGTAGTCTGTTTTTTATGCAAAATCTAATTTAATATATTGATATTTATATCATTTTACGTTTCT
CGTTCAGCTTTCTTGTACAAAGTGGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTGC
CATCCAGCTGCAGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAA
CAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCT
CGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGCTTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCA
ATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTAC
TCACCACTGCGATCCCCGGAAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGG
TTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGT
GATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAACTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGA
TTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCT
ATGGAACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATG
CTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGCAAGCTCATGACCAAAATCCCT
TAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACA
AAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATAC
TGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAG
TGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAG
CTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGT
AAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGA
GCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTC
ACATGTT

Key: *attP* sites; *ccdB* suicide gene; chloramphenicol resistance gene; kanamycin resistance gene; origin of replication

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pDONR201-JIP60 (wild type)(3059 bp) as shown in figure 5b

CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCTAGCCAGGAAGAGTTTGTAGAAACGCAAAAAGGCCATCCGTCAGGATGGCCTTCTG
CTTAGTTTGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACT
CAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTACTCTCGCG
TTAACGCTAGCATGGATCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGC
CAAGTTTGTACAAAAAAGCAGGCTTCATGGCTTTAGACAAAGTTGCTCCCATCGTTATAGTGACGCCGTTCAACGTGATGACGGACACGTACGATGATTTC
ATTGAGAAGGTACGCGCAGCTTTGGCCGGCAAAGTTCCTGACAGTCCCACGGTAGTAGGGCCAAAATCCGAGGTAGCACGCCCCGTGATGGACAAGGGG
ACGACGCCCGTGGAGCAGCCGCCGCGGTGGATCCACGTCGAGCTCCGCGGCAAGACGCAGGGAACGACAACGCCTAATCCCAAGGTGGCCATCCGAAG
CGACGACGCCTACATCATGGGTTTCACCAACAGCACAGGGAGGTGGTTCCAGCTGAGCAAGACGGGGACCAAGTACAAGCTCGTCGACGACAAGGCGGT
GATGGCAGGCTTCGAGGGCAACTACGACACGCTAGTCGGCGGTGTCAAACATCTGCCCGACTTGAACCTCAACAAGTTTAGCATGGCGCAAGCAGCTGCC
GCGCTCTGGAACAAGGCCATGCTAGACCCGGTAAAGCAAGCAGTGGCGACCCTCGCCGTCGCCTTCTGCGAGGCCGCGAGATTCATCCCTGTCTCCAATG
TCGTCAAGGAAGGTTGGAGCAAGGACAGGGTATCCGTCACACCCGACGAGGTCAACTACATCAGGGAGTGGGGTGACTTGTCCACCGCGCTGCTCAGCT
GGAAGAAGAAGGGTTACAAGGACGATGCAACCATTTTCAAAATATTCAATGGTATCGGGATAACCAACGGGGAACAAGCCTTGGCTGTGGTGCGGCTTG
TGAAGCGAGTCATCCGAAGCAACATGGCGGACGACCCAGCTTTCTTGTACAAAGTGGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACA
GGTCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGCAGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAATATAT
CATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCGAGGCCGCGATTAAATTCCAA
CATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGCTTGTATGGGAAGCCCGATGCGCCAGAG
TTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAA
GCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGAAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAA
ATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGC
AATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAACTTTT
GCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGT
CGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGA
TAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGAC
TTGACGGGACGGCGCAAGCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG
ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGA
AGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATAC
CTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGC
GGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCC
ACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT
ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCG
GCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTT

Key: *attL* sites; *JIP60*; kanamycin resistance gene; origin of replication

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pCR8 (2817 bp) as shown in figure 7

CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAG
TGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGA
AAGCGGGCAGTGAGCGCAACGCAATTAATACGCGTACCGCTAGCCAGGAAGAGTTTGTAGAAACGCAAAAAGGCCATCCGTCAGGATGGCCTTCTGCTT
AGTTTGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCA
GGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTACTCTCGCGTT
AACGCTAGCATGGATGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCT
GTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCCGAATTCGCCCTTAAGGGCGAATTCGACCCAGCTT
TCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTG
ATATCCCCTATAGTGAGTCGTATTACATGGTCATAGCTGTTTCCTGGCAGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAATA
TATCATCATGCCTCCTCTAGACCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGC
ACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGT
GGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGGGGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGG
GTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAAACATCATGAGGGAAGCGGTGATCGCCGAAG
TATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCCT
GAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAACTTCGGCT
TCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATT
TGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGC
GTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACTCGC
CGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTG
CCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCG
CGCAGATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAACCCTCGAGCCACCCATGACCAAAATCCCTTAAC
GTGAGTTACGCGTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACA
AAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATAC
TGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAG
TGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAG
CTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGT
AAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGA
GCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTC
ACATGTT

Key: M13 sites; *attL* sites; insertion site; spectinomycin resistance gene; origin of replication

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pCR8-SCRIPt (Genomic) (3255 bp) as shown in figure 19

CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAG
TGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGA
AAGCGGGCAGTGAGCGCAACGCAATTAATACGCGTACCGCTAGCCAGGAAGAGTTTGTAGAAACGCAAAAAGGCCATCCGTCAGGATGGCCTTCTGCTT
AGTTTGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCA
GGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTACTCTCGCGTT
AACGCTAGCATGGATGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCT
GTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCCGAATTCGCCCTTATGCTTTGATTCGGACGTTAC
TCACCTTTACCGTCTCGCCGATACACTTCATATCATTCACGCCGTCTCCAGTTAGATTCACATCCTTAGTTTCCGTTTGAATAACCGATTGCTCGTAATAATTG
TTAATCTAGAGCTCCATTTATTCTAATTTTGTTTCAGGTCTGAGAAATGAGACTACACAGGCACTCATGGAGAAGCTCGTTGTCGCAGATTTAGAGGTTGCT
CTGATCAGTATTTCTCTCTCTTTTCGGTTACTTTAAGAATTAGGCTATGATTAATAATTTTGCGTTATGTATTATCAGACGTTATGTTAATGTATTAAAATCT
GCATATACAGTGTATTATCAAAAAAACAAAATTACTACTCAGTTTGATTTTTAATTGGATTAAGATTTGTACTAATAACACCCTGGCCTATGTTAAATTTATC
TAAAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAAT
AAAATCATTATTTGCCATCCAGCTGATATCCCCTATAGTGAGTCGTATTACATGGTCATAGCTGTTTCCTGGCAGCTCTGGCCCGTGTCTCAAAATCTCTGAT
GTTACATTGCACAAGATAAAAATATATCATCATGCCTCCTCTAGACCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGC
ACACCGTGGAAACGGATGAAGGCACGAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCC
AGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTGGGGTACAGTCTATGCCTCGGGCATCCA
AGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAAACATCAT
GAGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTAC
GGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGATC
AACGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTA
TCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGC
TGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGA
AACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAA
ATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGACAAG
AAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAACCCTCGAGCC
ACCCATGACCAAAATCCCTTAACGTGAGTTACGCGTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTCTGC
GCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTC
AGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATC
CTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACG
GGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGG
AGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGT
CGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTC
CTGGCCTTTTGCTGGCCTTTTGCTCACATGTT

Key: M13 sites; *attL* sites; split insertion site; *SCRIPt* (genomic); spectinomycin resistance gene; origin of replication

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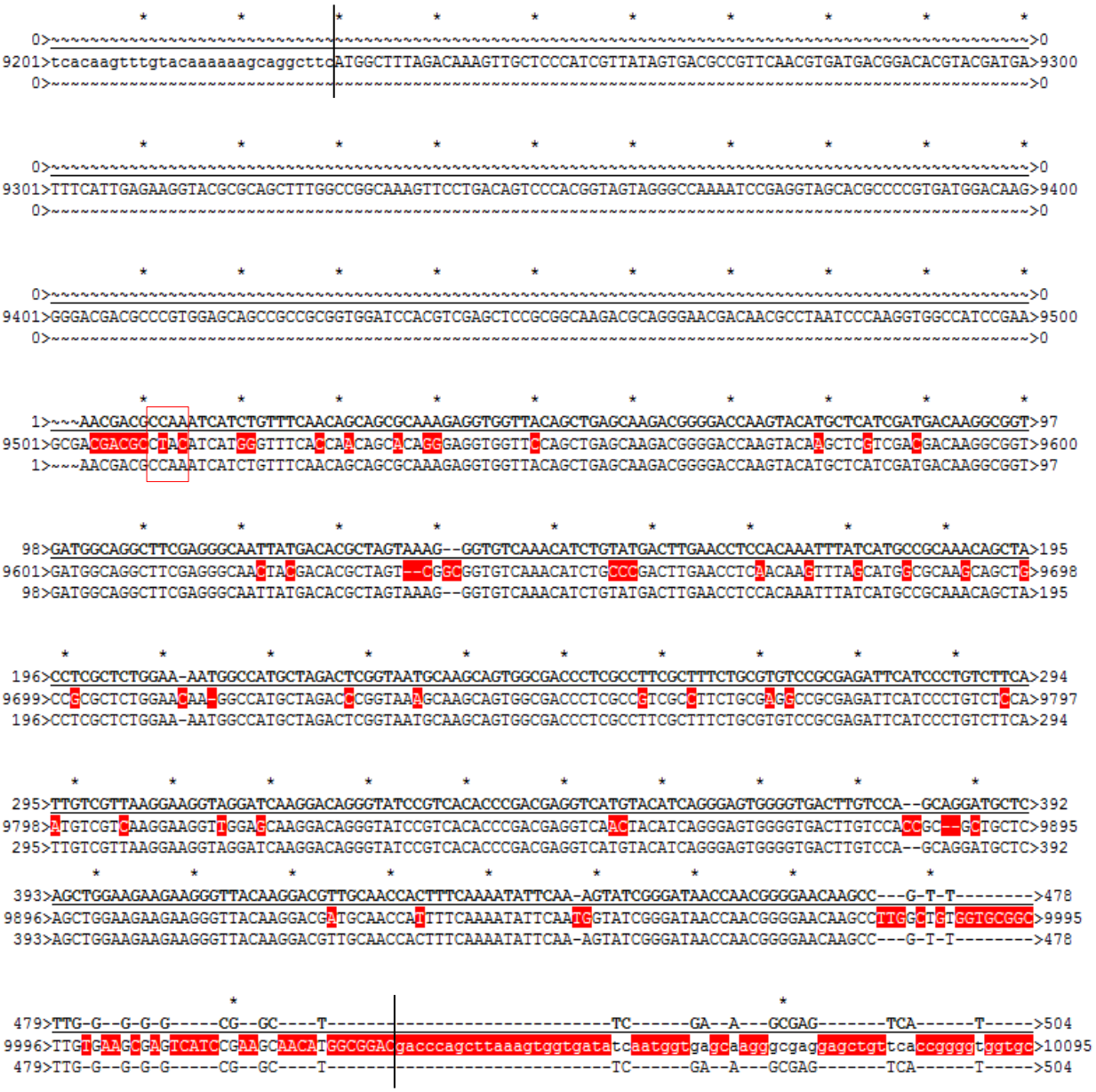
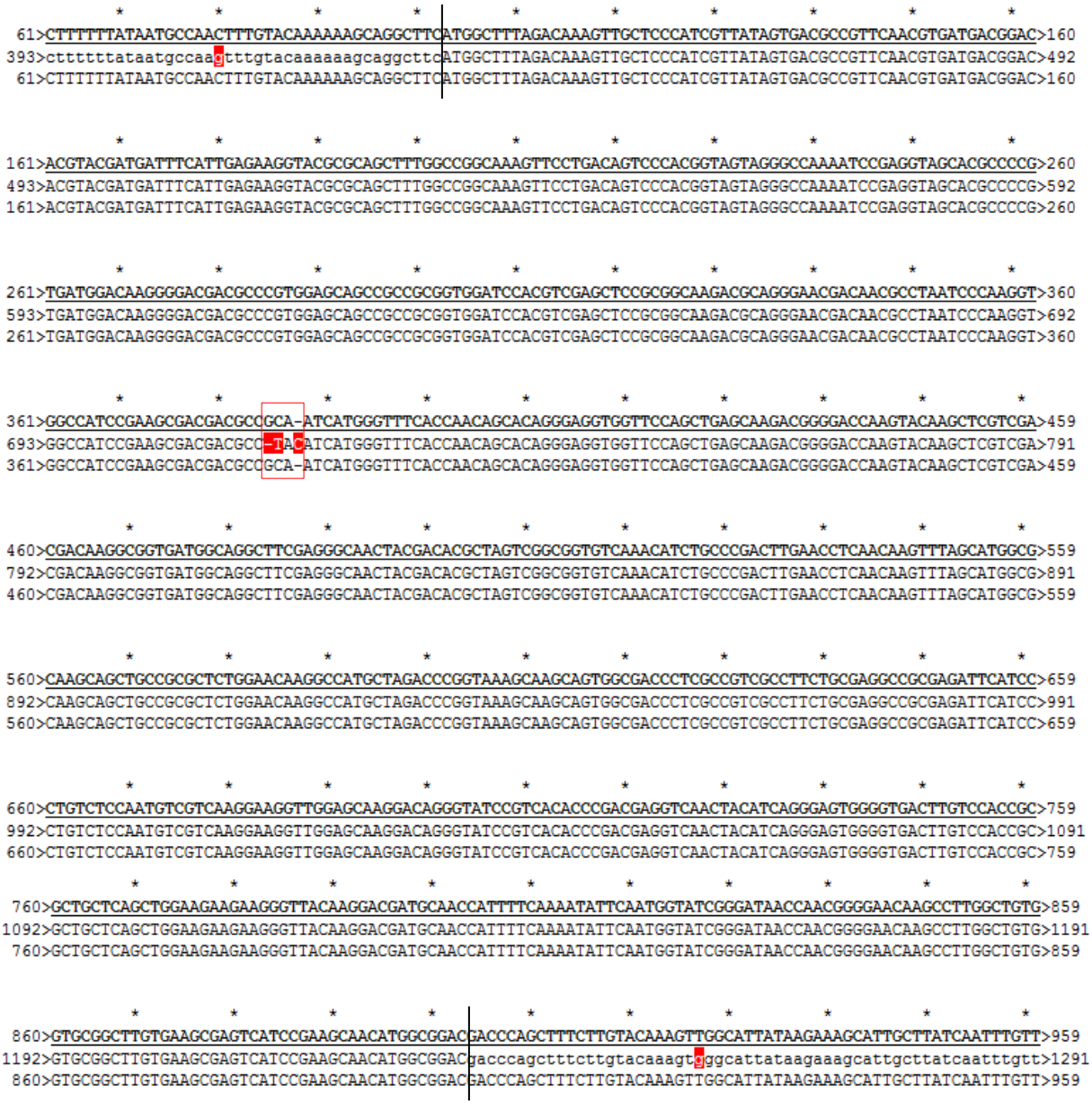
APPENDIX D: DATA FROM SANGER SEQUENCING – CONFIRMING PLASMIDS

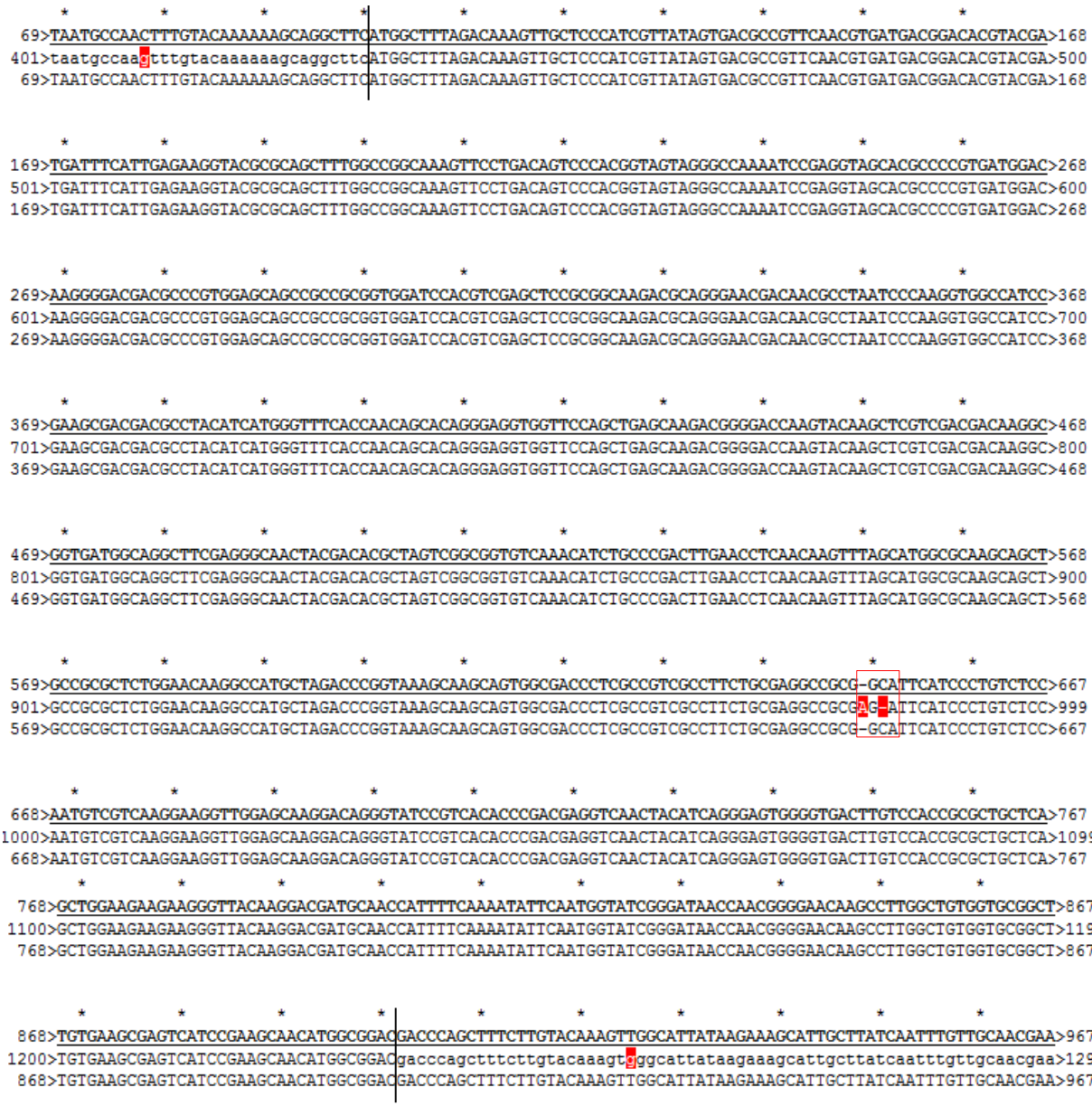
The Sanger sequencing was carried out by GATC Biotech. In the case of the pK7FWG2-*JIP60* plasmid constructs, reverse primers corresponding to *eGFP* were used, in order to gain sequence data for the *JIP60* insert part of the respective plasmid, as shown in the alignments below.



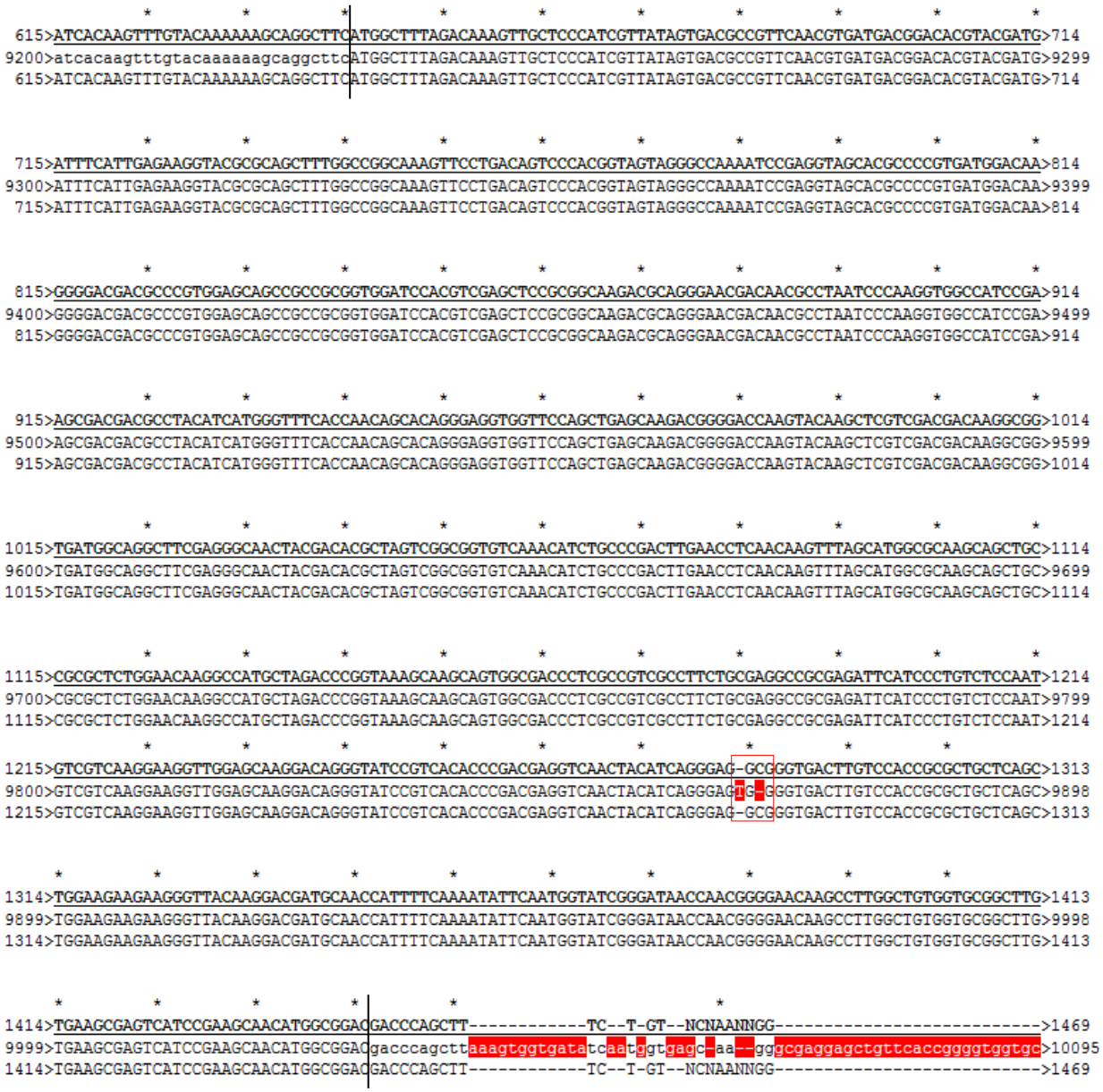
Appendix Figure D(i): Sequence Data Confirming the *JIP60* (Y96A)-pK7FWG2 Plasmid. Top row: alignment; middle row: expected sequence for pK7FWG2 *JIP60* (wild type) (with non-matches in red); bottom row: Sanger sequence of pK7FWG2 *JIP60* (Y96A). The start and end of the *JIP60* insert are indicated by solid vertical lines; the mutation site is indicated by a red box. This alignment does not convincingly show that the mutation has been successful, but did unequivocally show that the DNA sample in question was a pK7FWG2-*JIP60* mutant construct, rather than just empty pK7FWG2, which was the purpose of the sequencing. Therefore to prove that the mutation was successful, I have also included Sanger sequencing of pDONR201-*JIP60* (Y96A) (below).



Appendix Figure D(ii): Sequence Data Confirming the *JIP60* (Y96A)-pDONR201 Plasmid. Top row: alignment; middle row: expected sequence for pDONR201 *JIP60* (wild type) (with non-matches in red); bottom row: Sanger sequence ofpDONR201 *JIP60* (Y96A). The start and end of the *JIP60* insert are indicated by solid vertical lines; the mutation site is indicated by a red box.



Appendix Figure D(iii): Sequence Data Confirming the *JIP60* (R205A)-pDONR201 Plasmid. Top row: alignment; middle row: expected sequence for pDONR201 *JIP60* (wild type) (with non-matches in red); bottom row: Sanger sequence ofpDONR201 *JIP60* (R205A). The start and end of the *JIP60* insert are indicated by solid vertical lines; the mutation site is indicated by a red box.



Appendix Figure D(iv): Sequence Data Confirming the *JIP60* (W235A)-pK7FWG2 Plasmid. Top row: alignment; middle row: expected sequence for pK7FWG2 *JIP60* (wild type) (with non-matches in red); bottom row: Sanger sequence of pK7FWG2 *JIP60* (W235A). The start and end of the *JIP60* insert are indicated by solid vertical lines; the mutation site is indicated by a red box.

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