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(57) **Abrégé/Abstract:**

The invention relates to the field of gene expression in plants and describes methods and constructs using the first intron of a FAD2 gene in order to enhance gene expression.



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(54) Title: METHOD FOR ENHANCING GENE EXPRESSION IN PLANTS

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METHOD FOR ENHANCING GENE EXPRESSION IN PLANTS

BACKGROUND OF THE INVENTION

Efficient transformation systems are available in plants, in particular in
5 monocot species especially those of economic importance such as maize, wheat,
barley and rice. It is now possible to contemplate the introduction of a range of
traits by such technique. The most widely used techniques are the delivery of naked
DNA by micro projectile bombardment or the use of *Agrobacterium* as a vector. In
both cases the gene of interest and associated sequences are introduced into a
10 suitable target plant tissue and become stably integrated into the plant genome. The
target tissue has the potential to regenerate into a whole, fully-fertile plant.

In order for the introduced gene to be expressed, it must be driven by a
promoter. Promoters can be of different types; constitutive or tissue and temporally
specific. Widely use promoters include those derived from virus and bacteria;
15 including Cauliflower Mosaic Virus 35S and octopine and nopaline synthase and
RuBISCO, actin and ubiquitin from plant species.

The most widely used promoters are constitutive because they are generally
used to control the selectable marker gene and can also be used to drive the gene of
interest.

20 Although suitable promoters for monocot species are known, insufficient
numbers are available for use in biotechnology applications. Transformation may
require that one promoter drives the selectable marker and a separate promoter
drives the gene of interest.

It is indeed undesirable to use the same promoter for both genes for several
25 reasons. Initially the cloning and construct preparation becomes problematical.
Sequence duplications can lead to gene deletions and other corruptions which
would result in elimination of gene expression. Sequence duplication subsequently
in planta can result in gene silencing. In a longer term consideration of breeding
programmes, either gene stacking by retransformation or crossing of existing
30 transgenic lines, the lack of choice of promoters will become increasingly limiting.

Strong promoters are preferred as the efficiency of production of useful
transgenic plants is increased, in two ways. Firstly more plants are initially selected
in tissue culture due to sufficient expression of the selectable marker to survive the

selection conditions and secondly more plants thus selected will have a good phenotype resulting from the expression of the gene or genes of interest. In order to increase the number of suitable promoters it was discovered that the strength of a promoter can be increased by use of an intron.

5 Introns interrupt the coding regions of Eukaryotic genes and are removed post-transcription by the process of splicing. The expression of genes in animal cells has been known to be intron dependant since the late 1970s but the first demonstration of the effect of the inclusion of an intron in a plant species was not until 1987 (Callis et al , Genes and Development 1: 1183-1200).

10 The most widely used introns are the first introns of rice Actin and maize Ubiquitin, used in combination with the natural promoters. These promoters were the first strong promoters identified for monocot species. Rice actin was initially identified as a suitable candidate gene for provision of a strong constitutive promoter because actin is a fundamental and essential component of the eukaryotic
15 cell and cytoskeleton. A rice actin gene was identified that encoded a transcript that was abundant in all rice tissues at all developmental stages (McElroy et al 1990, Plant Cell 2:163.) Further characterisation revealed that the presence of the first intron was essential for the efficient function of the promoter *per se*. Similarly the maize ubiquitin promoter was identified as having a high level constitutive activity
20 dependant on the presence of its own first intron (Morris et al 1993, Plant Molecular Biology 21: 895 - 906).

 The enhancing effect of the rice actin first intron and the maize ubiquitin first intron can also be combined with heterologous promoter sequences which alone have minimal activity in monocot species. (Vain et al , 1996, Plant Cell
25 Reports 15: 489 - 494)

 A limited number of plant introns have been identified that can be combined with suboptimal heterologous promoters to create novel promoters sufficiently strong to use for biotechnological applications. Early comparisons of a range of introns revealed that not all have a significantly enhancing activity in combination
30 with a suboptimal promoter. (Vain et al, 1996)

 Nevertheless, intron duplication is undesirable for same reasons that promoter duplication should be avoided.

There is therefore a continuing need to identify new suitable introns to enhance expression in plants, and especially in monocot transformation systems. These introns need to be able to increase the level of expression when combined with various promoters.

5 At the present time there are very few such introns identified and this ultimately limits the number of genetic transformations that can be carried out in a given monocot species.

The invention now provides a new intron that is usable for increasing expression of an heterologous gene in a plant cell, and in particular a monocot plant
10 cell, such as wheat, maize, barley, or rice.

SUMMARY OF THE INVENTION

The inventors have indeed surprisingly observed that presence of the first intron of the FAD2 gene, which was believed to have no effect on expression, can
15 effectively enhance the efficiency of a promoter.

The FAD2 gene is the gene coding for the microsomal omega-6 fatty acid desaturase (FAD2/delta-12 desaturase), and its sequence is well known by the person skilled in the art, with the availability of the sequence of the chromosome 3 *Arabidopsis thaliana* genome.

20 Other FAD2 genes have been identified (for example *Brassica oleracea*, represented by AF181726 in GenBank, soybean with GenBank protein accession number P48628, *Brassica napus*, GenBank protein accession number P48627...). The identification of the first introns of FAD2 genes of other species is within the skill of the person in the art, by the differences between the cDNA and genomic
25 sequences.

FIGURES

Figure 1: Schematic representation of pJIT65del

Figure 2: Schematic representation of pWP443A

30 Figure 3 : Schematic representation of pWP461

Figure 4: Schematic representation of pWP464

Figure 5: Schematic representation of pWP480

Figure 6 : Schematic representation of pWP500

Figure 7 : Schematic representation of pWP514

Figure 8 : Transient expression comparison in wheat. Embryos bombarded with 3 promoters with and without FAD2 intron.

Figure 9 : Transient expression comparison in barley a) embryos bombarded with
5 pWP480 b) embryos bombarded with pWP500

Figure 10: Leaves from wheat stably transformed wheat with a) pBSV without FAD2 intron, or b) pBSV with FAD2 intron.

DETAILED DESCRIPTION OF THE INVENTION

10 The invention thus relates to a genetic construct for the expression of a polypeptide in a plant cell, comprising:

- (a) a promoter operative within said plant cell,
- (b) all or part of the first intron of a FAD2 gene,
- (c) a nucleotide sequence operably linked to said promoter, wherein said
15 nucleotide sequence encodes said polypeptide,
- (d) a polyadenylation site (PAA) operably linked to said nucleotide sequence.

Said polynucleotide is called "polynucleotide of interest" and is coded by a "gene of interest".

20 A "promoter operative in a plant cell" is intended to mean a sequence that directs the initiation of transcription in a plant cell. It can derive from one or multiple sources, be natural or synthetic.

In the preferred embodiment, the genetic construct of the invention is such
25 that said FAD2 gene is from *Arabidopsis thaliana* (GenBank AJ271841), wherein said first intron is represented by SEQ ID N° 1, or as indicated in SEQ ID N° 3, 5, 7 and 8. it is also envisaged that said FAD 2 intron has 80, 90 or 95 % identity with SEQ ID N° 1, over at least 500 or 1000 nucleotides.

Nevertheless, other FAD2 first introns from other species may also be used,
30 such as from maize, wheat, barley, rape, canola, sunflower, potato...

In the context of the present invention, "all or part of the first intron of a FAD2 gene" is to be considered as the full sequence of the first intron of a FAD 2

gene, or a fragment comprising at least 200, 500 or 1000 nucleotides of said sequence.

The promoter could be any promoter, as described above. In a preferred embodiment, said promoter is a tissue-specific promoter. In another embodiment, 5 said promoter is a constitutive promoter. Thus it is clear that the envisaged promoter is preferably a heterologous promoter, *i.e.* different of the natural FAD2 promoter.

Promoter sequences of genes which are expressed naturally in plants can be of plant, bacterial or viral origin. Suitable constitutive promoters include but are not restricted to octopine synthase, nopaline synthase, mannopine synthase derived 10 from the T-DNA of *Agrobacterium tumefaciens*; CaMV35S and CaMV19S from Cauliflower Mosaic Virus; rice actin, maize ubiquitin and histone promoters from plant species.

Tissue specific promoters include but are not restricted to rolC from *Agrobacterium rhizogenes*; RTBV from Rice Tungro Bacilliform Virus; LMW and 15 HMW glutenin from wheat; alcohol dehydrogenase, waxy, zein from maize and, AoPR1 from Asparagus.

All these promoters (and others) are well described in the art.

Whilst the most widely used promoters are constitutive or tissue specific it is also possible to contemplate the use of inducible promoters such as PinII or 20 AoPRT-L (WO 99/66057); more specifically light inducible including RUBISCO small subunit and chlorophyll a/b binding protein from a range of species and synthetic promoters eg EMU (US19900525866).

The genetic construct according to the invention is introduced within the plant cell on a vector. In an embodiment, said vector is maintained as an episomal 25 vector within the plant cell. In the preferred embodiment, said vector is capable of integration within the genome of said plant cell. Integration of a genetic construct within a plant cell is performed using methods known by those skilled in the art, for example transformation by *Agrobacterium*, or gun bombardment.

Agrobacterium tumefaciens has been widely and efficiently used to 30 transform numerous species dicots and including monocots such as maize, rice, barley and wheat (WO 00/63398). Alternative gene transfer and transformation methods include protoplast transformation through calcium, polyethylene glycol or electroporation mediated uptake of naked DNA. Additional methods include

introduction of DNA into intact cells or regenerable tissues by microinjection, silicon carbide fibres or, most widely, microprojectile bombardment. All these methods are now well known in the art.

5 The invention also relates to a method for enhancing the expression of a polypeptide in a plant cell, comprising the step of introducing the genetic construct of the invention within said plant cell, for example using the methods as mentioned above. Expression is enhanced as compared to expression obtained when the FAD2 intron is not present within the construct.

10 Expression relates to transcription and translation of a gene so that a protein is made.

In another aspect, the invention relates to a method for increasing the expression rate of a gene, under the control of a promoter in a plant cell, said method comprising the steps of:

- 15 (a) inserting the first intron of the FAD 2 gene between said promoter and the translation start of said gene, thereby forming an intron-modified gene; and
- (b) introducing said intron-modified gene into a plant cell such that said gene is expressed under the control of said intron in said plant cell,
- 20 whereby the expression rate of said gene is increased as compared to an unmodified gene.

Said gene is called "gene of interest".

In particular, said gene can be a selectable marker. These selectable markers include, but are not limited to, antibiotic resistance genes, herbicide resistance genes

25 or visible marker genes. Other phenotypic markers are known in the art and may be used in this invention.

A number of selective agents and resistance genes are known in the art. (See, for example, Hauptmann et al., 1988 ; Dekeyser et al., 1988 ; Eichholtz et al., 1987 ; and Meijer et al., 1991).

30 Notably the selectable marker used can be the bar gene conferring resistance to bialaphos (White et al., 1990), the sulfonamide herbicide Asulam resistance gene, sul (described in WO 98/49316) encoding a type I dihydropterate synthase (DHPS), the nptII gene conferring resistance to a group of antibiotics including kanamycin,

G418, paromomycin and neomycin (Bevan et al., 1983), the hph gene conferring resistance to hygromycin (Gritz et al., 1983), the EPSPS gene conferring tolerance to glyphosate (US 5,188,642), the HPPD gene conferring resistance to isoxazoles (WO 96/38567), the gene encoding for the GUS enzyme, the green fluorescent protein (GFP), expression of which, confers a recognisable physical characteristic to transformed cells, the chloramphenicol transferase gene, expression of which, detoxifies chloramphenicol.

In another embodiment, said gene encodes a protein to impart insect resistance, more preferably genes which encode for *Bacillus thuringiensis* (Bt) endotoxins (inter alia, U.S. Patent Nos. 5,460,963; 5,683,691; 5,545,565; 5,530,197; 5,317,096). The nucleic acids that are preferably embraced by the instant invention are cryI, cryII, cryIII, and cryIV genes. More preferably, the genes include: cryIA(a), cryIA(b); cryIA(c); and cryIIIA(a). Most preferably the gene is cryIA(a), cryIA(b) or cryIA(c).

Other genes of interest are

- the bacterial gene dapA for increasing the level of lysine;
- the gene for endotoxin Bt or for a protease inhibitor or for proteins extracted from bacteria such as *Photobacterium* (WO 97/17432 & WO 98/08932), for resistance to insects;
- among the proteins or peptides of interest which confer novel properties of resistance to diseases, mention will be made in particular of chitinases (WO 92/01792), glucanases (WO 93/02197), oxalate oxidase (WO 94/13790) or antibacterial and/or antifungal peptides, in particular the peptides of less than 100 amino acids rich in cysteins, such as plant thionins or defensins, and more particularly lytic peptides of any origins comprising one or more disulfide bridges between the cysteins and regions comprising basic amino acids, in particular the following lytic peptides: androctonin (WO 97/30082 and WO 99/09189), drosomicin (WO 99/02717), thanatin (WO 99/24594) or heliomycin (WO 99/53053). According to a particular embodiment of the invention, the protein or peptide of interest is chosen from fungal elicitor peptides, in particular elicitors (Kamoun et al., 1993; Panabières et al., 1995).

- genes involved in the biosynthetic processes which lead to a change in the quality of the products of the transgenic plant, such as the genes encoding enzymes for the biosynthesis or degradation of starch (i.e. synthases, starch-branching enzymes, etc.); genes encoding grain storage proteins (i.e. subunits of
5 glutenins, gliadins, hordeins); genes related to the strength of the grain in wheat (i.e. puroindolines).

- genes which modify the constitution of the modified plants, in particular the content and the quality of certain essential fatty acids (EP 666 918) or the content and the quality of the proteins, in particular in the leaves and/or the
10 grains of said plants. Mention will be made, in particular, of the genes encoding proteins enriched in sulfur-containing amino acids (Korit, A.A. et al.; WO 98/20133; WO 97/41239; WO 95/31554; WO 94/20828; WO 92/14822). The function of these proteins enriched in sulfur-containing amino acids will also be to trap and store excess cystein and/or methionine, making it possible to avoid the
15 possible problems of toxicity linked to an overproduction of these sulfur-containing amino acids by trapping them. Mention may also be made of genes encoding peptides rich in sulfur-containing amino acids, and more particularly in cysteins, said peptides also having antibacterial and/or antifungal activity. Mention will be made more particularly of plant defensins, and also lytic peptides of any origin, and
20 more particularly the following lytic peptides: androctonin (WO 97/30082 and WO 99/09189), drosomicin (WO 99/02717), thanatin (WO 99/24594) or heliomycin (WO 99/53053).

- genes for artificial male sterility (i.e. barnase, and PR-glucanase under the control of a suitable promoter) may also be used for the production of
25 hybrid seeds.

The invention also relates to a method as mentioned above wherein said intron has a nucleotide sequence as set forth in SEQ ID N° 1 or functionally equivalent duplications or modifications in length or minor sequence variations that
30 do not significantly affect function, which is expression enhancing. It is well within the skills of a person skilled in the art to modify the FAD 2 intron sequence and test new constructs for their ability to improve expression of a gene, various protocols in the art, and in particular the ones described in the examples.

In another aspect, the invention relates to the use of all or part of the first intron of the FAD2 gene for enhancing expression of a polypeptide in a plant cell.

In another aspect, the FAD2 first intron may be used to convert a tissue specific promoter into a constitutive promoter. For example, the strong seed specific
5 HMW glutenin promoter is converted into a strong/ useful promoter active in many tissue types, when used with the FAD 2 first intron.

EXAMPLES

All DNA modifications and digestions were performed using enzymes according to
10 the manufacturers' instructions and following protocols described in Sambrook and Russell, 2001; Molecular Cloning, A Laboratory Manual.

Example 1. Cloning of FAD 2 intron

The first intron of the FAD 2 gene was obtained by the polymerase chain
15 reaction (PCR) from *Arabidopsis thaliana* genomic DNA. The following pair of primers were used:

GGGCCAGGTCCGTCGCTTCTCTTCC FADfor (SEQ ID N° 9)
GGGTTTCTGCAGAAAACCAAAAGC FADrev (SEQ ID N° 10)

20 A standard PCR reaction was carried out, with amplification under the following conditions: 30 cycles of 97°C for 30 seconds, 57°C for 30 seconds and 74°C for 1 minute 40 seconds. The reaction products were separated by agarose gel electrophoresis.

25 The desired 1146 bp product was excised from the gel and ligated into the *Sma*I restriction site of pTZ18 (standard cloning vector; Pharmacia) to create pWP430. The fidelity of the clone was confirmed by sequencing.

Example 2. Preparation of GUS constructs

30 Several promoters, which are known in the field of plant biotechnology were identified, to be tested with and without the FAD2 intron. These included but were not limited to Cauliflower Mosaic Virus 35S (Odell et al, Nature. 1985 Feb 28-Mar 6;313(6005):810-2), Banana Streak Virus promoter of ORF1 (WO 99/43836), Sc4,

a member of the Plant Expression (PLEX) promoter family (EP 785999; US 6211431) and HMW glutenin (HMWG) promoter. Constructs were prepared from well-characterised genetic elements and cloned into widely used plasmid vectors.

5 *35S: pJIT65del*

The 35S promoter was cloned in a pUC vector, driving the GUS gene (Jefferson et al, PNAS 83: 8447-8451, 1986), to obtain plasmid pJIT65. This plasmid was modified by digestion with *Xba*I and *Eco*RI to removing intervening *Bam*HI and *Sma*I sites. This modified version was named as pJIT65del.

10

35S + *FAD2 intron:pWP443A*

The FAD2 intron was inserted between the 35S promoter and the GUS gene, specifically into the 5'UTR, by digesting pWP430 with *Sma*I and ligating the intron into *Sma*I digested pJIT65del, to create pWP443A.

15

BSV: pWP461

The BSV promoter was cloned into the *EcoRV* site of Bluescript KS to create pWP453B. The following primers were used:

GATGCTCTAGATCTGCTGG BSVFor (SEQ ID N° 11)

20 GGCCATGGCTTGCTCTGATACCAGACGGG BSVRev (SEQ ID N° 12)

pWP453B was digested with *Sst*I and *Sal*I to isolate the BSV promoter fragment which was then ligated into *Sst*I and *Sal*I digested JIT65del, thus replacing the 35S promoter to create pWP461.

25

BSV + FAD2 intron:pWP464

To create the combination of the BSV promoter with the FAD2 intron the 35S promoter of pWP443A was replaced by the BSV promoter from pWP461. Both plasmids were digested with SstI and BamHI and fragments ligated to produce pWP464.

Sc4:pWP480

A previously prepared construct, pKH2, having the Sc4 promoter driving the GUS gene, with the inclusion of the rice actin first intron in the 5'UTR, was digested with SstII and NcoI to remove the actin intron and create pWP480.

5 *Sc4 +FAD2 intron:pWP 500*

Similarly to the creation of pWP464, to combine the Sc4 promoter with the FAD2 intron the 35S promoter of pWP443A was replaced by the Sc4 promoter from pWP480 using suitable digests.

10 *HMWG + intron: pWP514*

A pUC clone of the HMWG promoter driving the GUS gene (Glu-1Dx5-GUS2) was obtained as described in Halford et al 1989 (Plant Science 62, 207-16). The FAD2 intron was inserted between the HMWG promoter and the GUS gene, specifically into the 5'UTR, to create pWP514.

15

Example 3 Transient expression in wheat

a) Initial assessment of functioning of constructs

A range of plant tissues and calli were bombarded with particles coated with one of the four promoter constructs including the FAD2 intron, according to methods known in the art. The tissues were immersed in a solution of the histochemical substrate, X-glucuronide, and a subjective assessment was made of the activity of the construct. In each case a significant number of blue spots were observed on each of the tissues tested.

	TRANSIENT EXPRESSION
pWP443A	Strong expression on embryo, leaf and callus
pWP500	Strong expression on embryo, leaf and callus
pWP464	Strong expression on embryo, leaf and callus
pWP514	Good expression on embryo, leaf and endosperms

25 It is especially worthy of note that there was significant expression in leaf and embryo tissues following bombardment with the HMWG construct (pWP514).

The HMWG promoter is naturally strong endosperm-specific promoter. The inclusion of the FAD2 intron has converted it into a strong constitutive promoter.

b) Comparison of constitutive promoters with and without introns

5 Having established that the promoters modified by the inclusion of the FAD2 intron were functioning a quantitative comparison was made between the promoters with and without the intron.

Isolated embryos were arranged in 4x4 arrays. Thirty-two embryos were bombarded with each construct.

10 Co-bombardment with a second construct containing the Green Fluorescent Protein (GFP) under control of the rice actin promoter, was performed. Expression of GFP can be non-destructively tested and hence the relative efficiency of each individual bombardment can be normalized to allow comparison between individual bombardments.

15

The level of expression for each construct was again visually assessed by histochemical assay for the GUS gene (figure 8) which enabled both a quantitative (counting the number of blue foci by 4x4 array), and a qualitative assessment (consideration of the size of blue foci).

20

The increase in expression due to inclusion of the FAD2 intron was 5.6 fold for the Sc4 promoter, 8 fold for the double 35S promoter and most remarkably 161 fold for the BSV promoter. Thus the expression of the weakest promoter (BSV) is increased to a level comparable with the maize ubiquitin promoter (pAAA) which is

25 widely used because it is known to be very strong.

	pSc4				pBSV			
	pWP480		pWP500		pWP461		pWP464	
GFP	1648	698	1501	1945	989	1015	1504	1384
GUS	610	142	2340	3101	36	14	5067	4287

Note on GUS spots	Very small spots, difficult to see with the naked eye		Big spots, well seen but no spreading nor covering		Very small spots (two big spots on two different embryos)		Very big, 'spreading' spots	
Ratio GUS/GFP	0.37	0.20	1.55	1.59	0.03	0.01	3.36	3.09
Average	0.28		1.57		0.02		3.22	
Ratio with/without	5.6				161			

	p35S				Positive Control	
	pJIT65del		pWP443A		pAAA	
GFP	470	922	711	687	1502	1390
GUS	93	296	1539	1279	7200	7200
Note on GUS spots	Very small dots, sometimes not seen with the naked eye, few big ones		Plenty of rather big spots		Very big spots, 'spreading' all over the embryo difficult to score	
Ratio GUS/GFP	0.19	0.32	2.16	1.86	4.79	5.17
Average	0.25		2.01		4.98	
Ratio with/without	8				/	

A negative control with only gold particles yielded no spots.

5

Example 4: Transient expression in barley

Other bombardment experiments were performed on barley embryos. The results also demonstrated that the presence of the FAD2 intron increases expression of the GUS protein in this species (figure 9). 2 plates of 16 embryos were

bombarded with either pWP480 or pWP500 plasmid. The number of blue foci was scored per embryo and averaged.

Expression of co-bombarded GFP was again used to assess the relative efficiency of each individual bombardment and used to normalize and allow comparison between individual bombardments.

	pWP480	pWP500
GFP	63.9	60.7
GUS	27.00	92.2
RatioGUS/GFP	0.42	1.52
Ratio +/- intron	3.61	

10 Example 5: Stable expression in wheat

Wheat embryos were stably transformed by bombardment (WO 98/49316). The embryos were bombarded with pWP461 or pWP464.

Transgenic plants were regenerated and grown, and expression of the GUS protein was studied by X-Gluc staining for glucuronidase activity. Following bombardment with WP461, no GUS expression was observed in regenerated transgenic plants. Following bombardment with WP464, strong constitutive expression could be observed in a range of tissues including leaf (Figure 10), root, male and female floral parts and seed.

20

Example 6: Stable transformation of wheat

A selectable marker gene was prepared consisting of the Sc4 promoter, FAD 2 intron, the nptII coding region and nopaline synthase terminator. A comparable selectable marker gene was prepared consisting of the more widely used rice actin promoter and the associated first intron, the nptII coding region and nopaline synthase terminator. Both versions of the selectable marker gene were cloned into a suitable vector for wheat transformation. Wheat embryos were stably transformed by the Agrobacterium Seed Inoculation Method (SIM; WO 00/63398) using either

25

Rice actin promoter + intron	Number of embryos	Transgenic events	Efficiency (%)
Experiment 6	192	1	0.5
Experiment 7	206	19	9.2
Experiment 8	220	3	1.4
Experiment 9	225	4	1.8
Experiment 10	145	1	0.7
Experiment 11	139	18	13
Total	1127	46	4.1

version of the selectable marker, and stably transformed transgenic plantlets selected on plant tissue culture media containing a suitable concentration of Geneticin G418. The number of independent transgenic events was recorded and the transformation efficiency calculated as the percentage of treated embryos
5 regenerating transgenic events.

Sc4 promoter + FAD 2 intron	Number of embryos	Transgenic events	Efficiency (%)
Experiment 1	265	11	4.2
Experiment 2	230	1	0.4
Experiment 3	270	8	3.0
Experiment 4	224	8	3.6
Experiment 5	230	19	8.3
Total	1219	47	3.9

10 The rice actin promoter including the first intron is a very strong promoter widely used to drive the selectable marker in monocot species. Transformation frequencies were observed between 0.5 and 9.2 % with an average of 4.1%. When the selectable marker was driven by the Sc4 promoter in combination with the FAD
15 2 intron, transformation frequencies of 0.4 to 8.3%, with an average of 3.9% were observed.

Surprisingly the combination of the very weak Sc4 promoter with the FAD2 intron is as efficient as the widely used rice actin promoter when used to drive the selectable marker needed for stable wheat transformation.

Example 7: Stable transformation of maize

Both versions of the selectable marker gene described in Example 6 were cloned into a suitable vector for maize transformation. Maize embryos were stably transformed essentially by the method of Ishida et al (1996) using either version of the selectable marker, and stably transformed transgenic plantlets selected on plant tissue culture media containing a suitable concentration of kanamycin. The number of independent transgenic events was recorded and the transformation efficiency calculated as the percentage of treated embryos regenerating transgenic events.

Sc4 promoter + FAD 2 intron	Number of embryos	Transgenic events	Efficiency %
Experiment 1	2656	62	2.33
2	1455	12	0.82
3	1196	21	1.76
4	2212	30	1.36
5	1306	8	0.61
6	1363	24	1.76
7	1247	24	1.92
8	1307	17	1.30
9	2297	18	0.78
10	2177	20	0.92
11	4306	67	1.56
12	2016	23	1.14
13	1218	6	0.49
14	1529	9	0.59
Total	26285	341	1.30

Rice actin promoter + intron	Number of embryos	Transgenic events	Efficiency %
Experiment 1	1177	4	0.34
2	870	6	0.69
3	900	3	0.33
4	685	4	0.58
5	730	1	0.14
6	698	1	0.14
7	2349	7	0.30
8	1895	23	1.21
9	2515	42	1.67
10	1863	17	0.91
11	1448	10	0.69
12	2231	9	0.40
13	2170	20	0.92
14	3067	30	0.98
15	1135	4	0.35
16	2287	5	0.22

17

	17	4132	54	1.31
	18	1218	6	0.49
	19	3250	12	0.37
	20	2024	20	0.99
	21	2013	20	0.99
Total		38657	298	0.77

Surprisingly the combination of the very weak Sc4 promoter with the FAD 2 intron is more efficient than the widely used rice actin promoter when used to drive the selectable marker needed for stable maize transformation (average 1.3% compared with 0.77%).

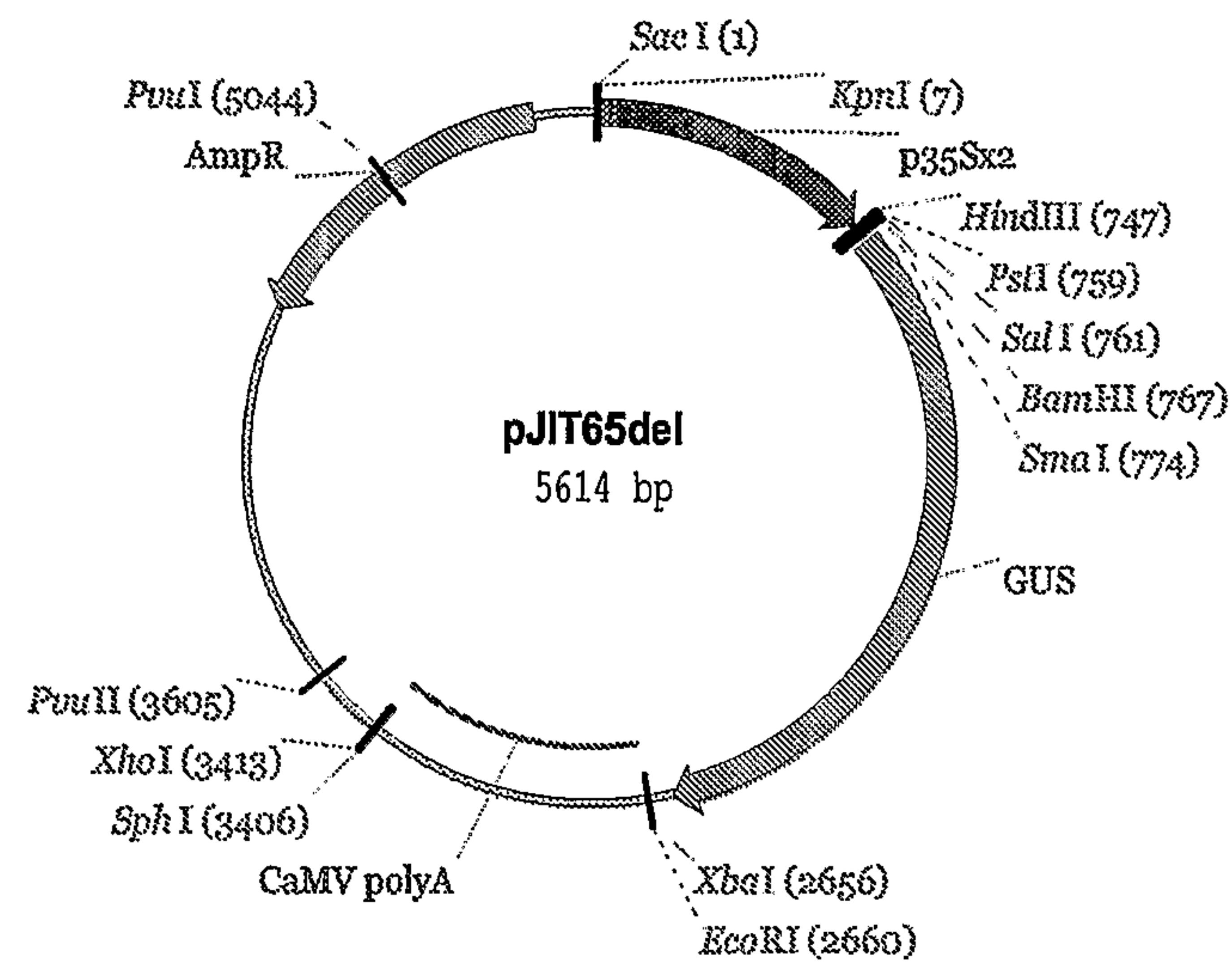
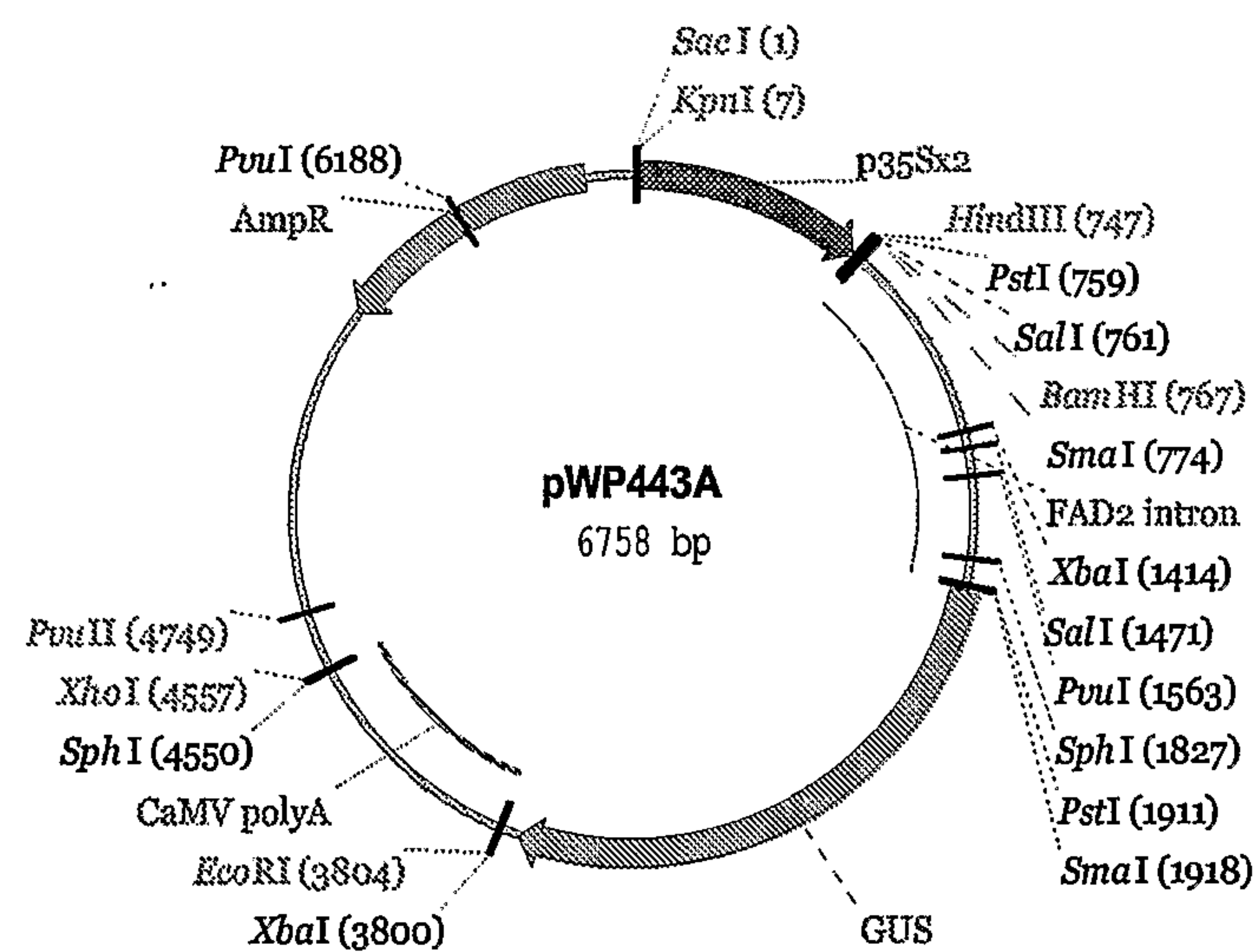
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Claims

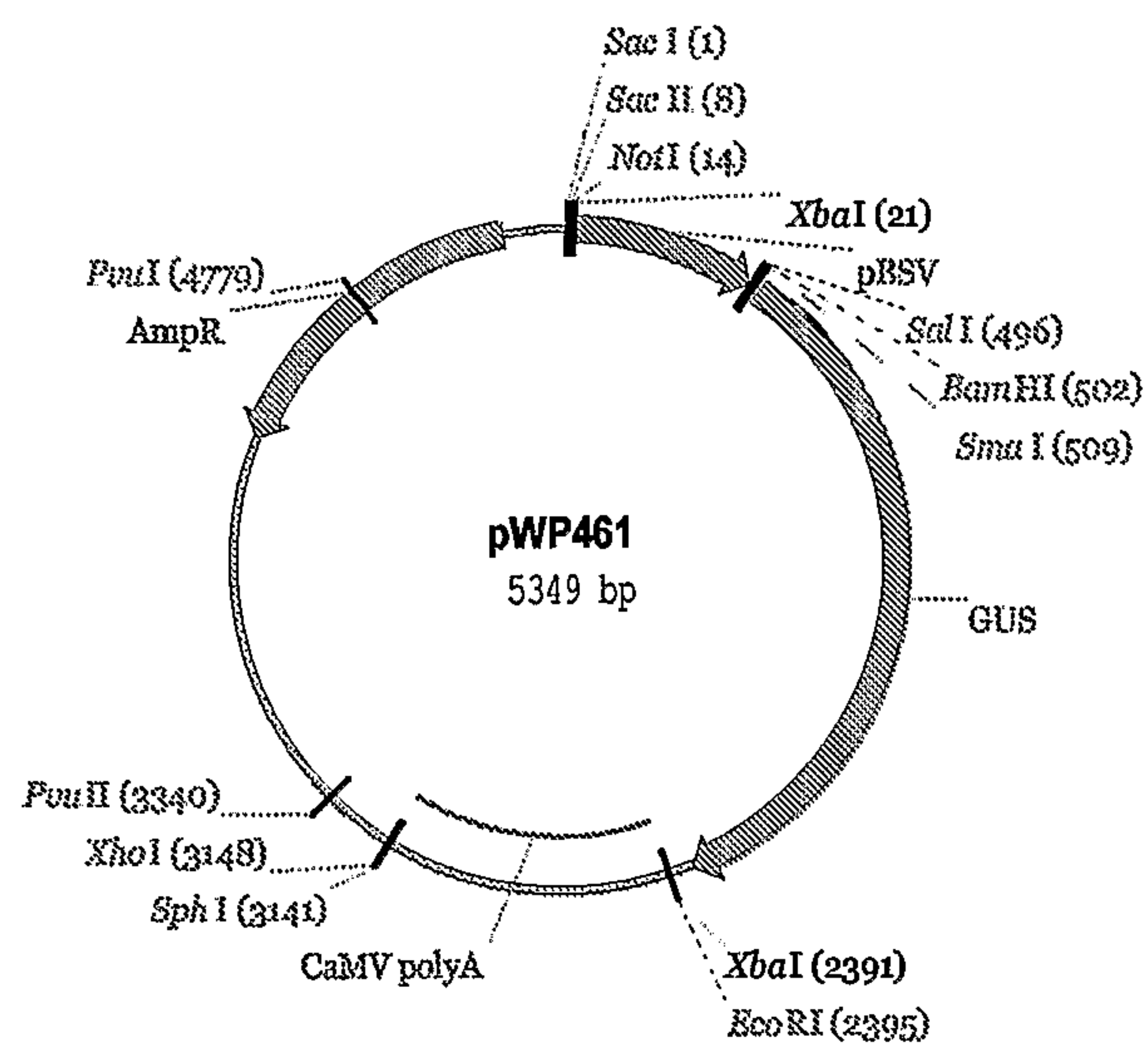
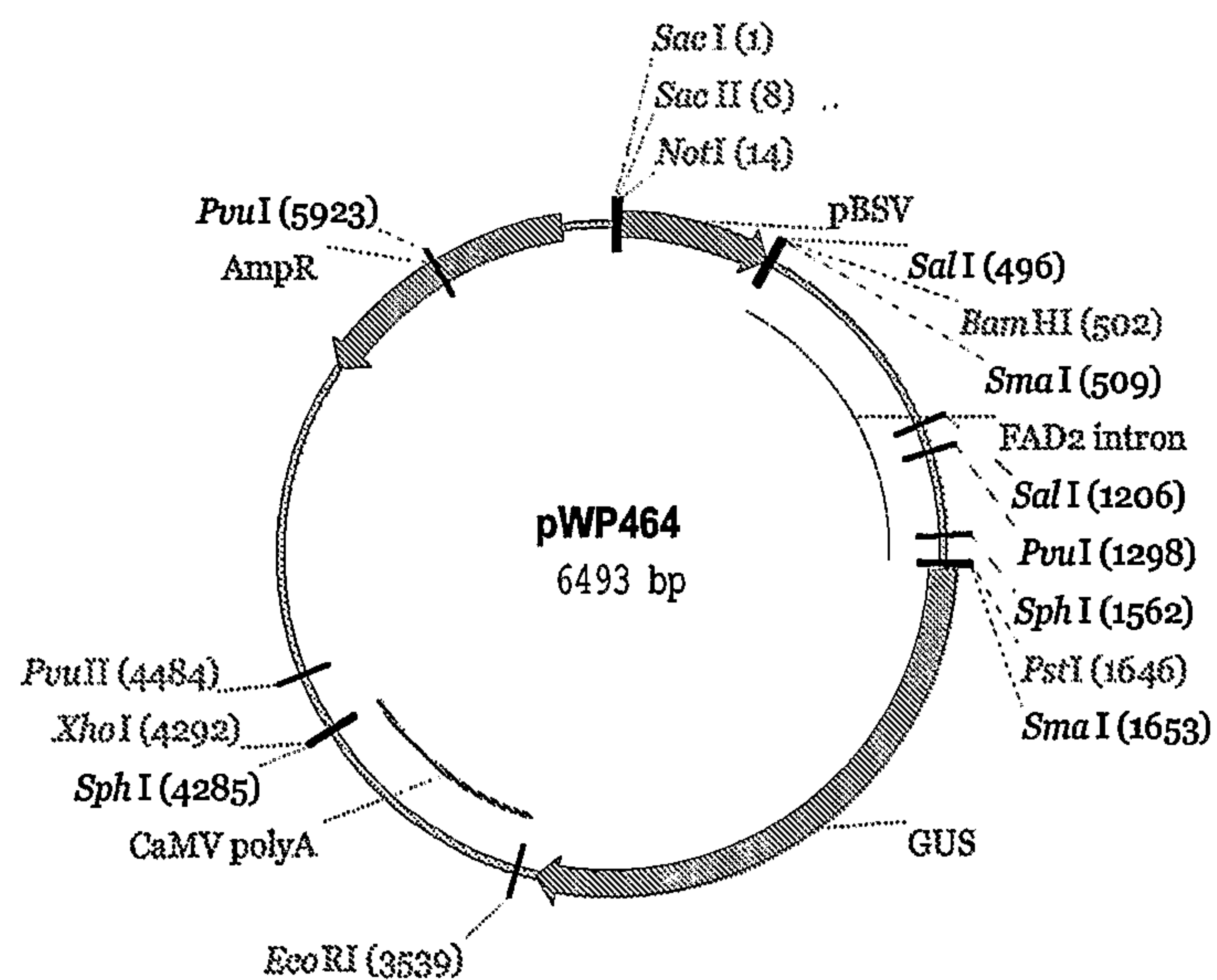
1. A genetic construct for the expression of a polypeptide in a plant cell, comprising:
 - (a) a promoter operative within said plant cell,
 - (b) the first intron of the ω -6 fatty acid desaturase (FAD2) gene, wherein said FAD2 gene is from *Arabidopsis thaliana*, and wherein said first intron is represented by SEQ ID No: 1,
 - (c) a nucleotide sequence operably linked to said promoter, wherein said nucleotide sequence encodes said polypeptide, and
 - (d) a polyadenylation site (PAA) operably linked to said nucleotide sequence;wherein said promoter is a promoter from a gene other than a FAD2 gene and wherein said intron is inserted between said promoter and the translation start of said nucleotide sequence.
2. The genetic construct of claim 1, wherein said promoter is a tissue-specific promoter.
3. The genetic construct of claim 1, wherein said promoter is a constitutive promoter.
4. The genetic construct of any one of claims 1 to 3, capable of integration within the genome of said plant cell.
5. A method for increasing, in a plant cell, the expression rate of a nucleotide sequence under the control of a promoter, said method comprising the steps of:
 - (a) inserting the first intron of the FAD2 gene, wherein said FAD2 gene is from *Arabidopsis thaliana*, and wherein said first intron is represented by SEQ ID No: 1, between said promoter and the translation start of said nucleotide sequence, thereby forming an intron-modified nucleotide sequence; and

- (b) introducing said intron-modified nucleotide sequence into a plant cell such that said nucleotide sequence is expressed under the control of said intron in said plant cell, whereby the expression rate of said nucleotide sequence is increased as compared to an unmodified copy of the nucleotide sequence under the control of said promoter;
wherein said promoter is a promoter from a gene other than a FAD2 gene.
6. Use of the first intron of the FAD2 gene in combination with a promoter from a gene other than a FAD2 gene to enhance expression of a polypeptide in a plant cell;
wherein said FAD2 gene is from *Arabidopsis thaliana*;
wherein said first intron is represented by SEQ ID No: 1;
wherein the polypeptide is encoded by a nucleic acid sequence under the control of the FAD2 intron and the promoter; and
wherein said intron is inserted between said promoter and the translation start of said nucleic acid sequence.
7. A plant cell comprising a genetic construct according to claim 1 integrated in its genome.
8. The plant cell of claim 7, which is a monocot plant cell.
9. The plant cell of claim 8, which is selected from the group consisting of wheat, barley, maize and rice.

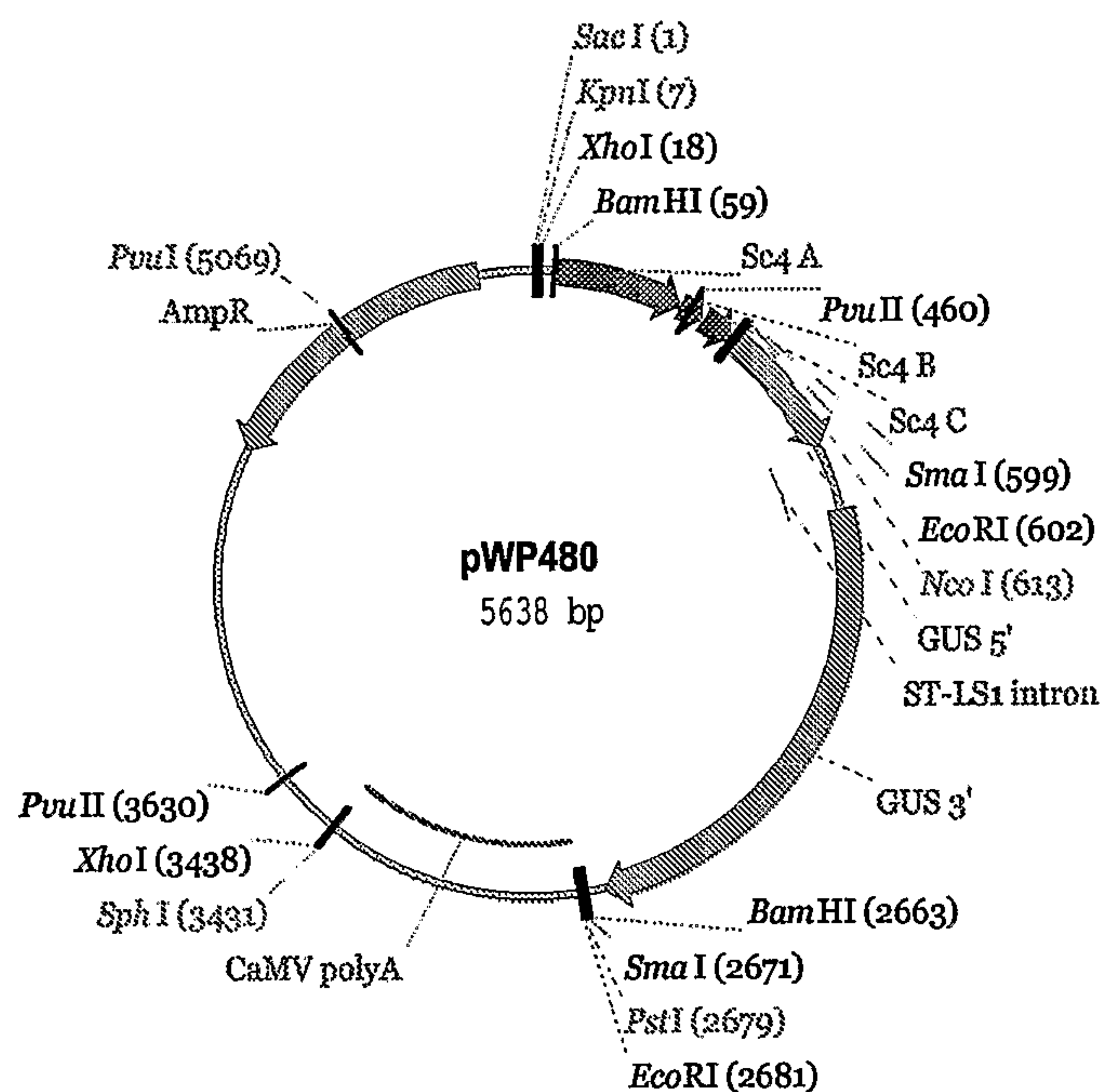
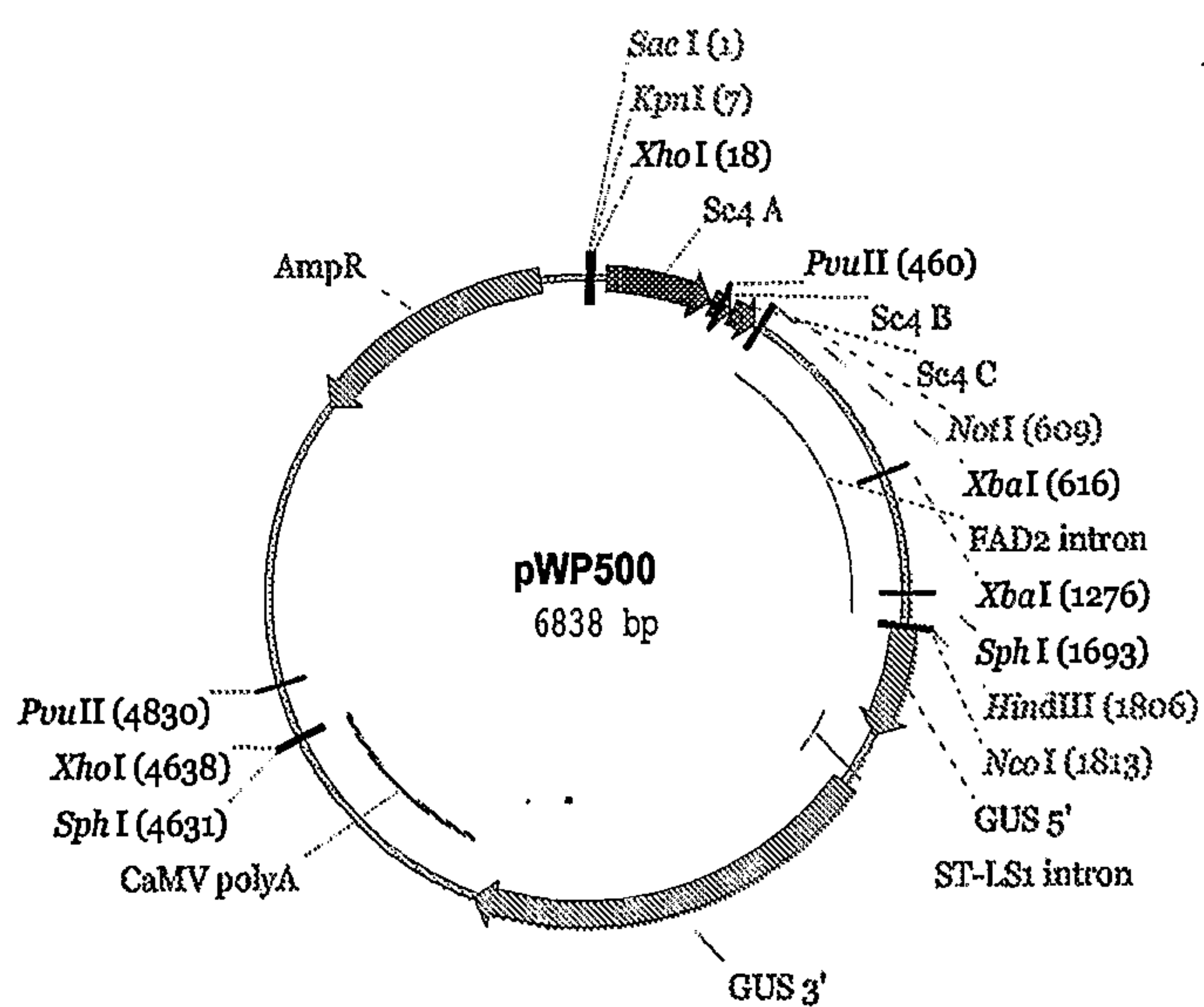
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**Figure 1****Figure 2**

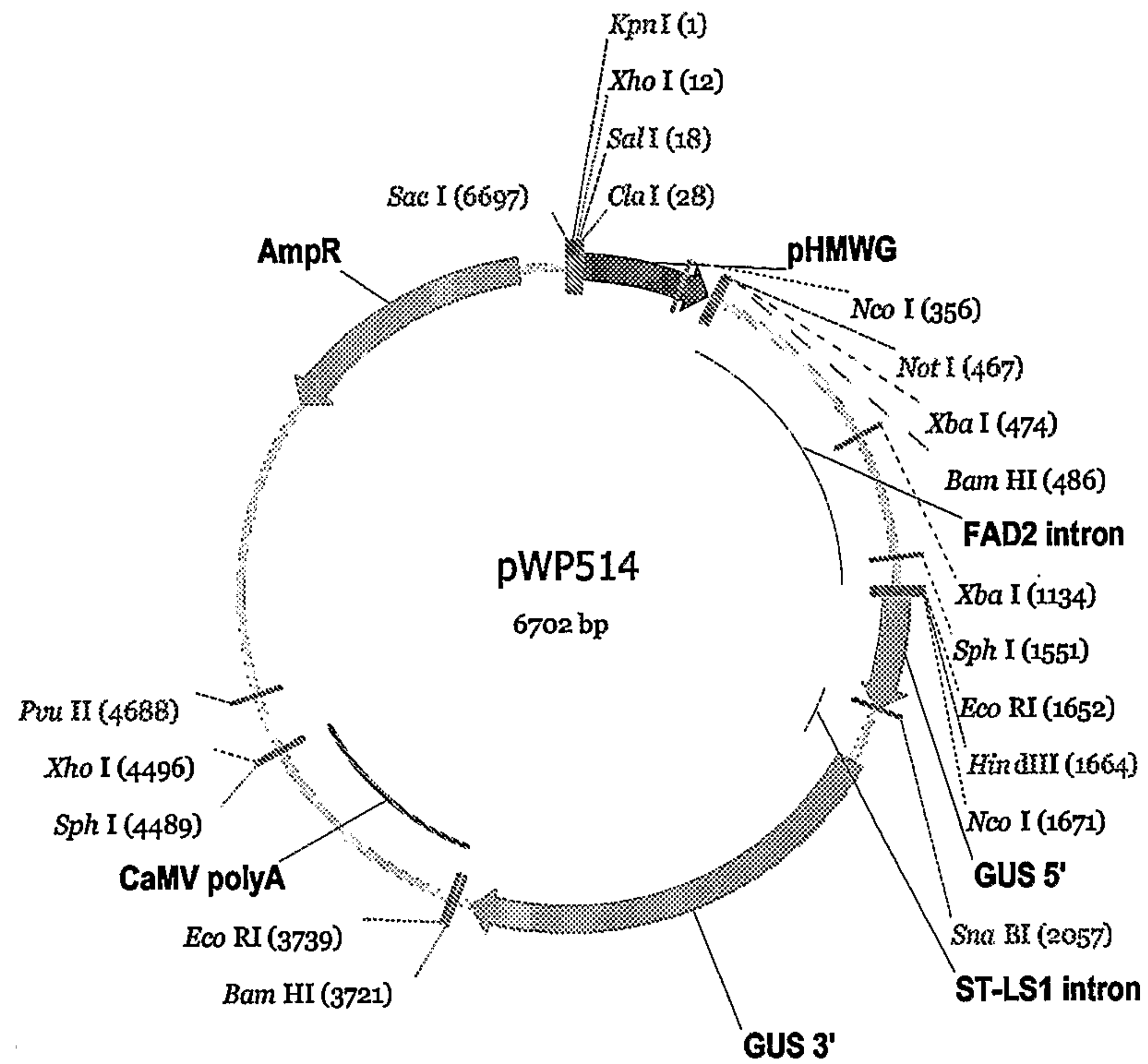
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**Figure 3****Figure 4**

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**Figure 5****Figure 6**

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**Figure 7**

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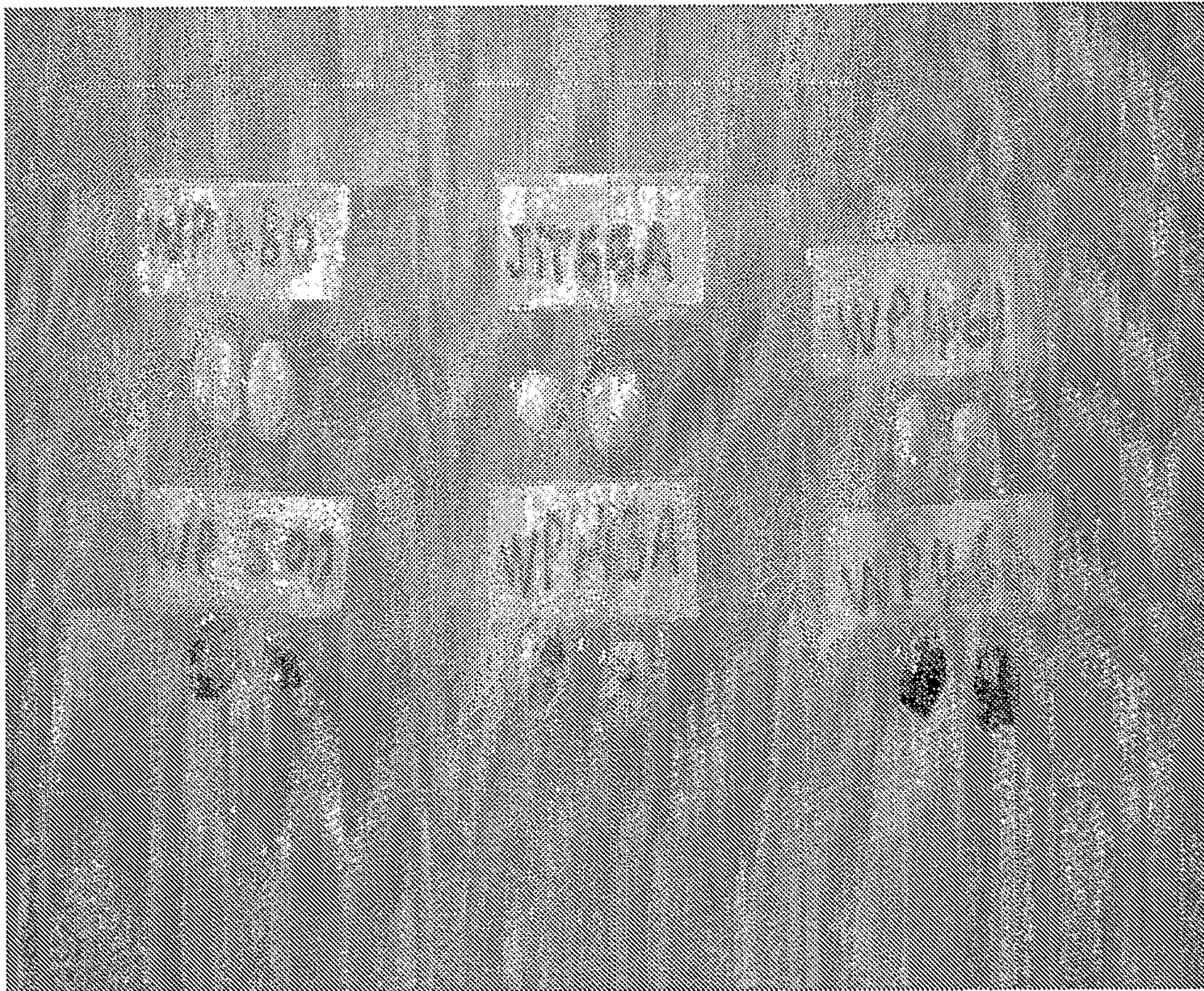


Figure 8

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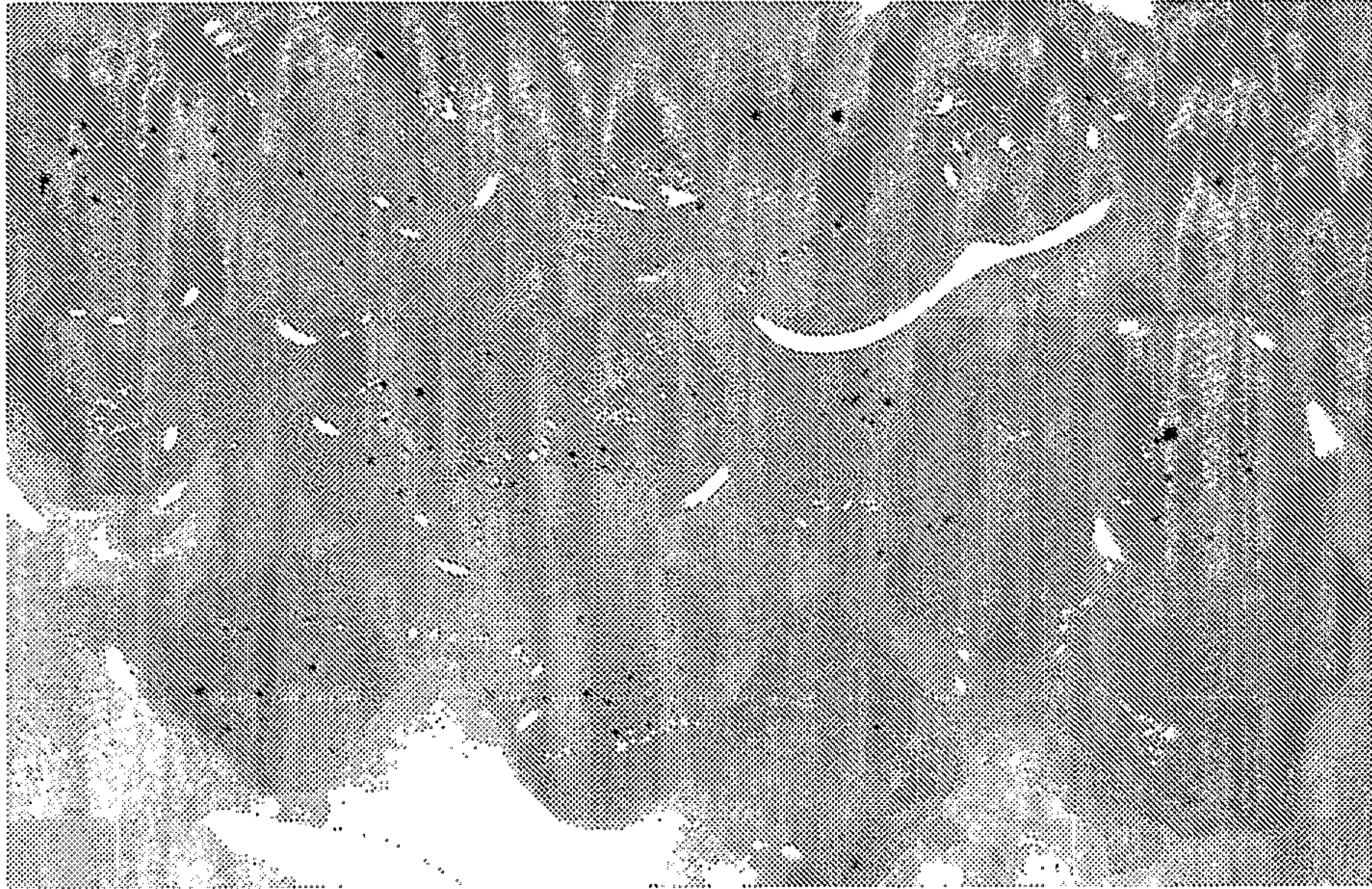


Figure 9a

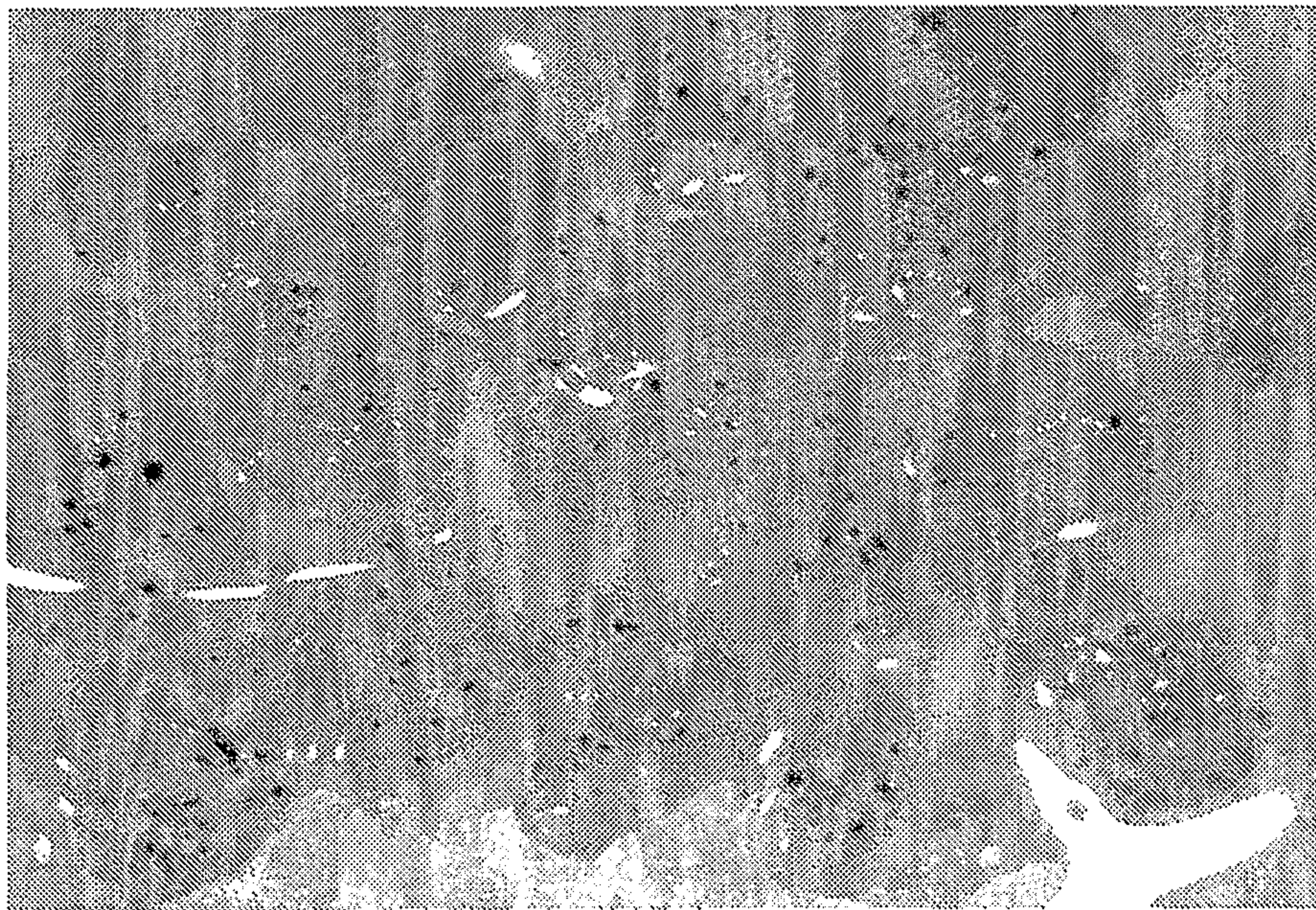


Figure 9b

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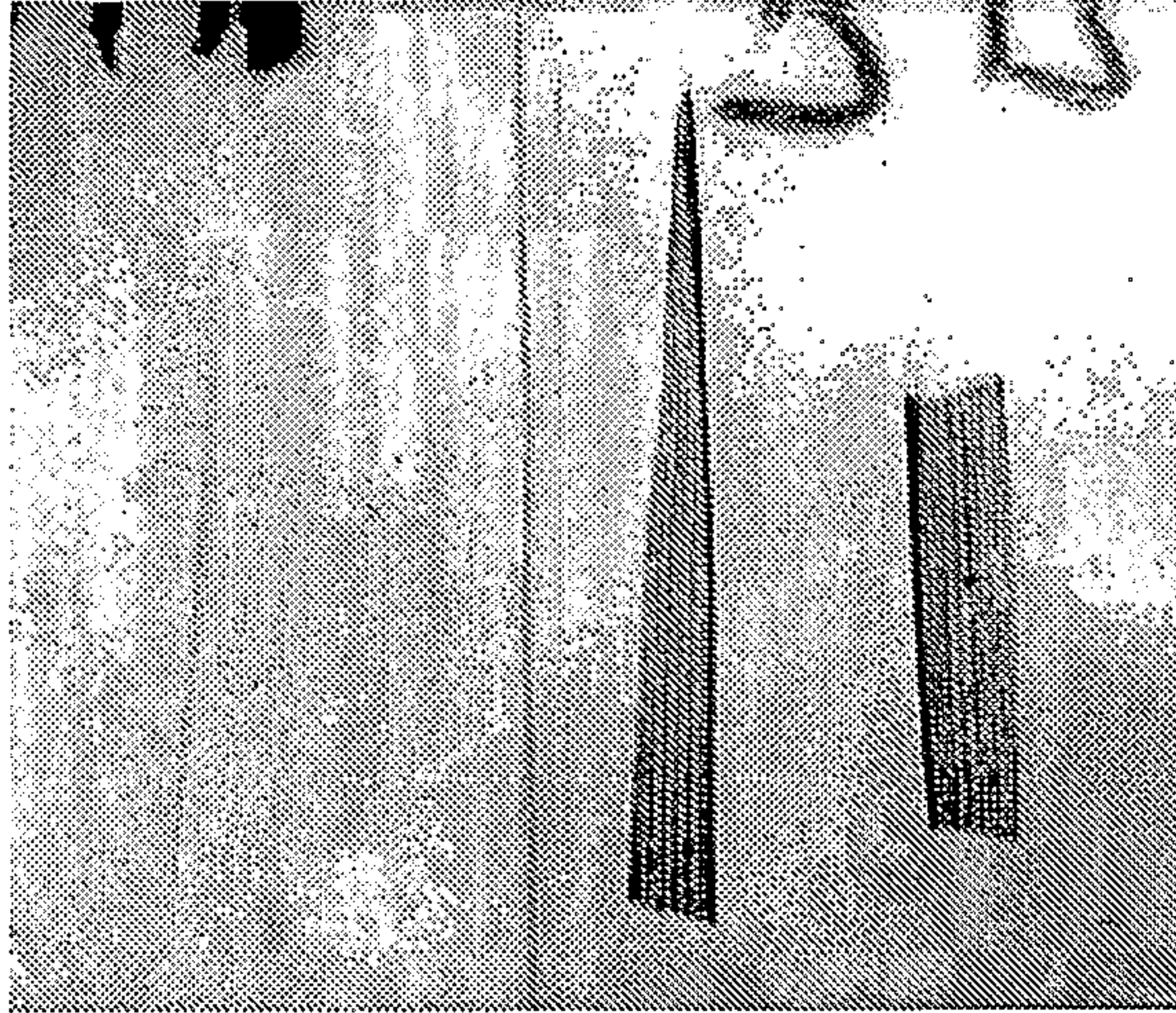


Figure 10