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June 6, 2019

Dr. Geoffrey Wong
Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: GRAS Notification for GraINzyme® Phytase produced by maize Event PY1203

Dear Dr. Wong,

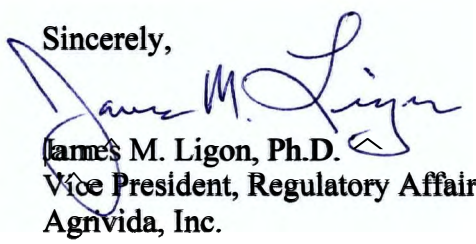
Under the Final Rule for the notification of self-determination of "Generally Recognized As Safe" (GRAS) for novel animal feed additives (21 CFR Parts 20, 25, 170 et al., Federal Register, vol. 81, No. 159, August 17, 2016) Agrivida, Inc. is hereby submitting a notification of the conclusion of Agrivida, Inc. that the 6-phytase enzyme, GraINzyme® Phytase produced by maize Event PY1203 is GRAS for use in the feed of poultry and swine. This enzyme releases phosphate groups from phytin and phytate that are present in plant based feed ingredients, thereby improving the availability of phosphorus in animal feeds. Previously, Agrivida, Inc. concluded that GraINzyme® Phytase produced by maize Event PY203 is GRAS for use in poultry and swine feed. Maize Event PY1203 produces a phytase enzyme that is identical to that produced by maize Event PY1203.

Based upon scientific procedures and information, Agrivida, Inc. had previously concluded that the use of GraINzyme® Phytase in poultry feed is GRAS and the Center for Veterinary Medicine has reviewed information supporting this conclusion and had no further questions (AGRN#21) related to this conclusion. Agrivida, Inc. has conducted further scientific investigation of the safety and functionality of the GraINzyme® Phytase in swine, the results of which support our conclusion of the GRAS status of this product for use in swine feed. A GRAS notice for the use of GraINzyme® Phytase in swine feed is currently under review by the U.S. FDA Center for Veterinary Medicine (AGRN#27).

Accompanying this letter is a compact disc (CD) with a file in PDF format that presents the data and information that supports Agrivida, Inc.'s conclusion on the GRAS status of GraINzyme® Phytase produced by maize Event PY1203. This document is a revised version of the earlier submission from Agrivida, Inc. that CVM refused to file based on deficiencies described in your letter of 12 March 2019. The deficiencies that you listed in your letter were addressed in this revised document. Also included on the CD are PDF files of the literature cited in the document that supports the scientific principles underlying our conclusions on the GRAS status of GraINzyme® Phytase produced by maize Event PY1203 for use in poultry and swine.

The complete data and original information that are the basis of this GRAS Notification are available to the Food and Drug Administration for review and copying upon request during normal business hours at our offices located at 78E Olympia Avenue, Medford, MA 01801.

Sincerely,



James M. Ligon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.

GraINzyme® Phytase

SAFETY AND FUNCTIONALITY OF PHY02 PHYTASE EXPRESSED IN MAIZE EVENT PY1203

SUMMARY of DATA SUPPORTING a NOTIFICATION of GRAS STATUS

Submitting Company:

Agrivida, Inc.
78E Olympia Ave
Woburn, MA 01801

May 31, 2019

This document was prepared by Jeffrey Stein and James Ligon

Please address correspondence related to this submission to:

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Executive Summary

Agrivida, Inc. has previously developed a new phytase feed enzyme product to improve phosphorus utilization in poultry and swine feeds. On May 6, 2016, Agrivida submitted to FDA a GRAS notice (AGRN 21) regarding the use of this new feed enzyme, designated Phy02 and marketed under the trade name of GraINzyme® Phytase and derived from maize Event PY203, in the feed of poultry [https://www.fda.gov/downloads/AnimalVeterinary/Products/AnimalFoodFeeds/GenerallyRecognizedasSafeGRASNotifications/UCM581398.pdf]. As part of this GRAS notice, Agrivida provided a report from a panel of independent experts that evaluated data and information from the literature and unpublished studies regarding the safety of this product. The GRAS panel concluded that ground corn grain containing Phy02 from Event PY203 is safe for its intended use in poultry feed. On May 23, 2017, FDA issued a letter stating it had no further questions regarding Agrivida's conclusion that ground grain derived from a corn variety expressing the altered *appA* phytase gene from *E.coli* K-12 is GRAS under its intended conditions of use.
[https://www.fda.gov/downloads/AnimalVeterinary/Products/AnimalFoodFeeds/GenerallyRecognizedasSafeGRASNotifications/UCM581397.pdf]. In 2018 Agrivida, Inc. submitted to FDA a second GRAS notice (AGRN 27) establishing the safety and functionality of the GraINzyme® Phytase in the feed of swine. This notice was accepted by FDA Center for Veterinary Medicine in a letter dated September 6, 2018.

Agrivida has developed a second maize transformation event, designated PY1203, that expresses the identical Phy02 protein as expressed in maize Event PY203. While the same *appA* phytase gene of *Escherichia coli* strain K12 has been introduced into this new maize event, the gene sequence has been codon optimized for maize, resulting in a much higher level of Phy02 expression. Event PY1203, produces 9,000 to 12,000 units of Phy02 phytase activity (FTU) per gram of grain, up to a 3-fold increase in Phy02 protein accumulation compared to Event PY203. Data presented in this submission shows that the Phy02 protein expressed in PY1203 is identical to the Phy02 protein expressed in Event PY203. The Phy02 product from Event PY1203 will be produced using common agronomic practices for the production of maize grain followed by milling to form a course meal. The Phy02 phytase product produced by maize Event PY1203 can be added to feed at a rate of 25 to 600g per ton of feed for poultry or 50 to 450g per ton of feed for swine to deliver an effective dose of phytase.

The *phy02* gene construct that was used in the Event PY1203 Agrobacterium-mediated transformation contains two copies of the codon optimized *phy02* gene, each under the control of a different monocot derived seed specific promoter. Characterization of the insert in Event PY1203 revealed that there are two complete copies of the T-DNA inserted at the same position in the maize genome. The complete DNA sequence of the insertion site including >10 kilobase pairs (kb) of flanking maize DNA was determined. No known maize genes were identified in the region of the insertion site. The absence in the maize genome of the antibiotic resistance gene and other elements of the transformation plasmid outside of the T-DNA was confirmed by Southern hybridization techniques. The stability of the two T-DNA inserts in Event PY1203 over multiple generations was also demonstrated.

The Phy02 phytase enzyme derived from Event PY1203 was characterized. Its molecular weight and amino acid sequence is identical to the previously approved Phy02 protein expressed in Event PY203, and the specific activity and kinetic properties of the Phy02 enzyme from both maize events are very similar.

Based on the above information that is supported by the data contained in this document, Agrivida, Inc. concludes that the Phy02 phytase expressed in Event PY1203 is identical to the Phy02 phytase expressed in Event PY203 and is therefore as safe and effective as the Phy02 phytase product derived from maize Event PY203 and that it is GRAS when used as intended in the feed of poultry and swine.

1.0 Signed statements and certification

1.1 Submission of a GRAS notice

Agrivida, Inc. is hereby submitting a GRAS notice in accordance with §170.225(c) of 21 CFR Parts 20, 25, 170 *et. al* (Federal Register, Vol. 81, No. 159, August 16, 2016) for a phytase enzyme produced in the grain of maize Event PY1203 for use in the feed of poultry and swine to improve the digestibility of phosphorus from phytic acid in the feed.

1.2 Name and address of notifier

Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801 USA
Tel: 781-391-1262

Person responsible for the dossier:
James Ligon, PhD
Agrivida, Inc.
VP, Regulatory Affairs and Stewardship
1023 Christopher Drive
Chapel Hill, NC 27517 USA
Tel: 919-675-6666; Email: jim.ligon@agrivida.com

1.3 Name of the notified substance

The substance that is the subject of this GRAS notice is a 6-phytase enzyme (E.C. 3.1.3.26) that is produced in the grain of *Zea mays*. The trade name of the phytase product is GraINzyme® Phytase.

1.4 Conditions of use of the notified substance

The GraINzyme® Phytase product produced by maize event PY203 is considered GRAS for use as a feed additive in the feed of poultry (AGRN #21, 2017) and a GRAS notice for its use in the feed of swine was submitted to the U.S. FDA Center for Veterinary Medicine (FDA/CVM) by Agrivida, Inc. and is currently under review. This GRAS notice is for the purpose of establishing the GRAS status of GraINzyme® Phytase that is produced by a different maize event herein referred to as maize Event PY1203 for inclusion in the feed of poultry and swine in order to increase the availability of phytin bound phosphorous in the feed. Maize event PY1203 produces approximately three-fold the amount of GraINzyme® Phytase that is produced by maize Event PY203. The recommended inclusion rate of the GraINzyme® Phytase produced by maize Event PY1203 is 25 – 600 g/ton of poultry feed to deliver a dose of 250 – 6000 FTU/kg and in swine feed it is 50 – 450 g/ton of feed to deliver a dose of 500 FTU to 4,500 FTU/kg feed where one FTU (phytase activity unit) is the amount of enzyme that releases 1 µmole of inorganic phosphorus per minute from phytate.

1.5 Statutory basis for conclusion of GRAS status

The conclusion that the GraINzyme® Phytase enzyme is GRAS for use in poultry and swine feeds is based on scientific procedures in accordance with §170.30(a) and (b) of 21 CFR Parts 20, 25, 170 *et. al* (Federal Register, Vol. 81, No. 159, August 16, 2016).

1.6 Substance is exempt from premarket approval

It is the opinion of Agrivida, Inc. that the GraINzyme® Phytase produced by maize Event PY1203 is exempt from the requirement for premarket approval under the Food, Drug and Cosmetic Act based on our conclusion that it is GRAS for its intended use in the feed of poultry and swine.

1.7 Data availability

The data that is the basis for the conclusion that the GraINzyme® Phytase produced by maize Event PY1203 is GRAS for its intended use will be made available to FDA either during or after its evaluation of the GRAS notice. Upon request of the FDA, Agrivida, Inc. will make all relevant data available for review or copying during customary business hours at its office in Woburn, MA. In addition, upon request by the FDA, Agrivida, Inc. will produce copies of requested information either in paper or suitable electronic form and provide these to the FDA.

1.8 Confidential business information in this GRAS notice

This document does not contain information that is considered by Agrivida, Inc. to be confidential business information.

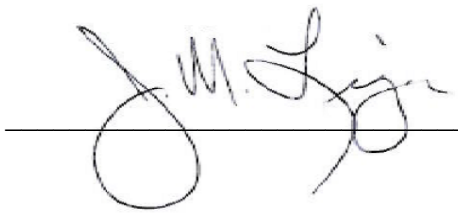
1.9 Certification

Agrivida, Inc. hereby certifies that to the best of its knowledge, this GRAS notice includes all relevant information, both favorable and unfavorable, that is pertinent to the safety and functionality of the GraINzyme® Phytase for its use in the feed of poultry and swine.

1.10 Signatory person

The following person will sign the GRAS notice on behalf of Agrivida, Inc.:

James M. Ligon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.



Date: May 31, 2019

1.11 Authorization to send trade secrets

If necessary, Agrivida, Inc. authorizes FDA Center for Veterinary Medicine to send information from this notification, including information considered by Agrivida, Inc. to be trade secret or CBI, to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture.

2.0 Identity, method of manufacture, specifications and technical effect

The identity, method of manufacture, specifications and technical effect of the GraINzyme® Phytase product is described in Part 2.0 of the GRAS notice for the use of GraINzyme® Phytase in the feed of swine that was submitted by Agrivida, Inc. to FDA/CVM and accepted for review in a letter from FDA/CVM dated September 6, 2018 (Animal Food GRAS Notice No. AGRN 27). Relevant information from this GRAS notice that is also applicable to the GraINzyme® Phytase produced by maize Event PY1203 as well as information from the Animal Food GRAS Notice No. AGRN 21 is incorporated herein by reference as specified in the sections below.

2.1 Identification of the notified substance

The identification of the notified substance is described in the GRAS notice for the use of GraINzyme® Phytase in the feed of swine Part 2, section 2.1, pg. 6.

2.2 Method of manufacture

The method of manufacture of GraINzyme® Phytase produced by maize Event PY1203 is described in the GRAS notice for the use of GraINzyme® Phytase in the feed of swine Part 2, section 2.2, pg. 6-7.

3.0 Target animal exposure and safety factor calculation

The target animal exposure and safety calculation for the use of GraINzyme® Phytase in poultry feed is described in the Animal Food GRAS Notice No. AGRN 21; section 4.3, pg. 35-38 and section 4.4; pg. 38-39.

Similar information on the target animal exposure and safety calculation for the use of GraINzyme® Phytase in swine feed is described in the GRAS notice for the use of this product in swine feed, Part 3.0, pg. 8-9.

4.0 Self-limiting levels of use

The self-limiting levels of use of GraINzyme® Phytase are described in the GRAS notice for the use of this product in swine feed (AGRN 27), Part 4.0, pg. 10-11.

5.0 Experience based on common use prior to 1958

The GraINzyme® Phytase product was not in use prior to 1958 and Agrivida, Inc.'s conclusion of GRAS status for the use of this product in swine feed is not based on its common use prior to 1958. Agrivida's conclusion that the GraINzyme® Phytase product is GRAS for use in poultry and swine feed is based on scientific principles. Therefore, the requirement to provide evidence of its use prior to 1958 is not applicable.

6.0 Safety and Functionality of the GraINzyme® Phytase in Poultry and Swine Feed

6.1. Safety of the maize production host

The safety of the maize production host is addressed in Animal Food GRAS Notice No. AGRN 21, section 4.0, pg. 31-39.

6.2 Safety of the Escherichia coli strain K12 gene donor

The safety of the *E. coli* strain K12 from which the *phy02* gene was isolated and derived through gene site saturation mutagenesis is addressed in Animal Food GRAS Notice No. AGRN 21, section 4.2.4, pg. 34-35.

6.3 Safety of the Phy02 phytase from Event PY1203 in poultry

The safety of the Phy02 phytase from Event PY203 in poultry is presented in Animal Food GRAS Notice No. AGRN 21, section 4.3, pg. 35-38. Since the Phy02 phytase that is produced in Event PY1203 is identical to that produced by Event PY203 the safety of the former is equivalent to that of the latter. The only difference in the use of the GraINzyme® Phytase product produced by maize Event PY1203 from that produced by maize Event PY203 is that the inclusion rate for the former will be decreased according to the measured phytase activity in the product to deliver a specified dose. Information about inclusion rates will be presented on the product label that accompanies the product.

6.4 Functionality of the GraINzyme® Phytase in Poultry and Swine

The functionality of the GraINzyme® Phytase product in poultry is described in the Animal Food GRAS Notice No. AGRN 21, section 5.0, pg 40-62 and its functionality in swine is described in the GRAS notice for use in swine (AGRN 27, section 6.7, pg 34-56).

6.5 Characteristics of the Phy02 Expression Construct of Event PY1203

The Phy02 phytase protein that is expressed in maize Event PY1203 is identical to the Phy02 phytase protein that is expressed in Event PY203 that is the subject of the Animal Food GRAS Notice No. AGRN 21. The expression construct containing the phytase genes in Event PY1203 is different from that used to create Event PY203. A transformation gene cassette (b) (4) was constructed containing two copies of the Phy02 phytase gene, each with a different monocot derived promoter. The genetic elements of plasmid (b) (4) are shown in Figure 1, and the individual genetic elements in plasmid (b) (4) are described in Table 1. This plasmid was introduced into maize by *Agrobacterium*-mediated transformation as described by Negrotto *et al*. (2000) and transformants were selected based on the presence of the plant selectable marker *manA* gene, as used in the selection of the Phy02 expressing Event PY203. Transformed maize plants were cultivated and the transformation event chosen as a development candidate was designated PY1203.

Figure 1. Diagram of (b) (4). See Table 1 for descriptions of individual genetic elements. Cleavage sites for the restriction enzyme *Bam*HI are indicated, as are nucleotide (nt) positions that are referenced elsewhere in this document

Table 1. Description of the genetic elements in the 16,596 bp plasmid (b) (4). Genetic elements highlighted in bold font are within the T-DNA borders.

Genetic Element	Description	Position*	Reference
(b) (4)			

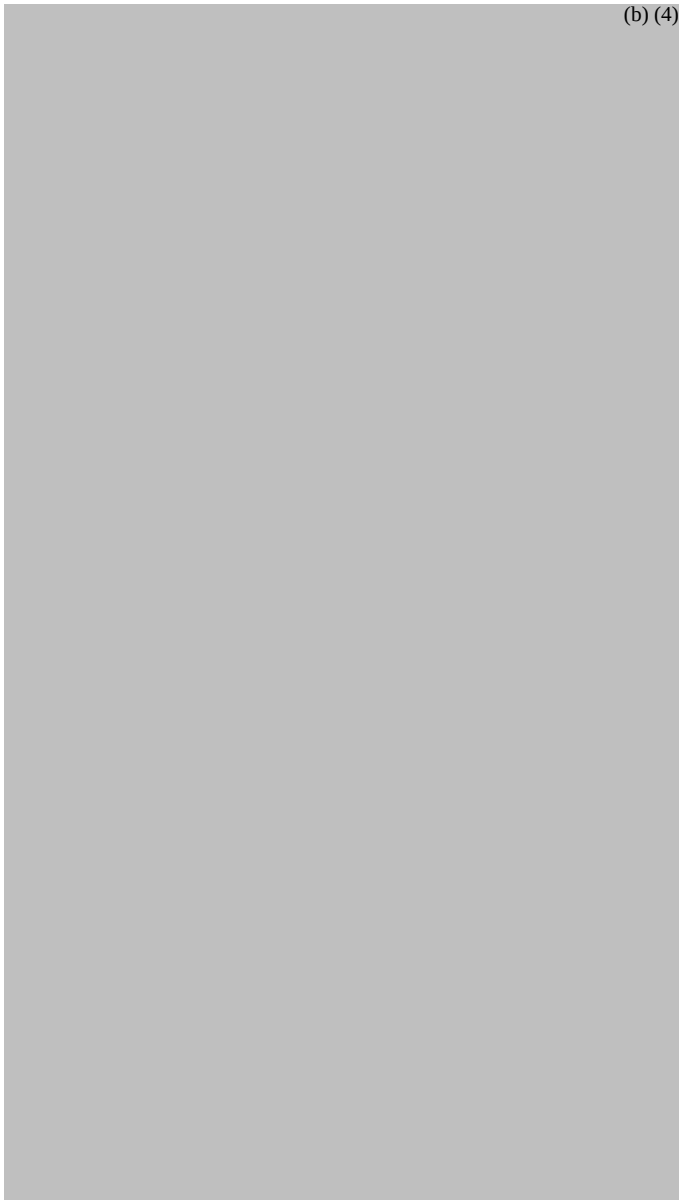

(b) (4)

Comparison Of Transformation Vectors (b) (4) and (b) (4)

The maize Event PY203 that is the production host for Phy02 phytase was previously reviewed by the Center for Veterinary Medicine and the Phy02 phytase product produced by it has GRAS status. Event PY203 was generated with the transformation vector (b) (4) (see GRAS Notice AGRN 21, Figure 3). Plasmid (b) (4), which was used to generate Event PY1203, is very similar to (b) (4) (Table 2). The most significant differences between these vectors are the following:

- (b) (4) lacks the third phytase expression cassette, which in (b) (4) was driven by the maize globulin promoter (ZmGlb1)
- The coding sequences for Phy02 have been optimized for improved expression in maize without changing the amino acid sequence of the mature Phy02 protein in (b) (4) relative to the amino acid sequence of the Phy02 protein encoded by the phy02 genes in (b) (4)
- Based on research conducted by Agrivida, Inc. that demonstrated that the (b) (4) improves expression levels in maize, (b) (4) codon was added in this position. Since the (b) (4) is part of the signal peptide, it is cleaved from the mature Phy02 phytase protein during transport into the ER.

Table 2. Comparison of features found in transformation vectors used to generate maize Event PY203 (b) (4) and Event PY1203 (b) (4)



Sequence Comparison Of T-DNAs From (b) (4) and (b) (4)

Differences in the DNA sequences of genetic elements common to both vectors (see Table 2) are confined to the Phy02 coding sequence in vector (b) (4) that has been optimized for expression in maize. There are no other sequence differences between (b) (4) and (b) (4).

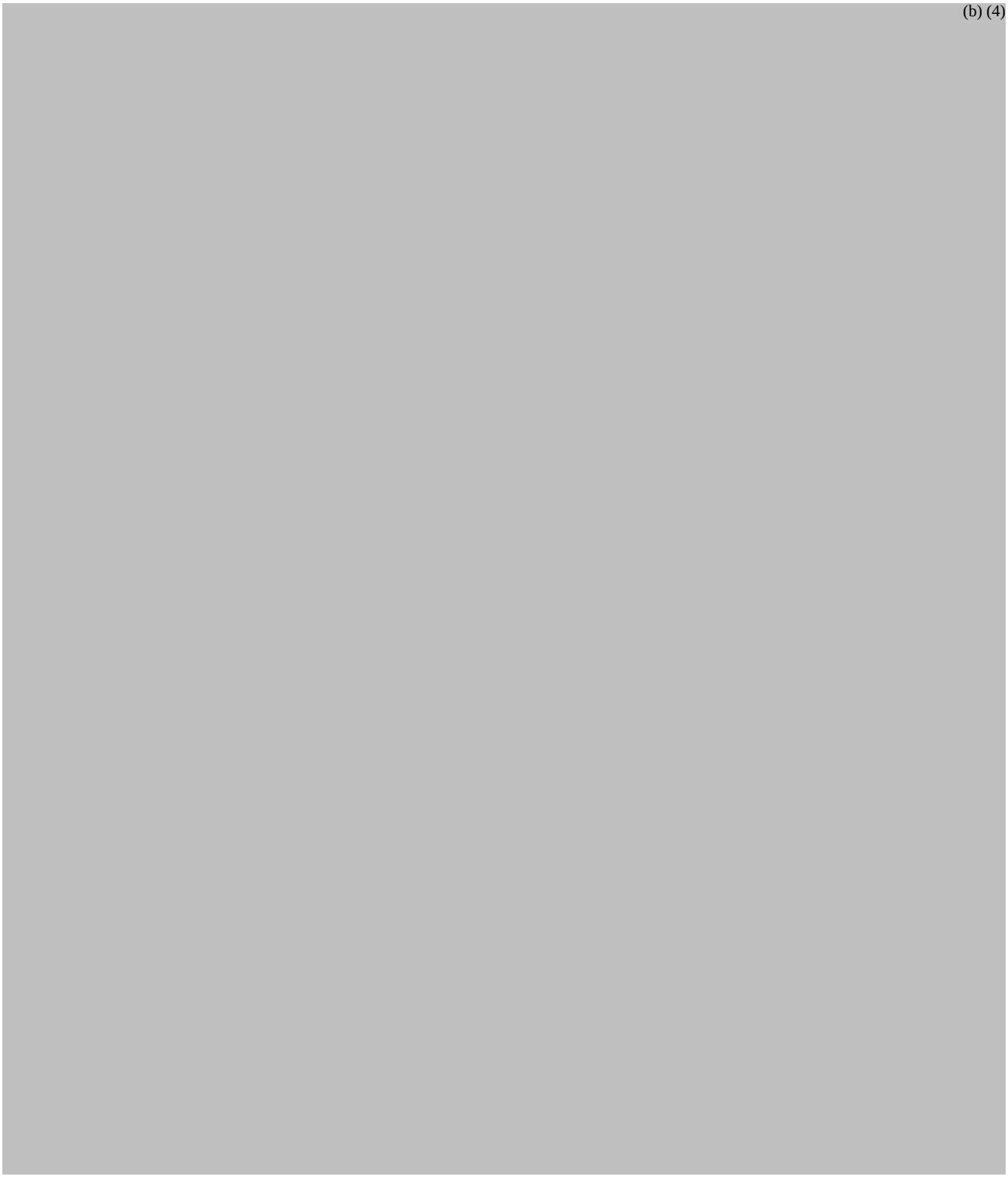
Figure 2 presents a DNA sequence alignment of the regions from (b) (4) and (b) (4) within the first expression cassette (adjacent to the (b) (4) promoter). This alignment shows that (b) (4) has (1) (b) (4) nucleotides relative to (b) (4) (as indicated by the

dashes) in the non-coding region (lowercase letters) upstream of the Phy02 coding sequence (uppercase letters), and (2) the intended nucleotide differences in the coding sequence of the Phy02 protein resulting from the codon optimization process. Translation and alignment of the polypeptides encoded by these sequences reveals that the only difference between them is (b) (4) (Figure 3) that is cleaved from the mature Phy02 protein during transport through the membrane of the endoplasmic reticulum. The presence of the (b) (4) residue in the signal sequence does not alter the amino acid sequence of the mature enzyme, as N-terminal sequencing of the phytase produced by Event PY1203 demonstrated that it is identical to that of the phytase produced by Event PY203 (see section 6.6.4).

Similarly, Figure 4 presents a DNA sequence alignment of the regions within the second expression cassette (adjacent to the (b) (4)). This alignment also shows that (b) (4) has (1) (b) (4) nucleotides relative to (b) (4) (as indicated by the dashes) in the non-coding region (lowercase letters) upstream of the Phy02 coding sequence (uppercase letters), and (2) the intended nucleotide differences in the coding sequence of the Phy02 protein resulting from the codon optimization process. Translation and alignment of the polypeptides encoded by these sequences reveals that the only difference between them is the addition (b) (4) (Figure 5) that is cleaved from the mature Phy02 protein.

In the alignment of the Phy02 coding sequences of both expression cassettes shown in Figures 2 and 4, (b) (4) These (b) (4) differences in sequence between the two cassettes does not result in any change in amino acid sequence in the Phy02 proteins produced from the two cassettes (Figure 5).

Figure 2. DNA sequence alignment of the regions from (b) (4) and (b) (4) that contain the coding sequence of the phytase expression cassette adjacent to the (b) (4) promoter. Lowercase letters indicate non-coding sequences. Dots in the lower line of each pair indicate identical nucleotides. Letters indicate nucleotides that differ. Dashes indicate gaps in one sequence relative to the other. In this figure, nucleotide (b) (4) corresponds to nucleotide (b) (4) in Figure 1. The *Bam*HI site is underlined.

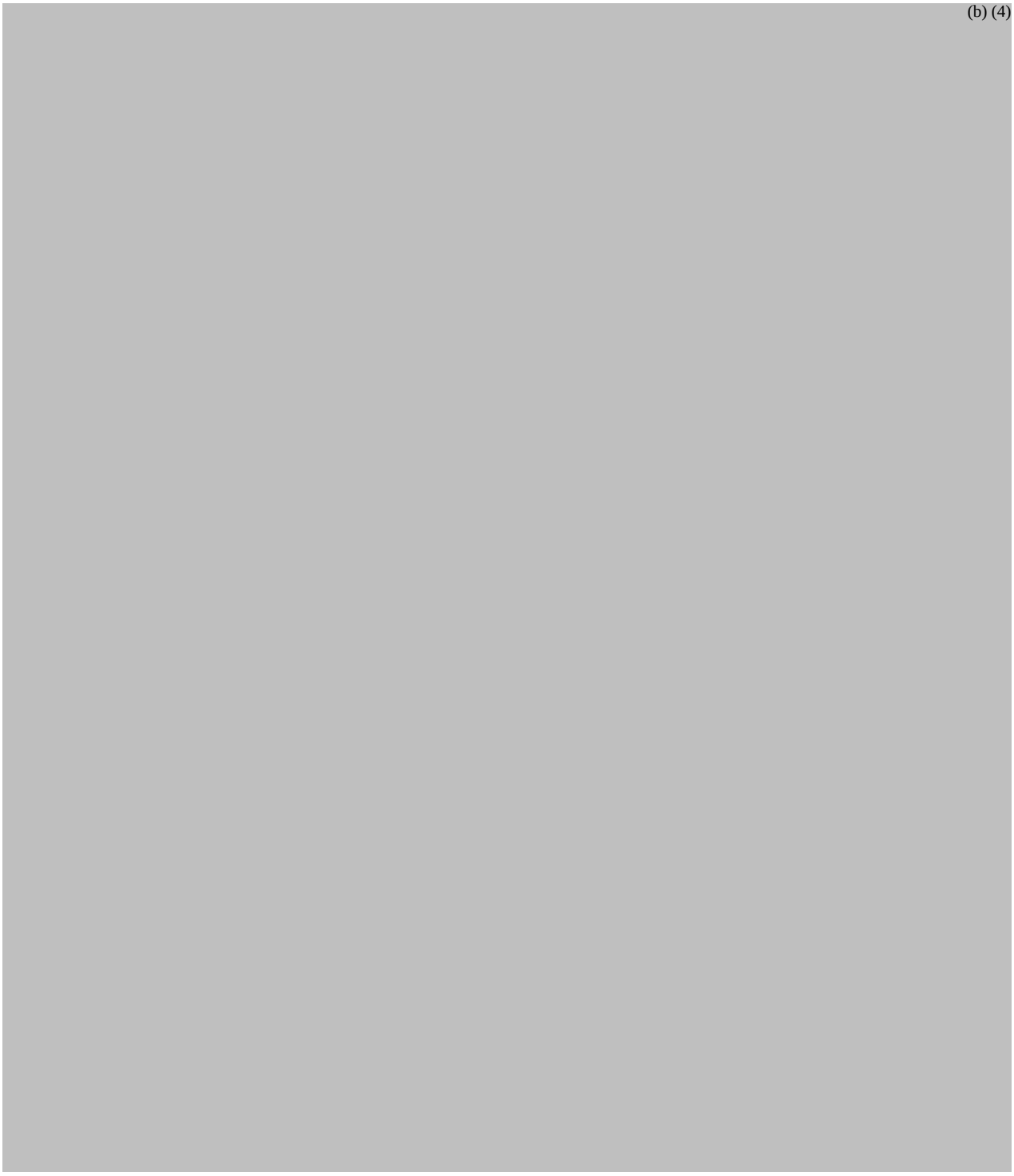



(b) (4)

Figure 3. Amino acid sequence alignment of the polypeptides encoded by the DNA sequences that are in Figure 2. The polypeptides differ only (b) (4) The endoplasmic retention signal at the C-terminus (SEKDEL) is underlined.

(b) (4)

Figure 4. DNA sequence alignment of the regions from (b) (4) and (b) (4) that contain the coding sequence of the phytase expression cassette adjacent to the (b) (4) promoter. Lowercase letters indicate non-coding sequences. Dots in the lower line of each pair indicate identical nucleotides. Letters indicate nucleotides that differ. Dashes indicate gaps in one sequence relative to the other. In this figure, nucleotide (b) (4) corresponds to nucleotide (b) (4) in Figure 1. The *Bam*HI site is underlined.




(b) (4)

Figure 5. Amino acid sequence alignment of the polypeptides encoded by the sequences that are indicated in Figure 4. Note, the polypeptides differ only (b) (4) The endoplasmic retention signal at the C-terminus (SEKDEL) is underlined.

(b) (4)

6.6 Characterization of the Insert in Event PY1203

6.6.1 Determination of Number of DNA Insertions

DNA sequencing, Southern blotting, and Mendelian inheritance analysis were used to characterize the T-DNA insert and surrounding maize genomic DNA in Event PY1203.

DNA Sequencing

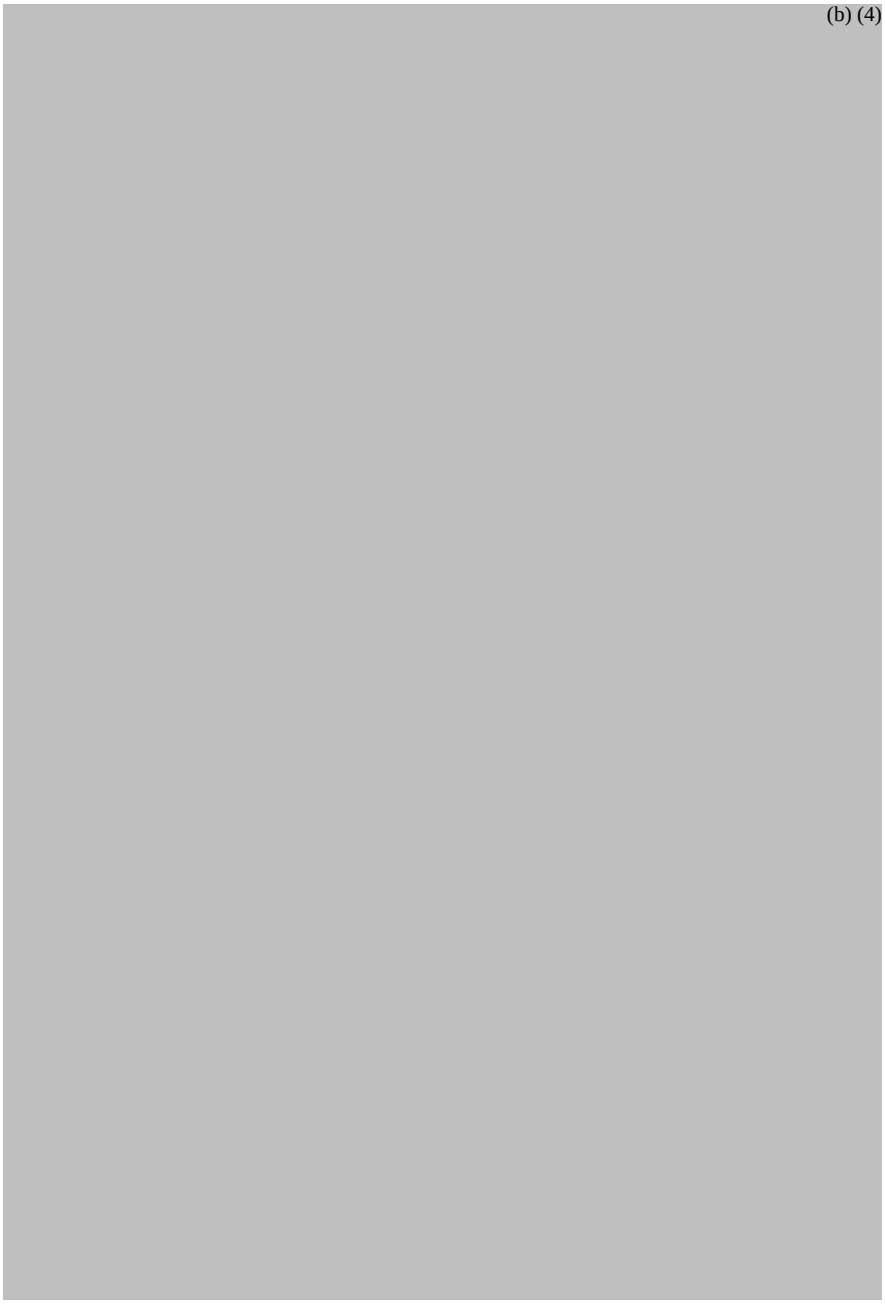
Combining several sequencing strategies such as target enrichment followed by PacBio sequencing, inverse PCR, long range PCR, and high-fidelity PCR for gap closure, the DNA sequence of the entire insert in Event PY1203 was elucidated and is presented in Appendix 1. The overall length of the PY1203 locus is (b) (4), including two complete T-DNA copies from the (b) (4) construct and maize genomic DNA flanks. The two T-DNAs in Event PY1203 are arranged as two inverted repeats and flanked by the maize genomic DNA sequences of (b) (4) (b) (4) at the "left side" of the insertion, as depicted in Figure 6, and (b) (4) (b) (4) at the right side of the insertion (Figure 6; Table 3). The left-most T-DNA ("T-DNA 1" in Figure 6) is composed of (b) (4) while the right most T-DNA ("T-DNA 2" in Figure 6) has a length of (b) (4). The (b) (4) sequence length difference between two T-DNAs is determined by differences in the amount of non-functional (spacer) sequence from the extreme ends of the T-DNA (up to and including the RB or LB) that was integrated at the locus. For example, the entire RB sequence was deleted from both copies of the inserted T-DNAs. The entire LB sequence as well as about (b) (4) of spacer sequence was deleted from the extreme left end of T-DNA 1 (as defined in Figure 6), and all but (b) (4) were deleted from the LB at the extreme right end of T-DNA 2.

The genomic organization of the PY1203 locus was confirmed by PCR and Southern Blot analyses. Analysis of nucleotide sequence identity between flanking sequences of the PY1203 locus and the reference maize genome B73 (version RefGen_v4, https://www.maizegdb.org) revealed that the T-DNA sequences were integrated into the maize genome between nucleotides (b) (4) on maize chromosome (b) (4) and displaced (b) (4) nucleotides of the wild type maize genomic DNA sequence. No additional vector sequences have been observed at this genetic locus or any other genomic locations as demonstrated by PCR, locus sequencing, and Southern Blot analyses. Furthermore, the PY1203 locus appeared to have no internal deletions or rearrangements of the genetic elements within the two integrated T-DNAs. The inserted T-DNA sequences are 100% identical to the T-DNA sequence of (b) (4), with the exception of two nucleotide substitutions (b) (4) and (b) (4), which occurred in a single codon of the (b) (4) selectable marker gene that is positioned on the T-DNA adjacent to the right maize genomic DNA flank. The identified mutations lead to a (b) (4) in the (b) (4) protein sequence. No annotated or predicted gene sequences were determined for the host genomic DNA sequence at the site of the T-DNA integration in the PY1203 locus.

Based on the recommendation of Ladics *et al*. (2011) that 30 amino acids is the minimum biologically relevant size of peptides capable of forming an allergenic epitope, the junction sequences between the maize genomic DNA flanks and the inserted T-DNAs at either side of the transgene were searched for the presence of new open reading frames (ORFs) of 30 or more amino acids. The results demonstrated that newly formed ORFs that span the junction sites and are equal to or greater than 30 amino acids were not formed at the junctions of the T-DNA and the maize genomic DNA.

Figure 6. Diagram of the T-DNA insertion locus within maize chromosome (b) (4) in Event PY1203. Descriptions and positions of individual genetic elements can be found in Table 3. For simplicity, (b) (4), and SEKDEL elements have been omitted from the figure. Cleavage positions for restriction enzymes *Hin*dIII, *Pme*I and *Pvu*II are indicated.

Table 3. Positions of key genetic elements within the PY1203 locus. The genetic elements are described in Table 1. The complete DNA sequence of this locus is presented in Appendix 1.



Southern Blot Analysis

Southern blotting provided further evidence of insert copy-number and demonstrated the stability of the T-DNA insertion locus over multiple generations. Genomic DNA was isolated from hemizygous plantlets from four consecutive generations of backcrossing into the "E" inbred variety (BC1E through BC4E; Figure 7). DNA was digested with either *Hind*III and *Pvu*II, to produce a single 21,937 bp fragment from the PY1203 locus, or *Hind*III, *Pme*I and *Pvu*II, to produce fragments of 11,288 and 10,628 bp from the PY1203 locus (see Figure 6). Following agarose gel electrophoresis, blots were hybridized with a 995 bp digoxigenin (DIG) labeled probe derived from the Phy02opt coding sequence (Table 4).

Table 4. Primers used to generate Phy02opt probe for Southern blotting

(b) (4)

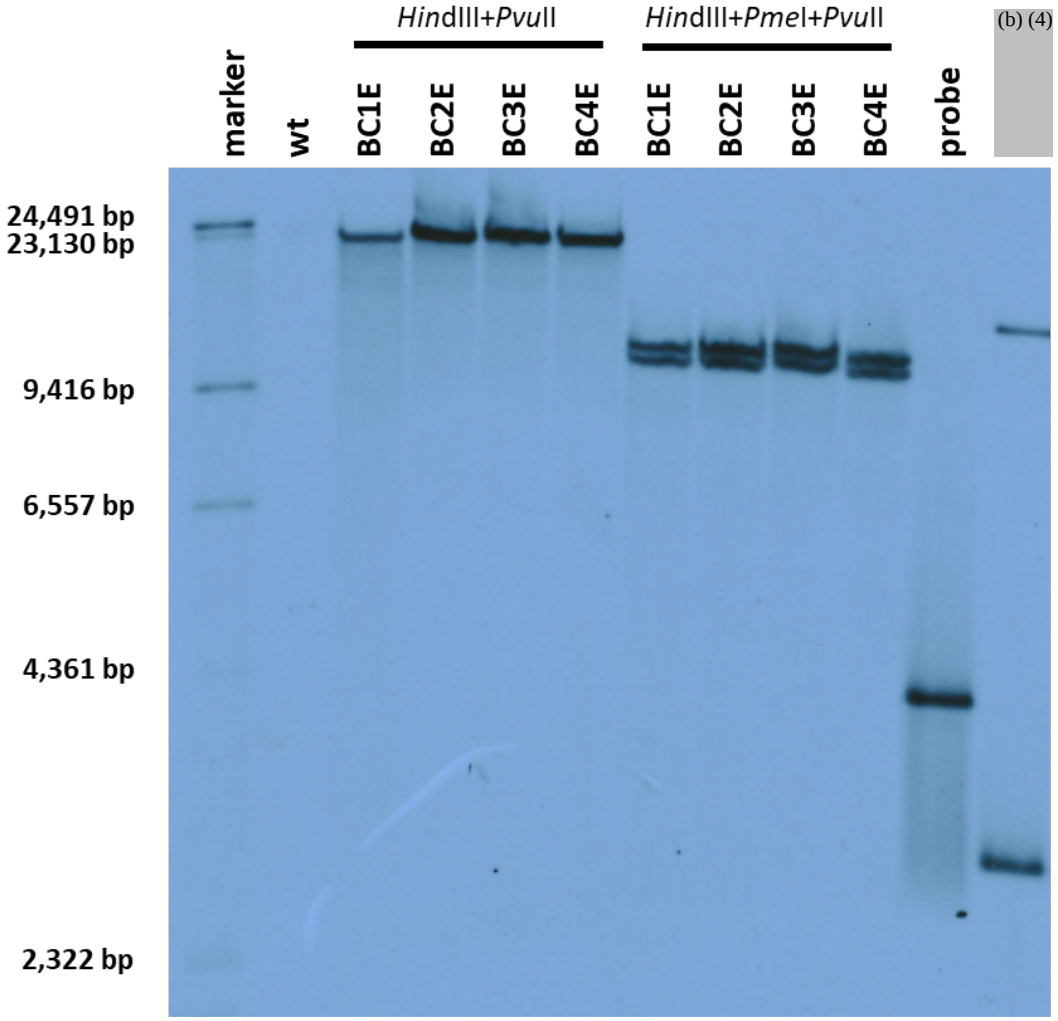
As shown in Figure 8, from each set of restriction enzyme digests, hybridizing fragments of identical size were evident across the four generations, indicating that (a) the two copies of T-DNA occupy a single locus in the genome of Event PY1203 (Figure 6), and (b) the two copies of the T-DNA are stably inherited as a single locus over 4 breeding generations.

Figure 7. Breeding diagram of PY1203 and progeny. (b) (4)

Multiple generations of continued backcrossing produced BC2E, BC3E and BC4E progeny.

(b) (4)

Figure 8. Southern blot of DNA derived from 4 backcross generations of Event PY1203. Marker: DIG-labeled molecular weight marker with corresponding sizes indicated to the left of the figure; wt: genomic DNA isolated from non-transgenic maize from inbred "E" cleaved with the restriction enzymes *Hind*III and *Pvu*II; BC1E-BC4E: genomic DNA from 4 generations of PY1203 plants, digested with either *Hind*III and *Pvu*II or with *Hind*III, *Pme*I and *Pvu*II, as indicated; probe: a positive control consisting of a linearized 3,586 bp DNA fragment carrying the Phy02opt coding sequence; (b) (4): the transformation vector (b) (4) digested with *Bam*HI, which produces fragments of 13,622 and 2,974 bp (see Figure 1).



6.6.2 Mendelian Inheritance of T-DNA Insert in Event PY1203

The inheritance of the T-DNA insertions in Event PY1203 was investigated by examining segregation ratios of the (b) (4) T-DNA element in progeny from an outcross between a hemizygous PY1203 plant and a non-transformed inbred (Figure 7). Genomic DNA was isolated from 50 seed from each of the 4 consecutive breeding generations (BC1E, BC2E, BC3E, BC4E). PCR primers that straddle the T-DNA/genomic DNA junction were used to detect the transgene in each DNA sample, while primers that lay down within the genomic DNA on either side of the T-DNA insertion site were used to detect presence of the wild type locus (i.e. absence of the T-DNA) (Figure 9).

Individual seed were scored as either "positive" (hemizygous) or null (wild type), as shown in Table 5. Chi squared analysis revealed that segregation of the transgene was very close to the expected 50:50 ratio (wherein a *p* value less than 0.05 would indicate divergence from the expected ratio).

Table 5. Segregation of the T-DNA in four successive generations of PY1203

Generation	Null	Positive	*p*
BC1E	25	25	1.00
BC2E	24	26	0.78
BC3E	25	25	1.00
BC4E	30	20	0.16

Figure 9. Diagram of PCR primers used to detect the presence or absence of T-DNA among progeny of Event PY1203. When the T-DNA is present, PCR with primers **a** and **b** produce a product of 86 bp. When the T-DNA is absent (the wild type locus), primers **a** and **b** will not generate a product. Conversely, primers **a** and **c** will generate a product of 119 bp with the wild type locus (absence of the T-DNA), while primers **a** and **c** will not produce a product when the T-DNA is present, as the ~21 kb of intervening sequence (the T-DNA insertion) is too large to synthesize a PCR product. When all three primers are used in a single reaction, PCR can distinguish hemizygous seed from nulls. Hemizygous ("positive") seed will produce PCR products of both 86 and 119 bp, corresponding to the transgenic and the wild type alleles, respectively. Null ("wild type") seed, however, carry only copies of the wild type allele, and PCR will produce only the corresponding 119 bp product.

6.6.3 Screening for plasmid backbone fragments

The absence in the genome of Event PY1203 of DNA fragments derived from outside of the T-DNA of vector (b) (4) was demonstrated by Southern blot analysis. Overlapping probes were generated from (b) (4) via PCR, which collectively represented the entire region of the plasmid vector from the LB to the RB, including the antibiotic resistance markers, *sat* and *aadA* genes, as well as the bacterial origin of replication and the *cos* site. The primers that were used to generate these PCR products that were used as probes are listed in Table 6, and the relative positions of these PCR products are depicted in Figure 10.

Genomic DNA samples from wild type (untransformed) *Z. mays* and Event PY1203 as well as the purified transformation vector (b) (4) were each digested with either *Bam*HI or *Eco*RI, separated via agarose gel electrophoresis, and subjected to Southern analysis using the PCR-generated DIG-labeled probes (Table 6). As

depicted in Figure 11, the probes readily detected the backbone fragments in lanes loaded with vector DNA (5, 6). The same probes, however, did not detect any fragments in either the wild type or Event PY1203 genomic DNA (lanes 1-4). These results indicate that no vector backbone-derived sequences are present in the genome of Event PY1203.

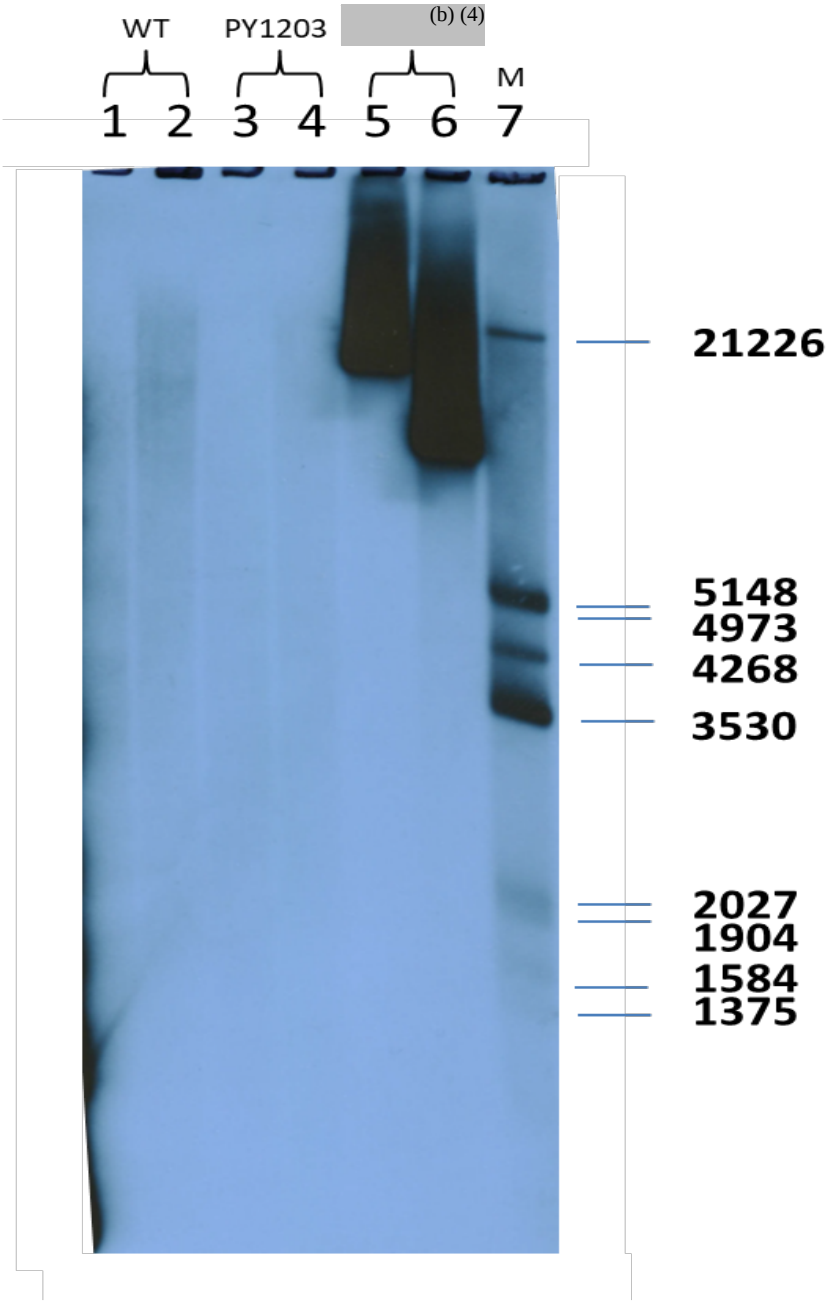
Table 6. PCR primers used to generate hybridization probes to detect presence of plasmid backbone sequences in Event PY1203.

Primer #	Sequence	From*	To*	sense	probe ID
	(b) (4)				

*Positions "from" and "to" are relative to the diagram of (b) (4) as shown in Figure 10. For reference, the LB corresponds to positions (b) (4) and the RB corresponds to positions (b) (4).

Figure 10. Positions of probes used for Southern blotting of vector backbone sequences. Probes a-f (red) were prepared by PCR, labeled with DIG, pooled, and used to probe genomic DNA derived from Event PY1203. Positions of *Eco*RI and *Bam*HI sites as well as the +1 position are indicated for reference.

Figure 11. Southern blot showing the lack of vector backbone-derived sequences in the genomic DNA of Event PY1203. Lane 1: wild type (WT) *Z. mays* DNA digested with *Bam*HI; lane 2: WT *Z. mays* DNA digested with *Eco*RI; lane 3: PY1203 DNA digested with *Bam*HI; lane 4: PY1203 DNA digested with *Eco*RI; lane 5: (b) (4) DNA, equivalent to one genome-copy equivalent, digested with *Bam*HI; lane 6: (b) (4) DNA, equivalent to one gene-copy equivalent, digested with *Eco*RI; lane 7: DIG-labeled molecular weight marker. The relative sizes (in base pairs) of the labeled fragments in lane 7 are indicated to the right.



6.6.4 Equivalence of Phy02 Phytase Protein Expressed in Maize Events PY203 and PY1203

Specific activity analysis

Phy02 protein extracted from maize grain derived from Event PY203 and Event PY1203 had phytase activities of 3437.88 ± 317.85 FTU/gram of flour and 11727.77 ± 767.64 FTU/gram of flour, respectively (Table 7). The amount of Phy02 protein quantified by ELISA from the protein extracts of PY203 and PY1203 were 8.06 ± 0.25 ug/mg of flour and 27.50 ± 1.67 ug/mg of flour (milled grain). The calculated phytase specific activities in grains of PY203 and PY1203 are listed in Table 7. While PY1203 grains had greater than three-fold higher Phy02 expression than PY203 grains, the expressed phytase had identical specific activity between these two events. The specific activity reported here for PY203 differs from that reported previously (AGRN 21). This difference reflects the fact that the earlier specific activities were measured as a function of the total protein present in aqueous extracts of milled grain, while the specific activities reported in Table 7 (below) are expressed as a function of the concentration of the Phy02 protein, specifically, as measured by ELISA. The latter method based on ELISA determination is more accurate.

Table 7. Specific activity of Phy02 phytase in grains of PY203 and PY1203

	Specific Activity	
	FTU per gram of milled grain	FTU per mg of Phy02 protein*
PY203	3437.88	426.58
PY1203	11727.77	426.48

*As determined by ELISA

Enzyme kinetic properties of Phy02 phytase from PY203 and PY1203 products

Enzyme kinetics of the Phy02 phytase from events PY203 and PY1203 were determined using protein extracts of milled grain. The K_m and other properties of the enzymes in these extracts were measured by testing phytase activity in the presence of different concentrations of phytic acid substrate, i.e. 0.11, 0.15, 0.23, 0.46, 0.91, 1.82, 4.55 and 9.1 mM within a reaction time of 20 minutes. Phytase protein was extracted from PY203 and PY1203 flour with sodium carbonate/bicarbonate buffer, pH 10.8 (w/v, 1/5) by shaking in flasks at 300 rpm for 60 minutes. Protein extracts were diluted such that the final phosphate released by phytase enzyme activity would fall within the linear range of detection of a phosphate standard curve. As shown in Table 8, V_{max}, K_m and K_{cat} values of Phy02 phytase from PY203 and PY1203 products were comparable. While the enzyme from PY1203 appears to have a slightly improved K_{cat}, the values for K_m/V_{max} of the enzymes from these two sources differ by only 2%.

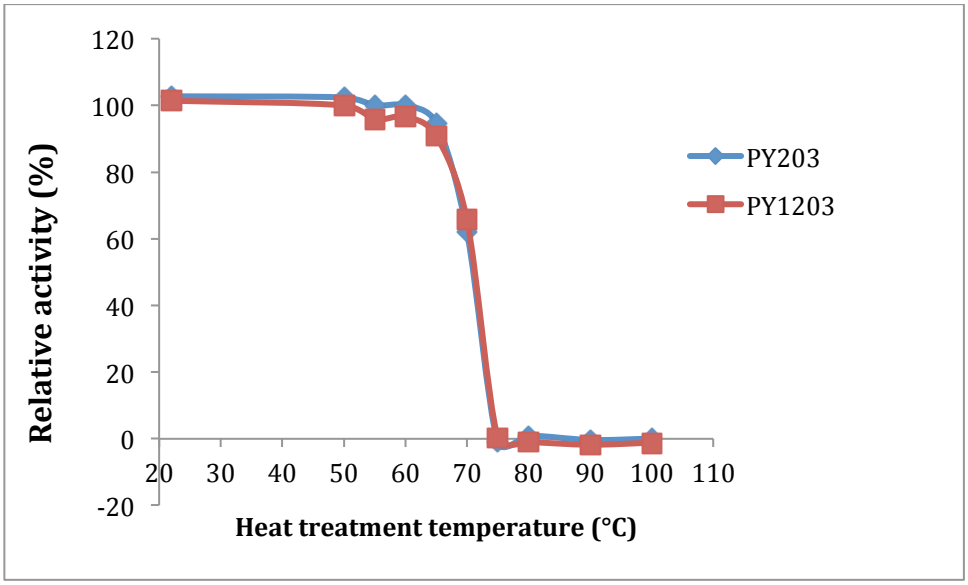
Table 8. Enzyme kinetic properties of Phy02 phytase derived from maize Events PY203 and PY1203

	PY203	PY1203
V_{max} (nmol/s)	0.12	0.14
K_m (uM)	605.23	686.19
K_m/V_{max}	4858.89	4963.24
K_{cat} (1/s)	258.90	345.33

Thermal stability of Phy02 phytase in extracts from PY203 and PY1203

The phytase activity in protein extracts from PY203 and PY1203 product was determined over a range of temperatures to determine the thermostability of the enzyme in solution. Protein extracts prepared from flour from each of the PY203 and PY1203 products were diluted 10-fold using phytase assay buffer. 400 µl of diluted protein was placed in a Thermo-Shaker MSC-100 at temperatures of 25, 50, 55, 60, 65, 70, 75, 80, 90, and 100°C. Heat treatment at each temperature was carried out for 5 min with shaking at 400 rpm. The temperature of the sample wells was checked using a Dual Channel Digital Thermometer (Fisher Scientific). After heat treatment and prior to analysis for phytase activity, the protein was further diluted in phytase assay buffer such that the final phosphate released by phytase enzyme activity would fall within the linear range of detection of a phosphate standard curve. The relative phytase activity of the Phy02 phytase in each of the PY203 and PY1203 product extracts at the different temperatures is presented in Figure 12. Phy02 in both PY203 and PY1203 product demonstrated above 97% activity at temperatures from 50 to 60°C relative to the temperature at which the highest activity was measured (25°C). About 91% and 95% activity remained following a 65°C heat treatment of the enzymes from PY203 and PY1203, respectively. Approximately 66% of the phytase activity remained following the 70°C heat treatment of both products. The activity rapidly decreased thereafter, and no significant activity remained following 5 minutes of incubation at or above 75°C. The results demonstrate that the phytase activity in the extracts from the PY203 and PY1203 products have nearly identical profiles over the range of temperature treatments.

Figure 12. Thermostability of Phy02 phytase in PY203 and PY1203 products

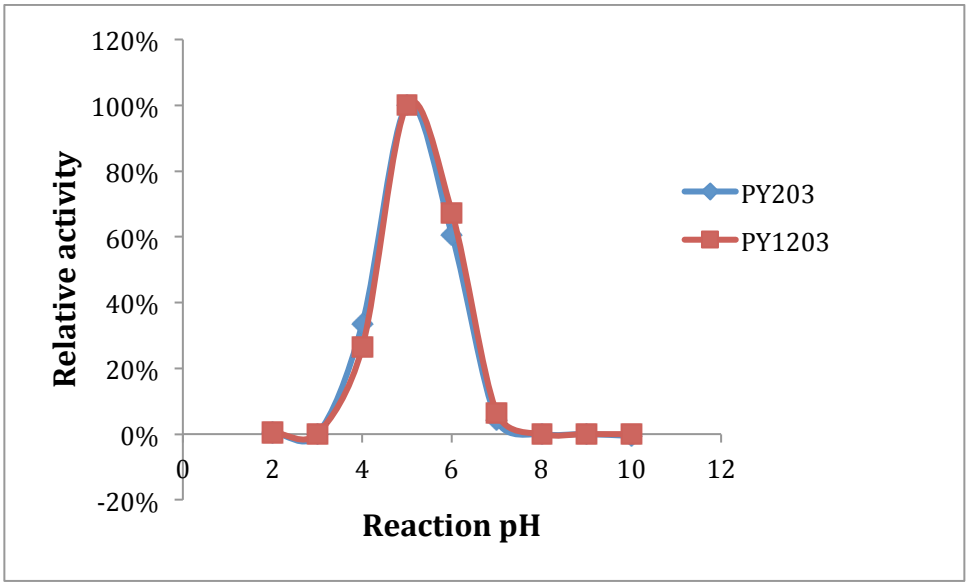


pH profile of Phy02 phytase derived from maize Events PY203 and PY1203

The phytase activity in protein extracts from PY203 and PY1203 products was compared over a range of pH to assess the optimal pH for phytase activity. The phytase enzymatic reactions were performed in 10x CCH (42.8 g/L citric acid, 92.1 g/L CHES, 79.4 g/L HEPES, pH 3) buffer that was diluted to 1x CCH buffer while adding either 1N HCl or 1N NaOH to adjust the pH from 2 to 10.

Extracts of flour from PY203 and PY1203 maize grain were diluted in 1x CCH buffer at each pH such that the amount of phosphate released by phytase activity from phytic acid substrate would be within the linear range of detection of a phosphate standard curve. Phytic acid substrate was prepared at a concentration of 9.1 mM and was dissolved in each of the 1x CCH buffers with different pH to ensure that upon mixing enzyme solution with the substrate the reaction pH did not change. Prior to analyses, the pH of the phytic acid substrate solution and each reaction buffer was verified with a standardized pH meter. Phytase reactions were initiated by adding diluted protein extract to the corresponding pH-adjusted substrate followed by incubation of the reaction mixtures for 60 minutes at 37°C. Reaction pH was monitored with colorpHast pH indicator strips (b) (4) following addition of enzyme**.** The results of the analyses of phytase activity are shown in Figure 13. The results demonstrate that the phytase activity in the extracts from the PY203 and PY1203 products have nearly identical activity profiles over the range of pH tested with highest activity at pH 5.0. About 67% and 25% phytase activity remained at pH 6 and pH 4 reaction condition for PY203 and PY1203 protein extracts, respectively. The phytase activity is lost rapidly and is absent at pH 3 and pH 8.

Figure 13. pH optima of Phy02 phytase derived from maize Events PY203 and PY1203



Molecular Weight and Immunoreactivity of Phy02 Phytase Derived from Maize Events PY203 and PY1203

Phy02 phytase expressed in grain derived from Event PY203 and Event PY1203 had the same apparent molecular weight of ~46 kDa (Figure 14). Further, Phy02 phytase expressed in both events also showed the same immunoreactivity to the polyclonal antibody raised against Phy02 protein (Figure 15).

Figure 14. SDS-PAGE coomassie stained gel of protein extracts from Events PY203 and PY1203. Lane 1: Positive control (PC), Phy02 phytase protein purified from PY203 corn grain; Lane 2: total protein extract from PY203 corn grain; Lane 3: total protein extract from PY1203 corn grain.

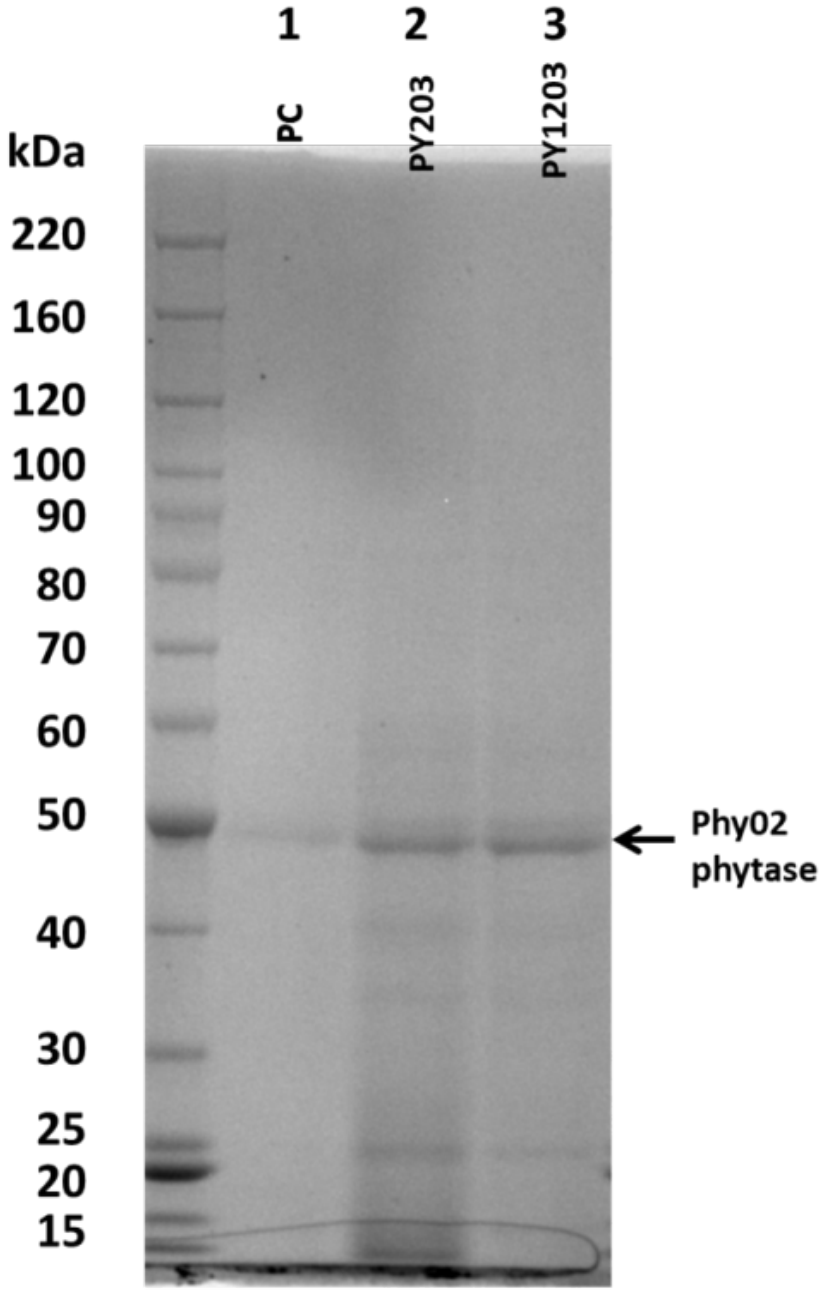
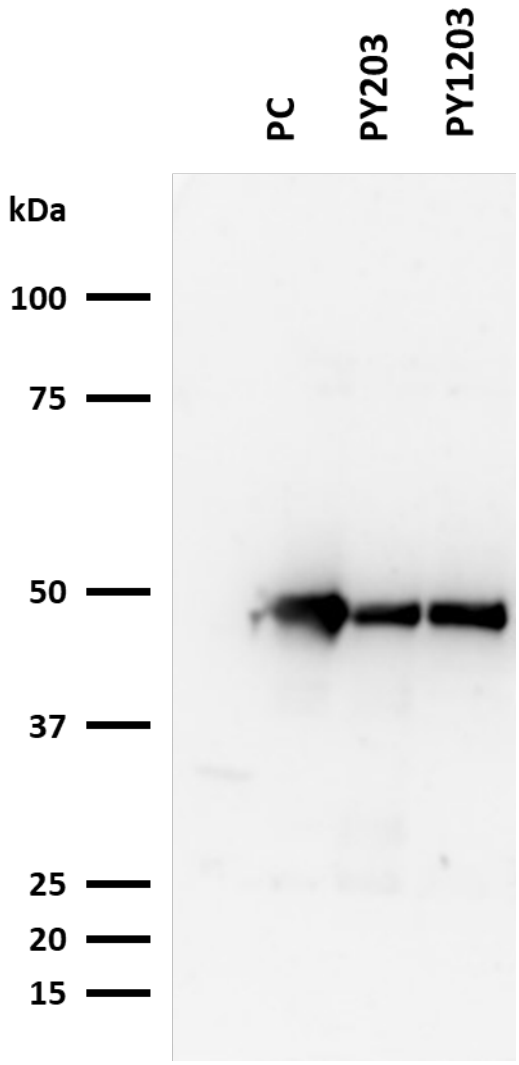


Figure 15. Western blot of protein extracted from Event PY203 and Event PY1203 using a polyclonal antibody against Phy02 phytase protein. Lane 1: PC, Phy02 phytase protein purified from PY203 corn grain; Lane 2: total protein extract from PY203 corn grain; Lane 3: total protein extract from PY1203 corn grain. Molecular weight markers: Precision Plus Protein™ Kaleidoscope™ Prestained Protein ladder (b) (4) loaded on the gel.



N-terminal sequence analysis of the Phy02 protein expressed in grain from Event PY1203

Phy02 protein from extracts of grain from maize Event PY1203 was subject to SDS-PAGE and transferred to a PVDF membrane that was stained with Coomassie Blue without heating to visualize the protein bands. The band corresponding to the

correct molecular weight of the Phy02 phytase was excised and the N-terminal amino acid sequence of the protein was determined by Edman degradation at (b) (4) (b) (4). The predicted cleavage site of the (b) (4) is immediately after the (b) (4) residue at position (b) (4) of the Phy02 phytase pre-protein (Figure 16) (b) (4) The results demonstrate that the (b) (4) at the N-terminus of the mature Phy02 phytase protein. The N-terminal amino acid sequence of the mature Phy02 phytase protein was shown to be either (b) (4) or (b) (4) (see Figure 16). In the case of the Phy02 phytase, it appears that the site of cleavage of the (b) (4) is not precise, and cleavage may occur between the two (b) (4) residues at positions (b) (4) to produce a mature protein that begins with the sequence (b) (4), but also between residues (b) (4) and (b) (4) to produce a mature protein that begins with the sequence (b) (4). These results confirm that the mature Phy02 phytase protein that is produced in the grain of maize has the N-terminal amino acid sequence that is expected from the coding sequence of the *phy02* gene with the exception of the slight variability due to variable cleavage of the (b) (4). The N-terminal amino acid sequence of the Phy02 protein from Event PY203 also demonstrated the identical variation in cleavage of the (b) (4) and thus the N-terminal amino acid sequence of the Phy02 phytases from grain of maize Events PY203 and PY1203 is identical (AGRN 21).

Figure 16. Comparison of the N-terminal sequences of the predicted Phy02 pre-protein (with the sequence of the (b) (4) underlined), and two forms of the mature Phy02 protein that were detected in extracts from grain of Event PY1203.

```
Phy02 pre-protein          (b) (4)
Phy02 mature protein #1
Phy02 mature protein #2
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6.7 Product Characterization

Three separate representative product batches of the Phy02 phytase were produced from grain of maize Event PY1203. Planting the seed, cultivation of the maize, and harvest of the grain were performed using commonly used agronomic practices for maize. Cultivation of the Phy02 producing maize PY1203 also utilized common agronomic practices for maize including the use of fertilizers, herbicides and pesticides approved for use on maize. After harvest, the grain was dried on the cob for three days until the grain moisture was below 15% at which time it was shelled and placed in labeled containers. The grain was shipped to Agrivida, Inc. (Medford, MA) and stored in separate storage bins prior to being milled in a Retsch SM100 cutting mill.

Each of the three representative Phy02 phytase product batches of Event PY1203 were analyzed to demonstrate that they meet the purity, chemical and microbial specifications established for enzyme preparations, as outlined in the specifications established for enzymes used in food processing, as proposed by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO 2006). Physical, chemical, and microbial characteristics were determined for each of the Phy02 phytase product batches by (b) (4) The results of these analyses are presented in Table 9.

Examination of the results of the analysis of key product characteristics as presented in Table 9 demonstrate that all three Phy02 phytase product batches meet or exceed all JECFA specifications established for enzyme preparations that are used in food and/or feed with the exception of total bacterial count and the number of coliform colony forming units (cfu). All three product batches had no detectible presence of either *Salmonella* or *E. coli* bacteria. Coliform bacteria are defined as rod-shaped Gram negative, non-spore forming, motile or non-motile, bacteria that can ferment lactose with the production of acid and gas when incubated at 35–37°C (Brenner, 1992; Bettelheim, 1992). While coliforms themselves are not normally causes of serious illness, their presence has been used to indicate that other pathogenic organisms of fecal origin may be present (Krentz *et al*., 2013). Typical genera in the coliform group include: *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, and *Escherichia* (Brenner, 1992; Bettelheim, 1992).

The JECFA specifications for food enzyme preparations have been traditionally applied to enzyme products that are produced by sterile fermentation followed by purification of the enzyme in a sanitary laboratory environment. Under these conditions it is feasible to produce a purified enzyme product that meets the JECFA specifications for the presence of microbes in the product. However, the Phy02 phytase product is produced in the same manner as the production of maize grain that is widely used as a major component of human food and animal feed. It is produced in agricultural fields in the environment where bacteria are present in the soil, air and water and on the surfaces of plants, including the maize that produces the Phy02 phytase containing grain. Therefore it is reasonable to expect that the Phy02 phytase product would contain levels of bacterial presence that is typical for maize grain produced by typical agricultural practices. All of the three Phy02 phytase product batches exceeded the JECFA specification of 30 cfu/g product for coliform bacteria with coliform numbers of 10,000, >200,000 and 4,500/g (Table 9). However, these numbers are consistent with studies of microbial presence in maize grain and in animal feed. Tabib *et al*. (1981) surveyed feeds and feed ingredients, including maize, in the feed of broilers, layers and turkeys and found that the numbers of coliform bacteria ranged from 450 – 910,000 cfu/g. Similar studies have also reported equivalent levels of coliform bacteria in cattle feed (Sanderson *et al*., 2005) and tortillas made from corn meal (Gomez-Aldapa *et al*., 2013). From these reports it is evident that the level of coliform bacteria in the three Phy02 product batches is similar to those reported as normal for maize grain and other commonly

used feed ingredients. Since the numbers of coliform bacteria found to be present in two of the three Phy02 phytase product batches are typical for those found in maize grain and other animal feed ingredients and since known pathogenic bacteria such as *Salmonella* and *E. coli* were absent from the product batches, the higher level of coliforms in the Phy02 product compared to the JECFA specifications for food enzyme products is considered to be safe. Similar results for coliforms and total bacterial count were also demonstrated for Phy02 phytase produced by maize Event PY203 (Animal Food GRAS Notice No. AGRN 21, section 6.0, pg. 63).

6.8 Safety of human consumption of meat produced by animals treated with GraINzyme® Phytase

The safety of meat that is produced by animals treated with GraINzyme® Phytase and consumed by humans is addressed in the GRAS notice for use in swine (AGRN 21; section 6.3, pg. 20).

Table 9. Physical, chemical, and microbial characteristics of three independent Phy02 phytase product batches produced by maize Event PY1203 compared to JECFA specifications (JECFA, 2006) for enzyme preparations used in food and feed. Values preceded by "<" indicate that these were below the lower limit of quantitation of the assay.

			Event PY1203 Phy02 Phytase Product Batch			
	Method	**Unit**	**AV_Phy02_GPS**	**AV_Phy02_EPS**	**AV_Phy02_OHG**	**JECFA Specification Limit**
Physical Characteristics						
Phytase Activity	Agrivida, Inc. SOP	FTU/g			(b) (4)	NA
Density	USP 616	g/ml	0.5	0.5	0.6	NA
Micron particle size	MF-2051 Evaluating Particle Size, KSU 2002	um	632	624	584	NA
Chemical Characteristics						
Cadmium	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	<0.010	30 max
Mercury	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	<0.010	30 max
Lead	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	<0.010	5 max
Arsenic	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	0.022	3 max
Microbial Characteristics						
Coliforms	AOAC 991.14	cfu/g	10,000	>200,000*	4,500	30 max
Salmonella	AOAC 2003.09	#/25g	negative	negative	negative	Absent
Aerobic Plate Count	BAM Chapter 3	cfu/g	130,000*	>570,000*	5,700	50,000 max
E. coli	U.S. Pharmacopeia Chapter 62	#/10g	negative	negative	negative	Absent
Mycotoxins						
Aflatoxin	Commercial Test Kit (ELISA)	ppb	<5	<5	<5	Nondetectible
T-2 Toxin	Commercial Test Kit (ELISA)	ppb	<25	<25	<25	Nondetectible
Ochratoxin	Commercial Test Kit (LC-MS)	ppb	<1	<1	<1	Nondetectible
Sterigmatocystin	(b) (4)	ug/kg	<10	<10	<10	Nondetectible

*Estimated

6.9 Product Stability

The characteristics of the Phy02 phytase enzyme that is produced in the grain of maize Events PY203 and PY1203 are nearly identical. The specific activities and enzyme kinetic properties of the Phy02 phytase derived from grain of maize Events PY203 and PY1203 are nearly identical (section 6.6.4). The profiles of phytase activity of the Phy02 phytase from the two maize sources at different temperatures and pHs are also identical (section 6.6.4). The molecular weights of the Phy02 phytases and their immunoreactivity with Phy02 specific antibody are identical, as is the N-terminal amino acid sequence. The analyzed product characteristics of three typical product batches of the Phy02 phytases from maize Events PY203 and PY1203, including product density and chemical and microbial characteristics also demonstrate that the products from the different maize events share the same product characteristics (section 6.4.5 and section 6.0, pg. 62-64 of AGRN 21). Other than the higher concentration of Phy02 phytase produced in grain of maize Event PY1203 compared to grain produced by maize Event PY203 that is the subject of previous GRAS notices for the use of the Phy02 phytase in poultry feed (AGRN 21) and swine feed (AGRN 27), the Phy02 products derived from the grain of Events PY203 and 1203 are identical. The GraINzyme® Phytase product produced by maize Event PY203 was demonstrated to maintain good product stability for up to 3 months at ambient temperatures (section 7.1, pg. 65-66 of AGRN 21). Agrivida, Inc. continued this stability study and demonstrated that the Phy02 phytase produced in grain of Event PY203 maintains up to 80% of its initial activity after 18 months of storage at ambient temperatures. The GraINzyme® Phytase product was also demonstrated to maintain 98% of its activity in feed mixtures after 10 weeks of storage at ambient temperatures (section 7.3, pg. 69-72 of AGRN 21). The stability of the phytase activity in GraINzyme® Phytase product produced by maize Event PY203 was demonstrated to be tolerant to high temperatures experienced by the treated feed during typical pelletizing operations. GraINzyme® Phytase had 76% of its original activity after pelleting at 90°C (section 7.4, pg. 72-76 of AGRN 21).

Since the Phy02 phytase enzyme produced in the grain of maize Event PY1203 is identical in enzyme kinetic and product characteristics to the Phy02 phytase produced in the grain of Event PY203, and since the Phy02 phytase is packaged in the same environment in the grain from both maize events (e.g., in the endosperm of the maize grain), it is reasonable to conclude that the Phy02 phytase in the grain of Event PY1203 is equally as stable as it is in the grain of Event PY203. Based upon this Agrivida, Inc. concludes that the Phy02 phytase activity of product derived from maize Event PY1203 is as stable as the Phy02 phytase activity from grain of Event PY203.

6.10 Other information relevant to this GRAS notice

As required by 21 CFR 570.250(c), Agrivida, Inc. hereby certifies that to the best of its knowledge, this GRAS notice includes all relevant information, both favorable and

unfavorable, that is pertinent to the safety and functionality of the GraINzyme® Phytase for its use in the feed of poultry and swine. Agrivida, Inc. is not aware of any other relevant information regarding the safety or functionality of the Phy02 phytase from maize Event PY1203 that is contradictory to the information presented herein.

6.11 Summary

Maize Event PY1203 was genetically engineered to produce the Phy02 phytase enzyme that is identical to that produced by maize Event PY203. In order to increase the expression of the Phy02 genes in maize, the genes of the (b) (4) expression construct that encode the Phy02 phytase were codon optimized to reflect the codon preference of maize without changing the amino acid coding sequence. In addition, to further increase expression, a (b) (4) codon was inserted at the end of the (b) (4) that mediates the transport of the Phy02 phytase protein into the endoplasmic reticulum. Since this signal sequence is cleaved during transport through the membrane of the endoplasmic reticulum, this (b) (4) residue is not retained in the mature Phy02 protein. The amount of Phy02 phytase produced by Event PY1203 was measured and was determined to be 10000 to 12000 FTU/g of maize flour, approximately three fold the level produced by Event PY203. The specific activity, apparent molecular weight and immunoreactivity of the Phy02 phytase from Events PY203 and PY1203 were compared. The Phy02 phytase produced by Event PY1203 was demonstrated to be identical in apparent molecular weight, specific activity, and enzyme kinetic parameters to the Phy02 phytase produced by Event PY203 and it reacted equally with an antibody raised against the Phy02 produced by Event PY203. The Phy02 phytases produced by Event PY203 and PY1203 had nearly identical activity profiles over a range of temperatures and pH. These findings demonstrate that the Phy02 phytase produced by both Events PY203 and PY1203 are identical to one another.

The nucleotide sequence of the T-DNA insertion of Event PY1203 and flanking maize genomic DNA was determined and analyzed. The analysis determined that the T-DNA insertion was in maize chromosome (b) (4) and that it consisted of an inverted repeat of two T-DNAs derived from (b) (4) The region of maize chromosome (b) (4) where the T-DNA inserted contained no known maize genes and no putative ORFs were created at the junctions of the T-DNA and maize genomic DNA. The T-DNA insertion of Event PY1203 was shown to be stable and to be inherited in a Mendelian manner over multiple generations. Finally, it was demonstrated by Southern hybridization that genetic elements derived from the transformation construct (b) (4) outside the T-DNA borders are absent from the genome of Event PY1203.

The Phy02 phytase that is produced by maize Event PY203 is GRAS for use in the feed of poultry to improve the availability of phosphorus (AGRN 21). Agrivida, Inc. has determined that the Phy02 phytase produced by Event PY203 is also GRAS for the same purpose when included in the feed of swine and the GRAS notice supporting this conclusion (AGRN 27) is currently under review by FDA/CVM. Agrivida, Inc. has

herein demonstrated that the Phy02 phytase produced by Event PY1203 is identical to the Phy02 phytase produced by Event PY203 and that the molecular characterization of the T-DNA of the former revealed no concerns of safety. Based on the fact that the Phy02 phytase produced by Events PY203 and PY1203 are identical, Agrivida, Inc. concludes that the Phy02 phytase produced by maize Event PY1203 is also GRAS for use in poultry and swine feeds to improve the availability of phosphorus.

7.0 References

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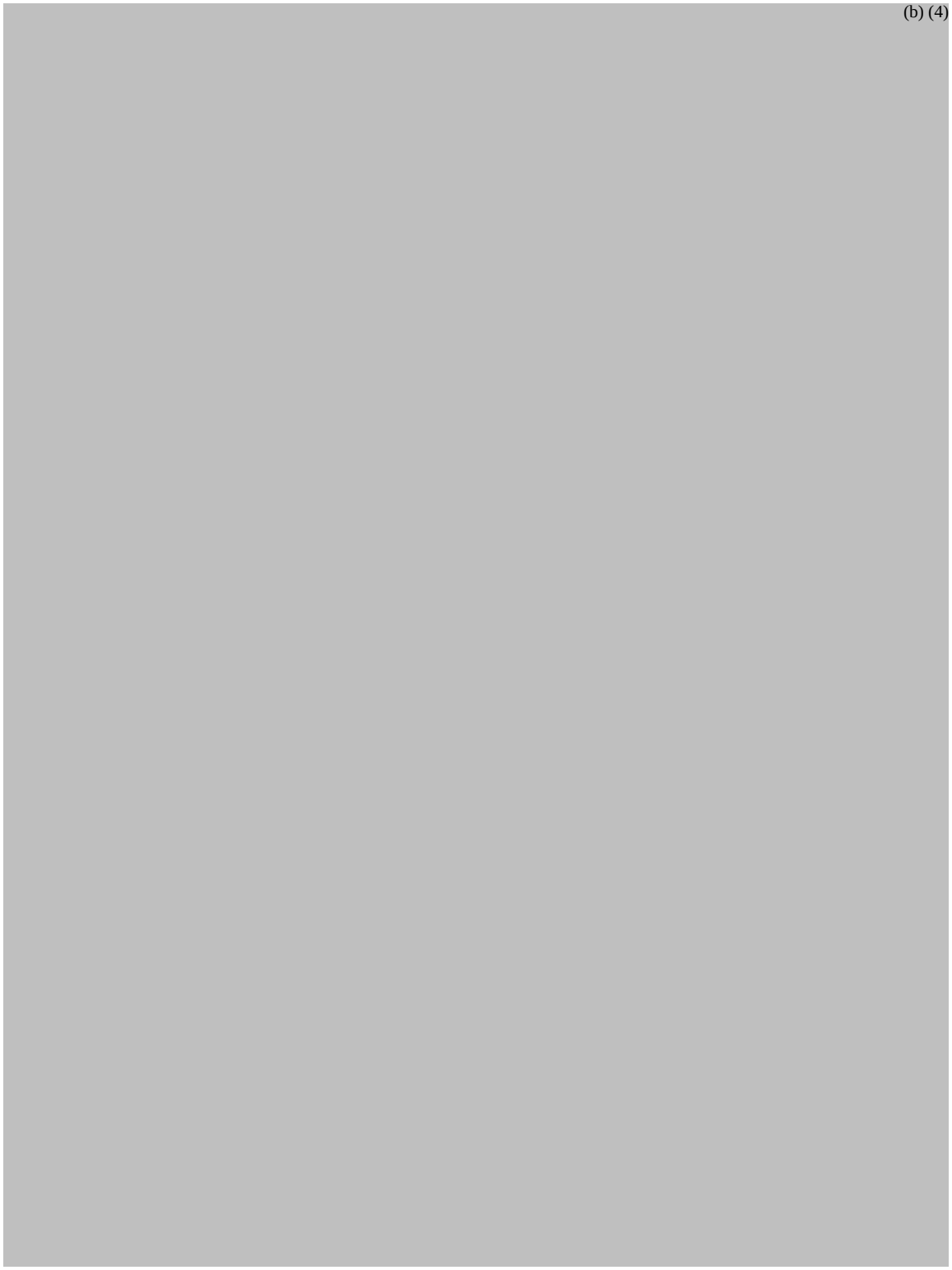
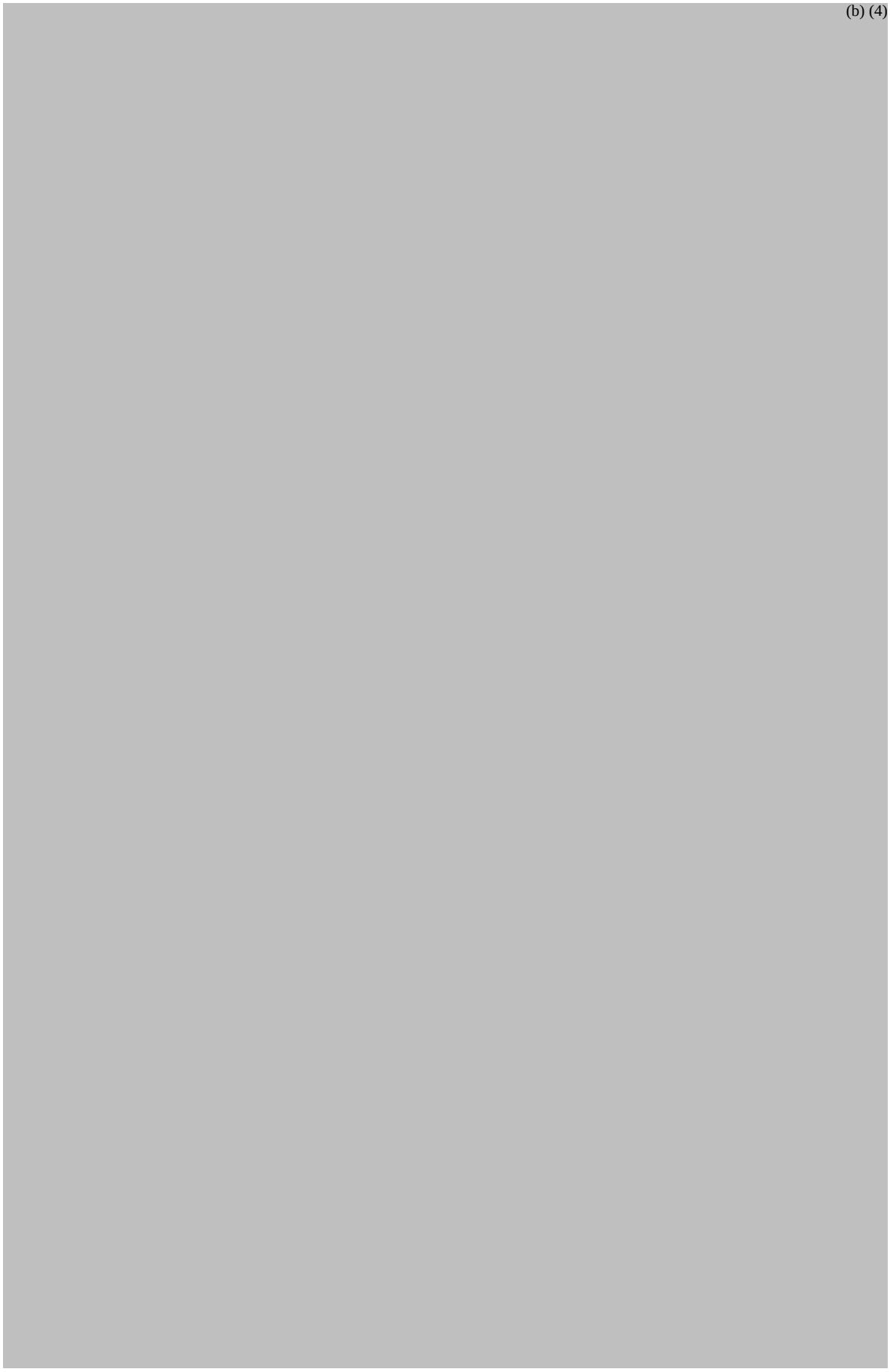
Appendix 1

Nucleotide sequence of the locus of T-DNA insertion and the flanking maize genomic DNA in Event PY1203**.** Maize genomic DNA sequence is presented in lower case letters while the sequence of the T-DNA insert is presented in upper case. Table 3 indicates the positions of key elements with respect to the locus sequence.

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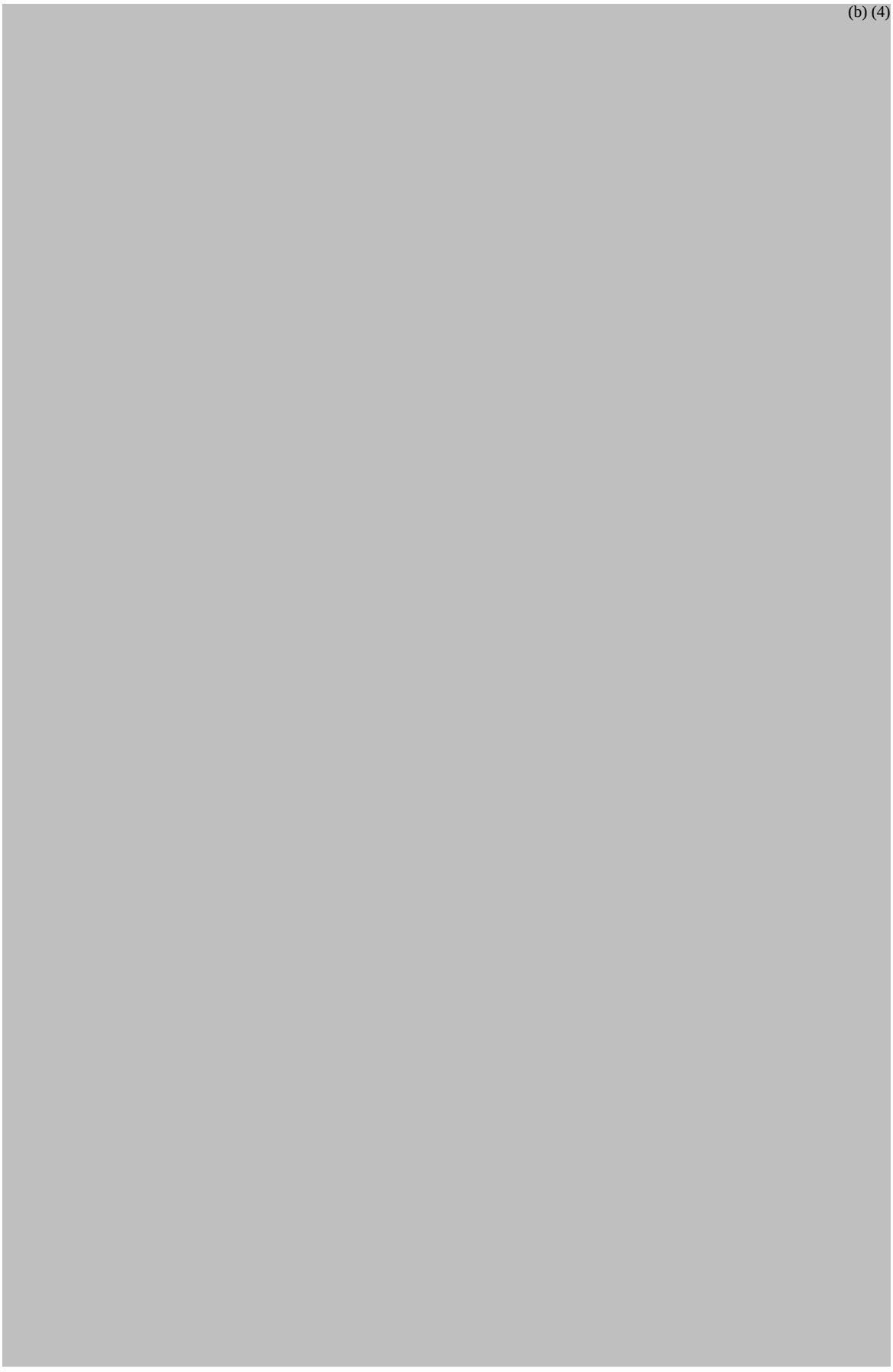
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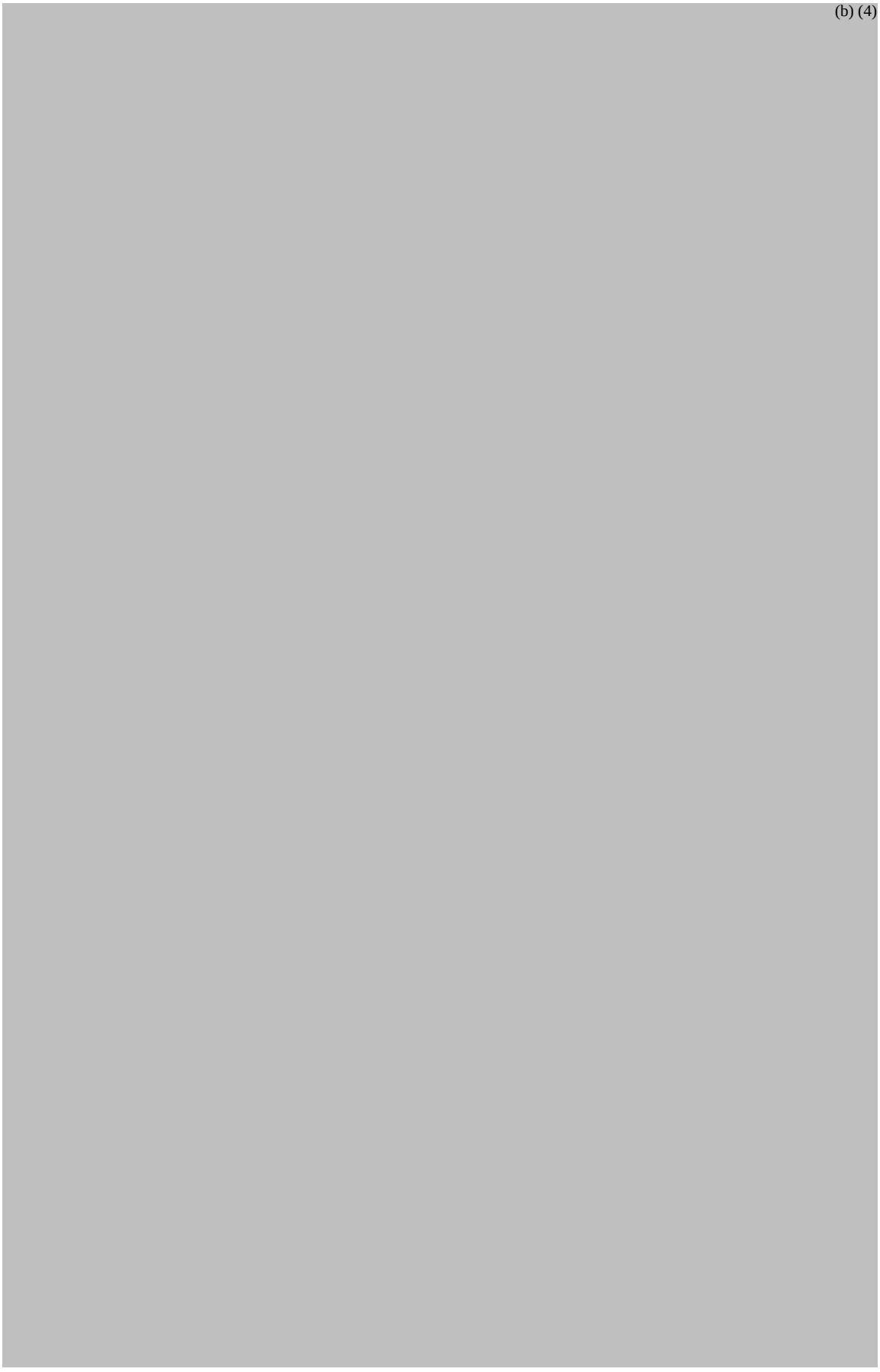
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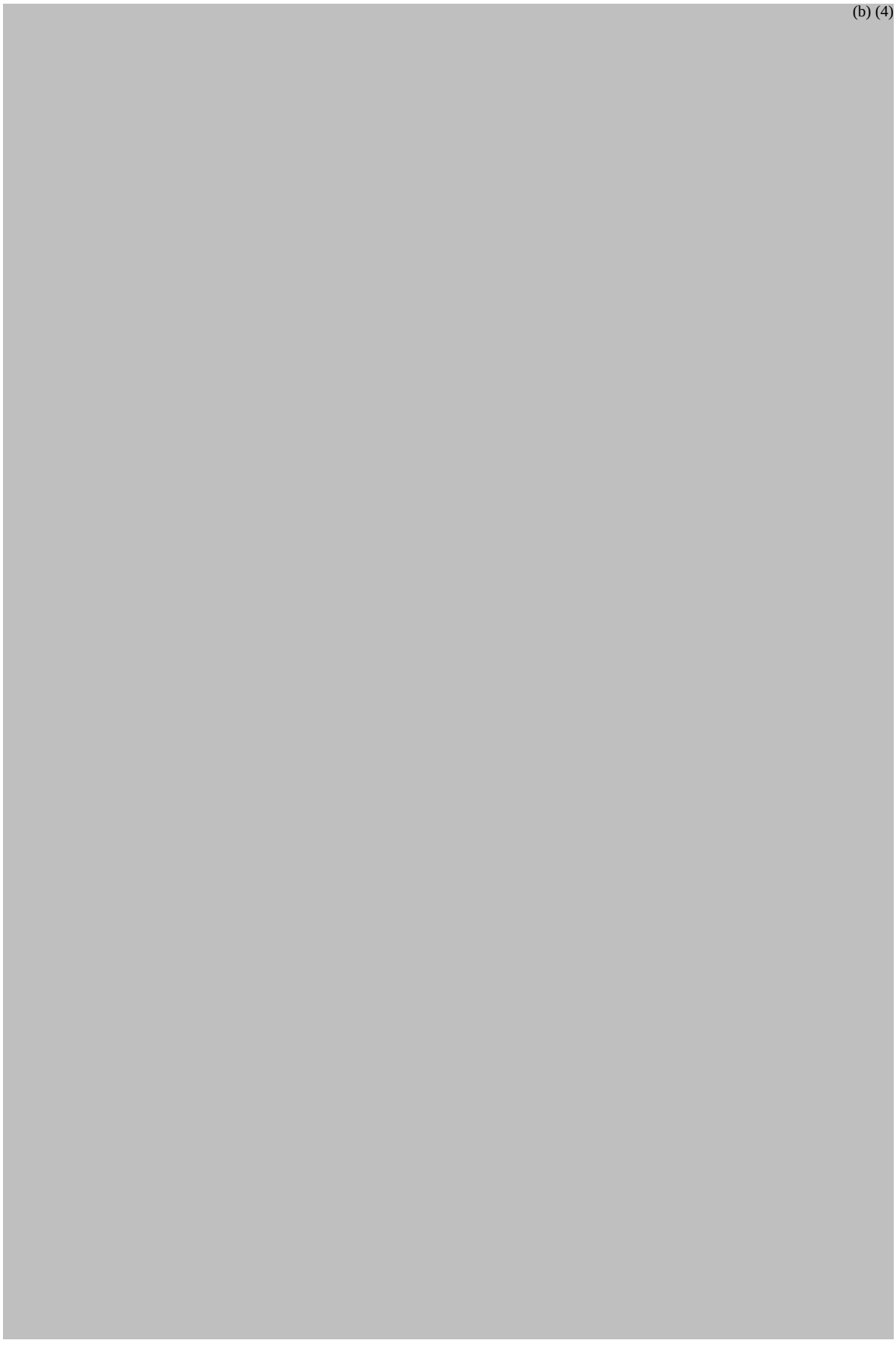
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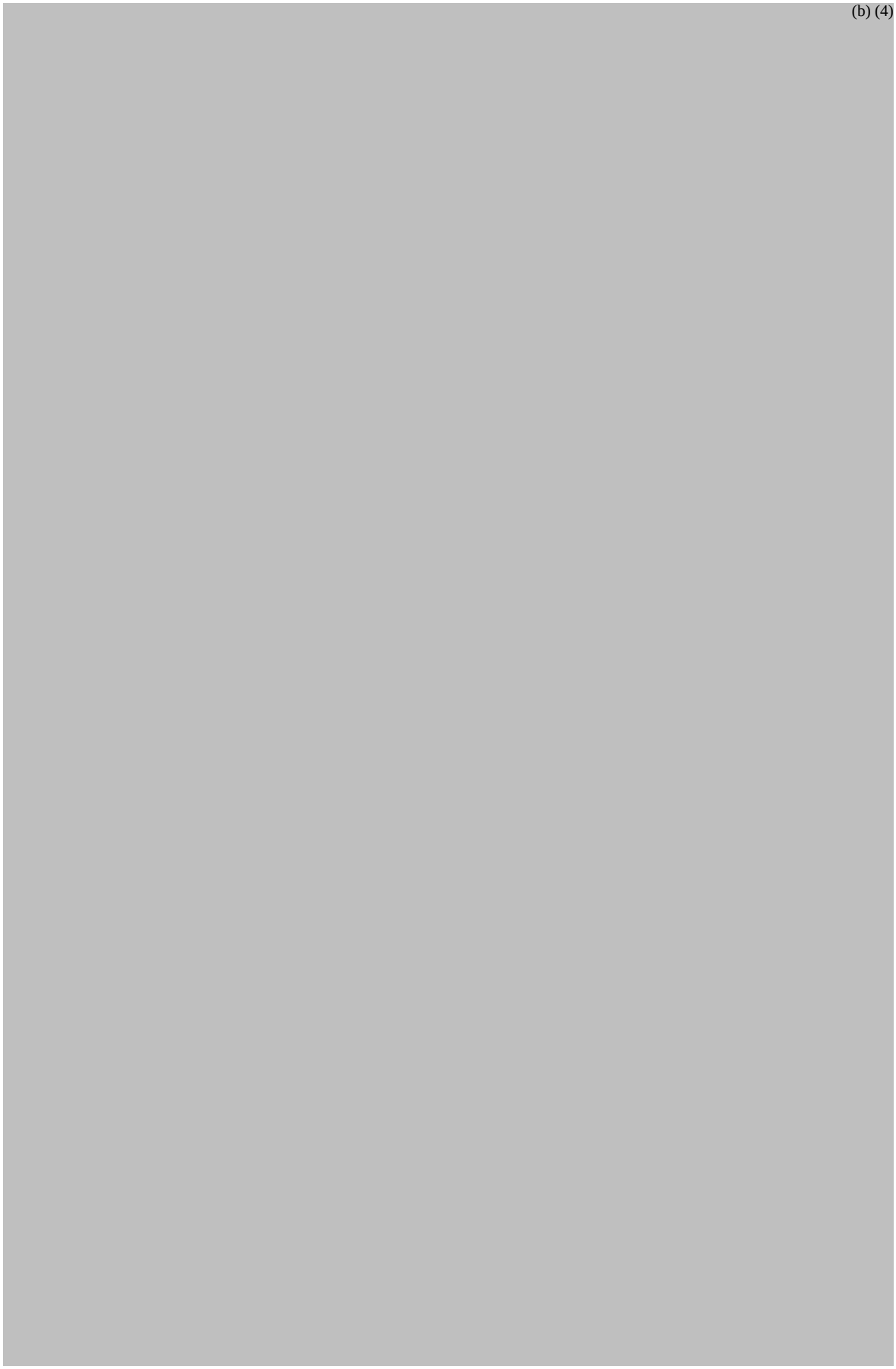
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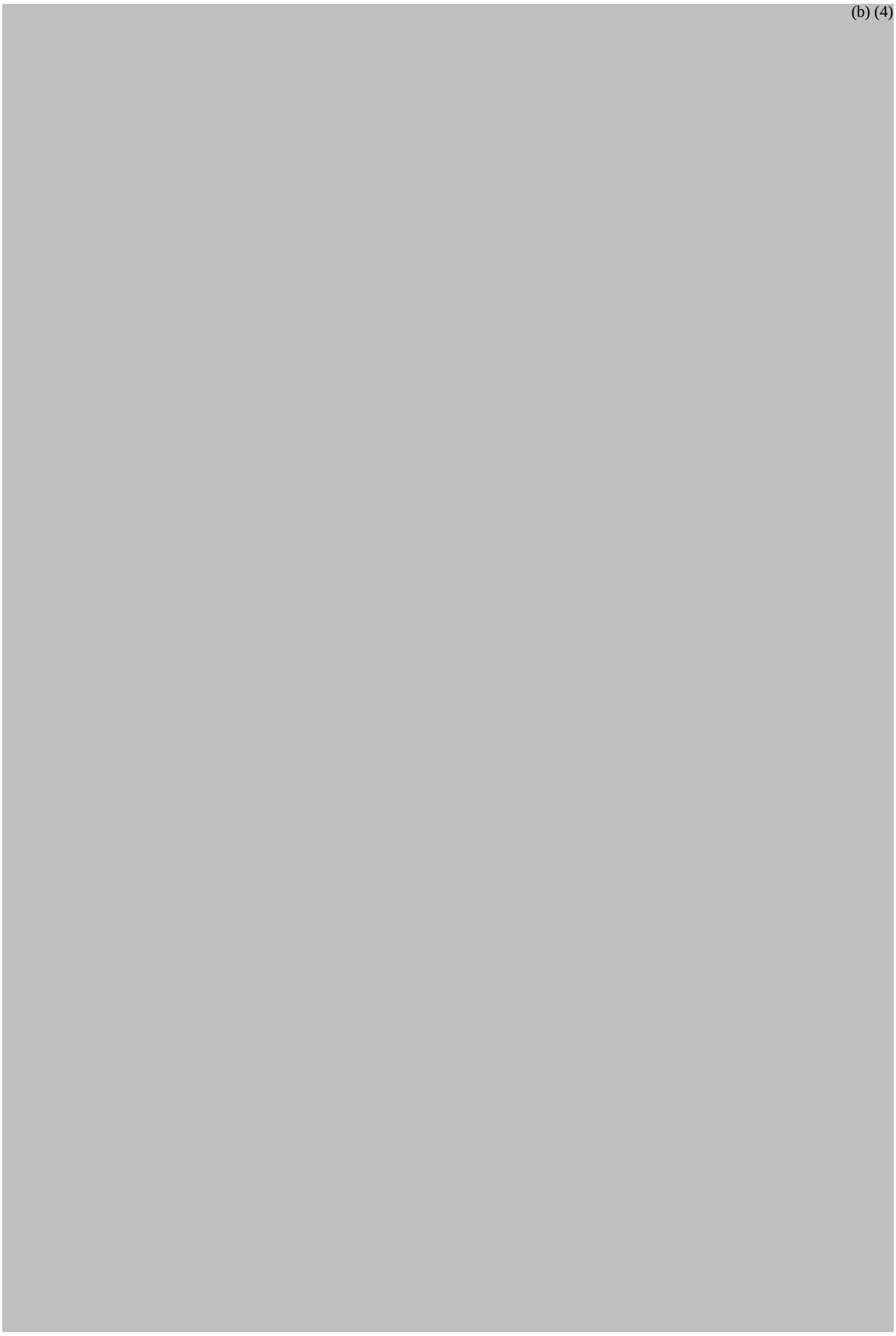
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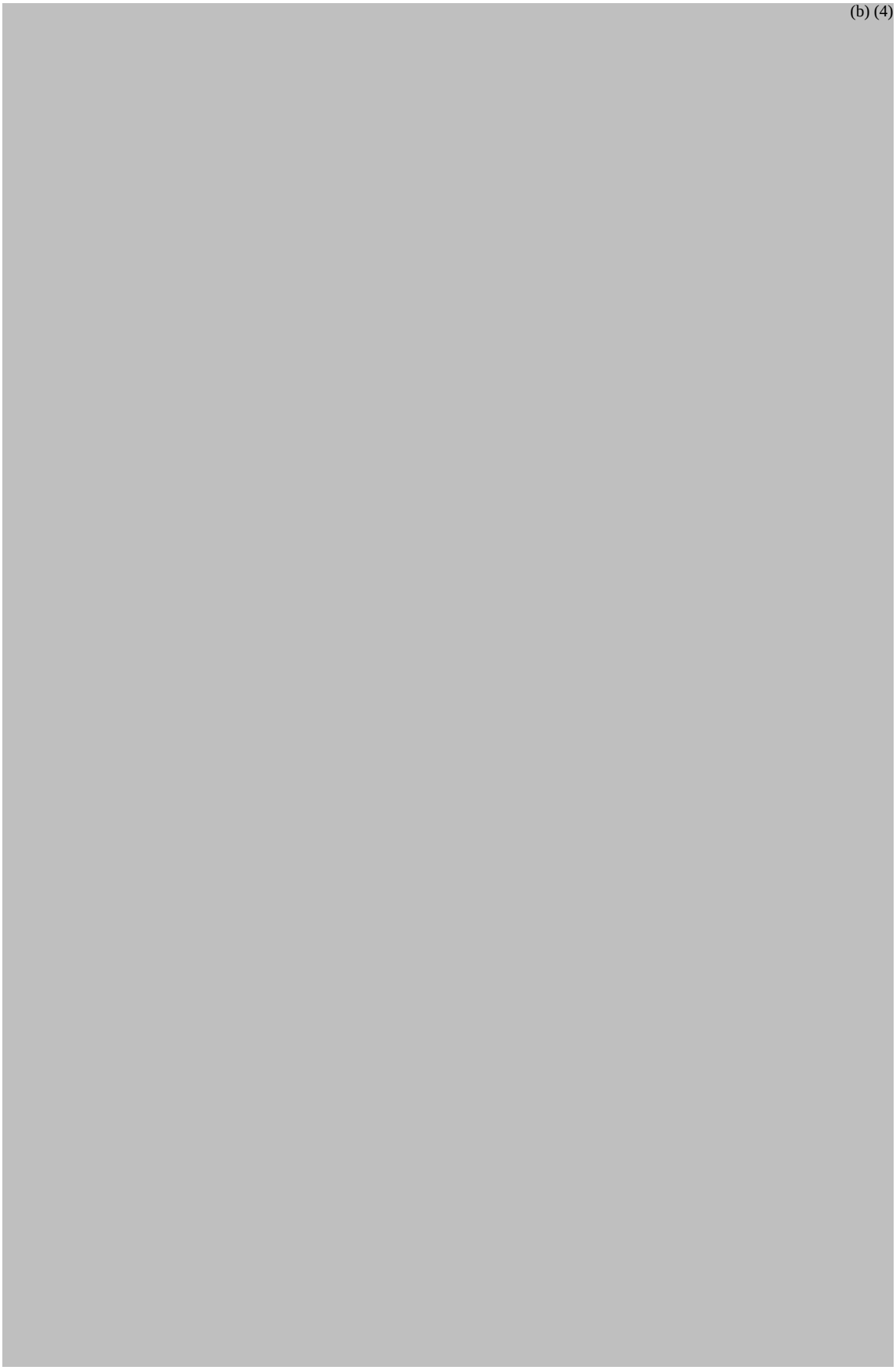
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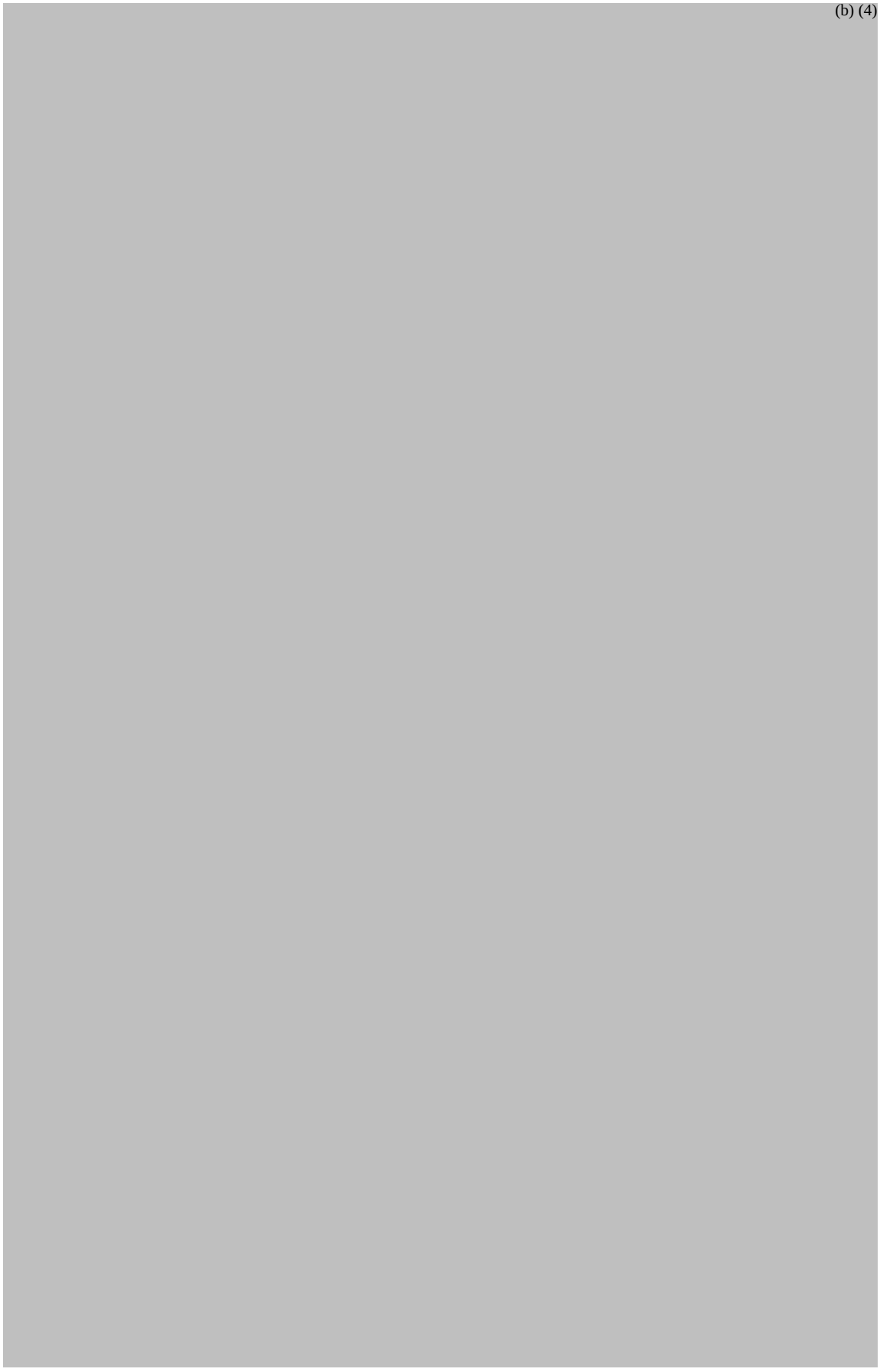
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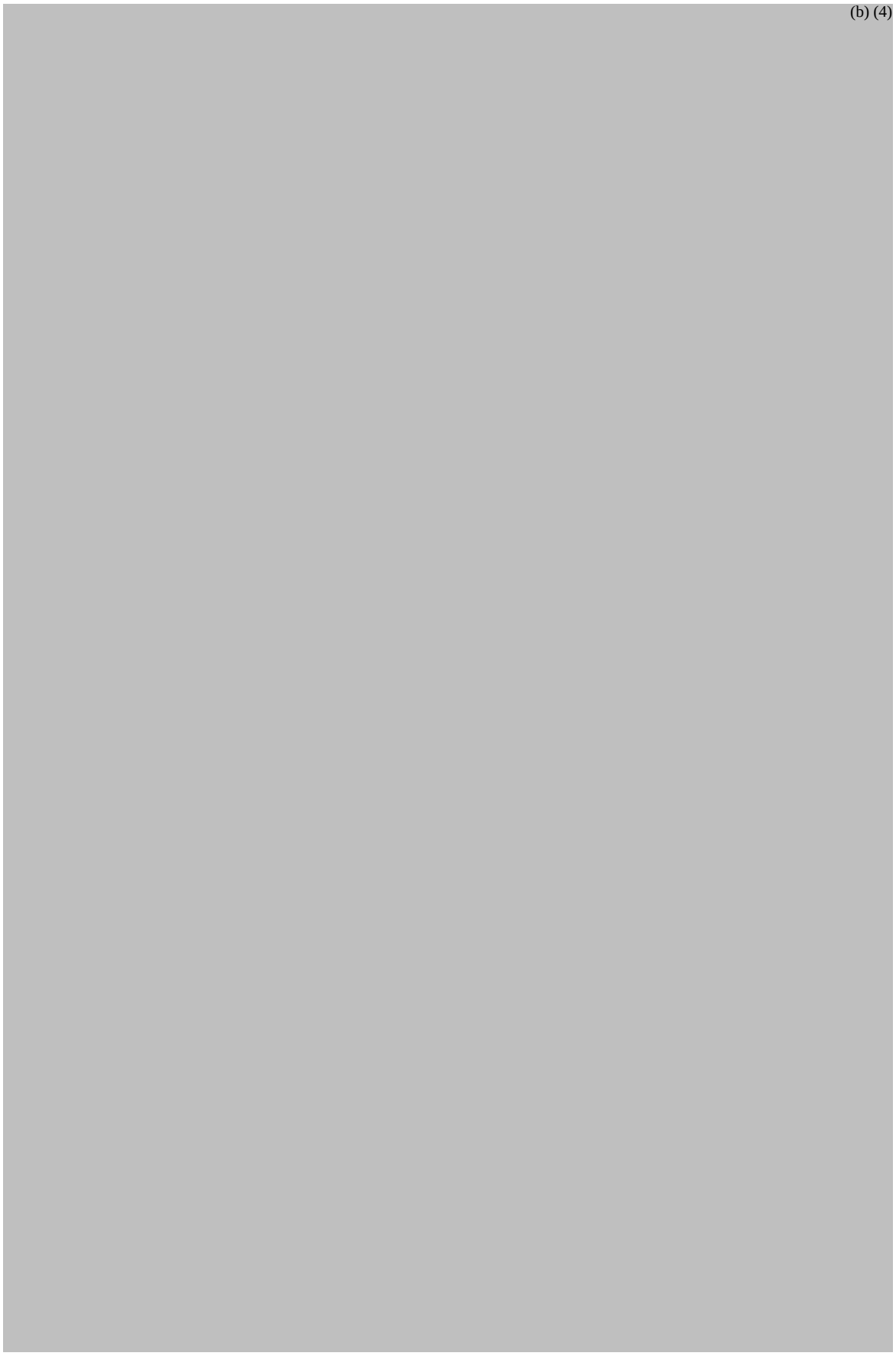
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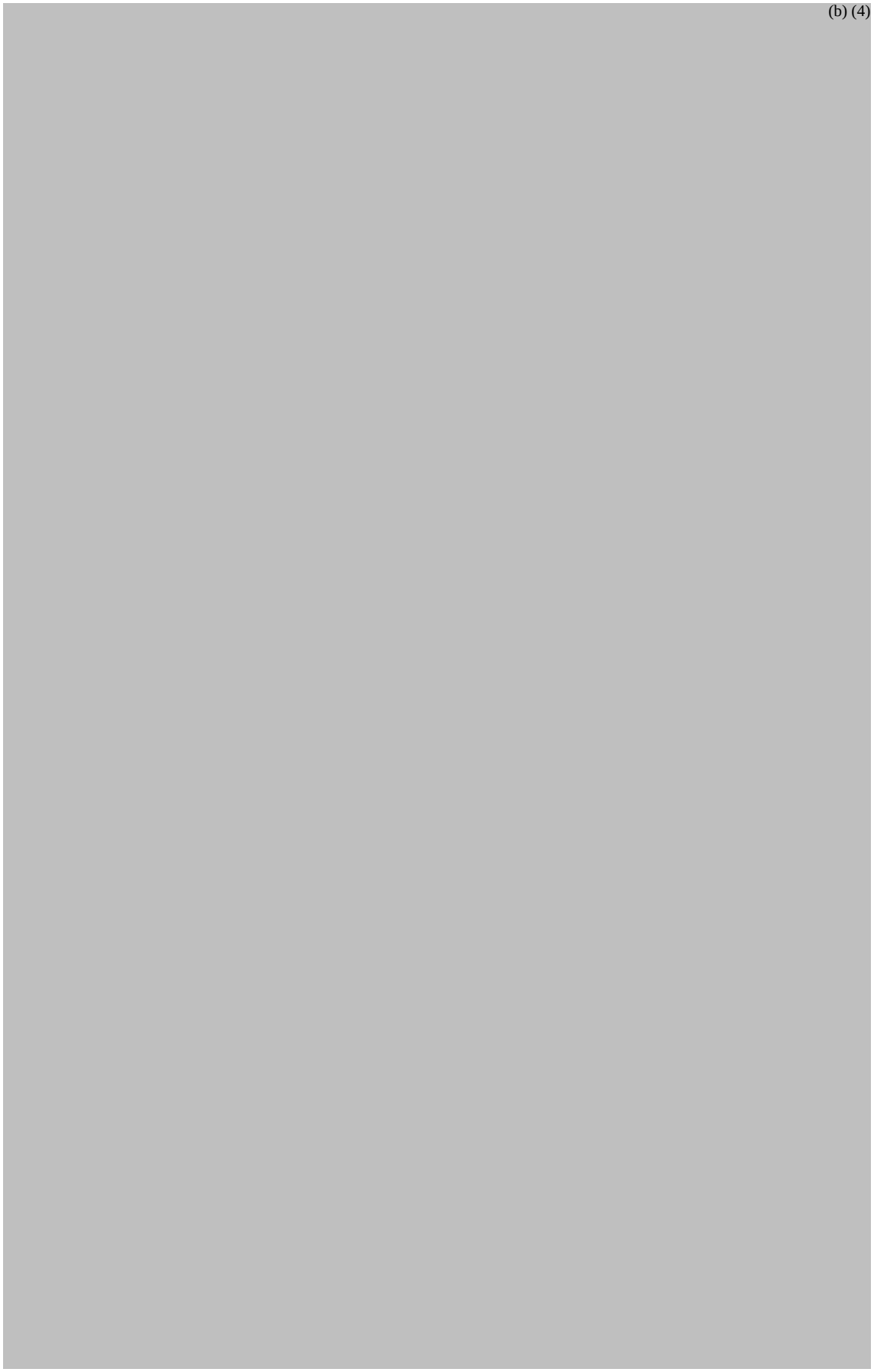
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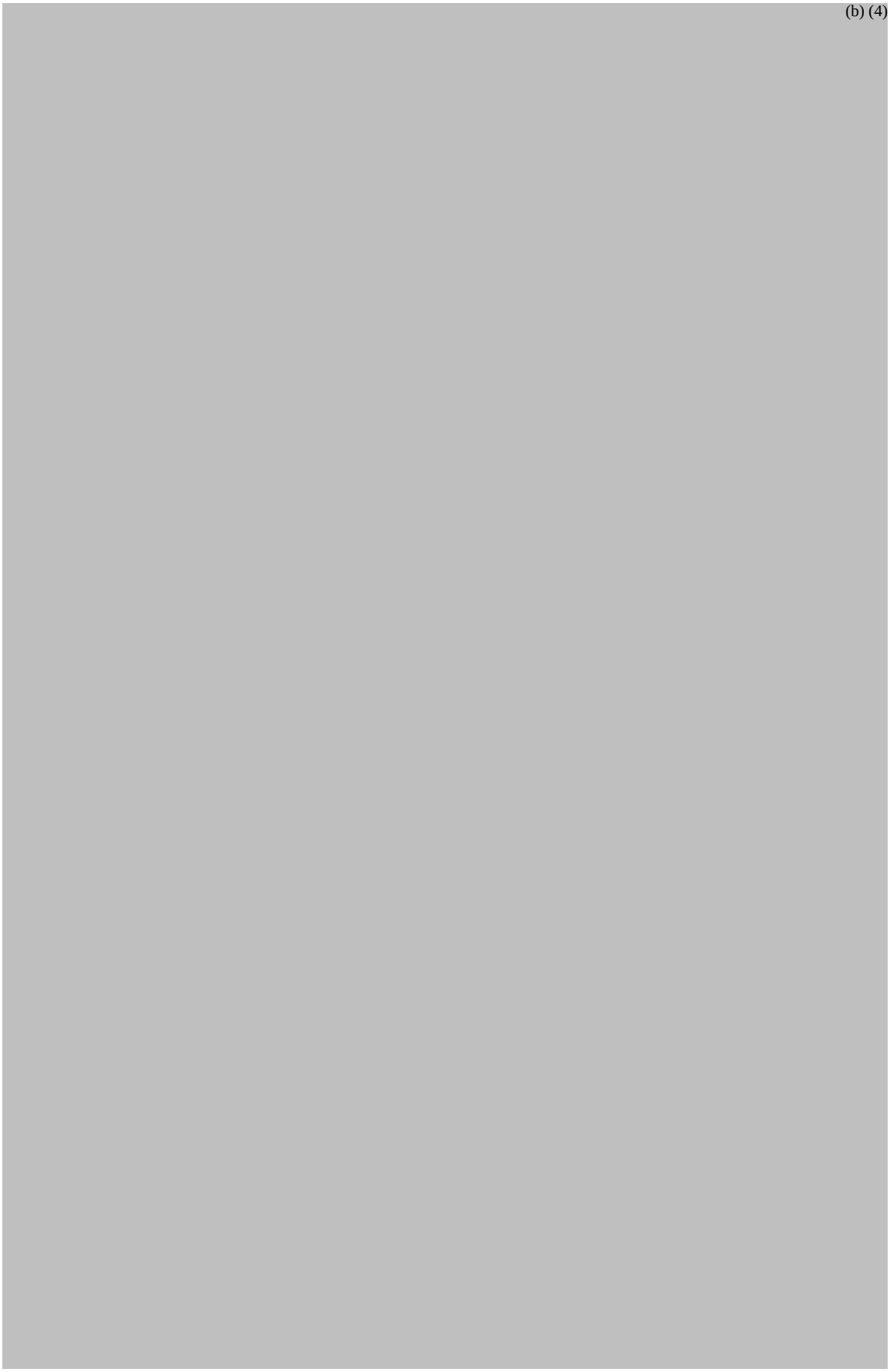
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(b) (4)

T-1

From:	jim.ligon(S)aGrivida.conn
To:	Tang, Lei
Cc	Wong, Geoffrey K; Michael Raab R. Ph.D.; Phil Lessard
Subject:	Re: GRAS submission for phytase com event PY1203
Date:	Tuesday, July 16, 2019 11:15:20 AM
Attachments:	SuDPlementary Info to PY1203 GRASn 16Jul19.Ddf

Dear Dr. Tang and Mr. Wong,

In response to the email I received yesterday from Dr. Tang, I have prepared supplementary information relative to Agrivida's GRAS Notice for the use of the Phy02 phytase in poultry and swine feed that is produced by maize Event PYI203 . The attached supplementary document contains a revised section 6.3 from the earlier GRAS Notice document. In this revised version of section 6.3 I have added references to the AGRN No. 27 GRAS Notice that support the safety of the Phy02 phytase in swine feed. I hope that this will adequately address the issue that Dr. Tang relayed in her email message of yesterday.

As always, if you need any further information to support our conclusions that the Phy02 phytase produced by maize Event PYI203 is GRAS for use in poultry and swine feed, please let me know.

Jim Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
Agrivida, Inc.
www.agrivida.com

jim:lignn@agrivida.com
919-675-6666

1023 Christopher Drive
Chapel Hill, NC 27517

On Jul 15, 2019, at 5:17 PM, Tang, Lei <Lei.Tang@fda.hhs.gov> wrote:

Dear Dr. Ligon,

We are currently conducting a pre-filing evaluation on your submission dated June 6, 2019 for the ground corn grain containing phytase (event PY1203). We have identified one issue that we hope can be resolved in a timely manner.

In our letter of March 12, 2019 responding to your previous submission for this same phytase corn (event PY1203), we state "if your target animal safety assessment is based on the information provided in other GRAS notices, you should provide specific references." In your current submission dated June 6, 2019, section 6.3 is titled "Safety of the Phy02 phytase from Event PY1203 in

poultry". In this section, you reference specific information provided in GRAS notice AGRN 21 to support the safety of the intended use for poultry. However, you do not reference any specific information from your previous GRAS notices to support the safety of the intended use for swine. Please provide specific references or a clear narrative to address the safety of the intended use for swine

You can either mail the requested information to Mr. Geoffrey Wong or email me and Mr. Geoffrey Wong a pdf version. If you have any questions, please don't hesitate to contact me.

Best regards,

Lei Tang, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal feeds
U.S. Food and Drug Administration

Tel: 240-402-5922
lei.tang@fda.hhs.gov

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SUPPLEMENTARY INFORMATION to a NOTIFICATION of GRAS STATUS for the USE of PHY02 PYTASE PRODUCED by MAIZE EVENT PY1203 in the FEED of POULTRY and SWINE

Submitting Company:

Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801

Submitted by:

James M. Ligon, Ph.D.
V.P., Regulatory Affairs and Stewardship
Agrivida, Inc.
1023 Christopher Drive
Chapel Hill, NC 27517
919-675-6666
jim.ligon@Agrivida.com

July 16, 2019

This document contains supplementary information to the GRAS Notification that Agrivida, Inc. has submitted for the use of GraINzyme® Phytase produced by maize Event PY1203 in the feed of poultry and swine. It contains a revised section 6.3 with additional information supporting the safety of this phytase in swine feed.

6.3 Safety of the Phy02 phytase from Event PY1203 in poultry and swine

The safety of the Phy02 phytase from Event PY203 in poultry is presented in Animal Food GRAS Notice No. AGRN 21, section 4.3, pg. 35-38. The safety of the Phy02 phytase in swine feed is supported by a tolerance study with 45,000 FTU Phy02 phytase/kg feed in weaned piglets as described in Animal Food GRAS Notice No. 27, section 6.4, pg. 20-23 and by its substantial equivalence to two commercial phytases that are known to be safe in swine as described in Animal Food GRAS Notice No. 27, section 6.5, pg. 24-32. The overall safety of the Phy02 phytase produced by maize Event PY203 in swine feed is summarized in Animal Food GRAS Notice No. 27, section 6.6, pg. 33-34. Since the Phy02 phytase that is produced in Event PY1203 is identical to that produced by Event PY203 the safety of the former is equivalent to that of the latter. The only difference in the use of the GraINzyme® Phytase product produced by maize Event PY1203 from that produced by maize Event PY203 is that the inclusion rate for the former will be decreased according to the measured phytase activity in the product to deliver a specified dose. Information about inclusion rates will be presented on the product label that accompanies the product.

T-3

From: jim.ligon@agrivida.com
To: Tang, Lei
Cc: Wong, Geoffrey K; Michael Raab R. Ph.D.; Phil Lessard
Subject: Re: GRAS AGRN 32 - event PY1203 phytase corn
Date: Tuesday, February 11, 2020 9:57:16 AM
Attachments: AGRN32 Amendment_10Feb20.pdf

Dear Dr. Tang,

We have completed an amendment to our GRAS Notice for the use of the Agrivida Phytase produced by maize Event PY1203 in poultry and swine feed (AGRN-032). This amendment contains responses to the issues and questions that were presented in the email you sent us on 3 February this year. I have attached a copy of the amendment in PDF for your information. None of the information contained in this amendment is considered by Agrivida, Inc. to be confidential business information.

If you or others on the review team have further questions related to AGRN 032, please feel free to contact me.

Best regards,

Jim Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
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www.agrivida.com

jim.ligon@agrivida.com
919-675-6666

1023 Christopher Drive
Chapel Hill, NC 27517

On Feb 3, 2020, at 10:22 AM, Tang, Lei <Lei.Tang@fda.hhs.gov> wrote:

Dear Dr. Ligon,

We are currently evaluating Agrivida's GRAS Notice AGRN 32. In order for us to complete our evaluation, please provide an amendment to clarify following issues regarding the bioengineering process of production corn strain:

1. Agrivida makes the following statement on page 32 of the notice:
   "Genomic DNA samples from wild type (untransformed) *Z. mays* and Event PY1203 as well as the purified transformation vector (b) (4) were each digested with either *Bam*HI or *Eco*RI, separated via agarose gel electrophoresis, and subjected to Southern analysis using the PCR-generated DIG-labeled probes

(Table 6). As depicted in Figure 11, the probes readily detected the backbone fragments in lanes loaded with vector DNA (5, 6). The same probes, however, did not detect any fragments in either the wild type or Event PY1203 genomic DNA (lanes 1-4). These results indicate that no vector backbone-derived sequences are present in the genome of Event PY1203."

We note that Figure 11 legend does not specify which of the six probes is shown in the depicted Southern blot. The notice would be more complete if the Southern blots for each of the six probes were included in the notice. However, the two most important Southern blots are that ones that demonstrate that the *aadA* and *sat* genes (the antimicrobial resistance determinants) were not incorporated into the genome of Event PY1203. Please address this issue.

2. During our evaluation, using the B73 RefGen_v4 sequence in maizegdb.org, we noted that upstream and downstream sequences that bordered the site of insertion mapped to a region on chromosome (b)(4). However, approximately half of the corn genome nucleotide sequence provide in Appendix 1 did not map to the above mentioned region on chromosome (b)(4) Please provide an explanation for this discrepancy.

Please provide the requested amendment within two weeks from today.

Best regards,

Lei Tang, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal feeds
U.S. Food and Drug Administration

Tel: 240-402-5922
lei.tang@fda.hhs.gov

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sender immediately at Lei.Tang@fda.hhs.gov.

A phytase feed enzyme produced by *Zea mays* Event PY1203 expressing a phytase gene derived from *Escherichia coli* K12

AMMENDMENT TO GRAS NOTICE No. AGRN 000-032

Submitting Company:
Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801

Please address correspondence related to this submission to:

James M. Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
Agrivida, Inc.
1023 Christopher Drive
Chapel Hill, NC 27517

Tel: 919-675-6666
Email: jim.ligon@agrivida.com

February 10, 2020

Introduction

The FDA Center for Veterinary Medicine (CVM) is reviewing GRAS Notice No. AGRN 000-032, submitted by Agrivida, Inc. in May 2019 for its GraINzyme® Phytase product produced in the grain of maize Event PY1203. During the review, CVM has developed questions related to this GRAS notice. These questions were presented to Agrivida in an email from Dr. Lei Tang of CVM dated February 3, 2020. Agrivida has carefully considered the questions from CVM and has formulated responses to address each of them. These responses are contained in this amendment to the GRAS Notice No. AGRN 000-032. In this amendment, the issue raised by CVM is stated, followed by Agrivida's response.

Question 1. Agrivida makes the following statement on page 32 of the notice: "Genomic DNA samples from wild type (untransformed) *Z. mays* and Event PY1203 as well as the purified transformation vector (b) (4) were each digested with either *Bam*HI or *Eco*RI, separated via agarose gel electrophoresis, and subjected to Southern analysis using the PCR-generated DIG-labeled probes (Table 6). As depicted in Figure 11, the probes readily detected the backbone fragments in lanes loaded with vector DNA (5, 6). The same probes, however, did not detect any fragments in either the wild type or Event PY1203 genomic DNA (lanes 1-4). These results indicate that no vector backbone-derived sequences are present in the genome of Event PY1203."

In the email of 3 February CVM states: "We note that Figure 11 legend does not specify which of the six probes is shown in the depicted Southern blot. The notice would be more complete if the Southern blots for each of the six probes were included in the notice. However, the two most important Southern blots are that ones that demonstrate that the *aadA* and *sat* genes (the antimicrobial resistance determinants) were not incorporated into the genome of Event PY1203. Please address this issue."

Agrivida's response:
All six of the probes described in Figure 10 and Table 6 were used simultaneously in the Southern blot that is depicted in Figure 11. This was stated in the legend to Figure 10, where Agrivida stated "Probes a-f (red) were prepared by PCR, labeled with DIG, pooled, and used to probe genomic DNA derived from Event PY1203." Agrivida, Inc acknowledges that it would be helpful to point this out more explicitly in the legend to Figure 11. The amended legend to Figure 11 is presented below:

> **Figure 11. Southern blot showing the lack of vector backbone-derived sequences in the genomic DNA of Event PY1203.** Six overlapping probes, which together correspond to the entire backbone portion of (b) (4) including the *sat* and *aadA* selectable markers (as shown in Figure 10), were prepared by PCR, labeled with DIG, pooled, and used to probe genomic DNA derived from WT maize and Event PY1203. Lane 1: wild type (WT) *Z. mays*

DNA digested with *Bam*HI; lane 2: WT Z. mays DNA digested with *Eco*RI; lane 3: PY1203 DNA digested with *Bam*HI; lane 4: PY1203 DNA digested with *Eco*RI; lane 5: (b) (4) DNA, equivalent to one genome-copy equivalent, digested with *Bam*HI; lane 6: (b) (4) DNA, equivalent to one gene-copy equivalent, digested with *Eco*RI; lane 7: DIG-labeled molecular weight marker. The relative sizes (in base pairs) of the labeled fragments in lane 7 are indicated to the right.

Question 2. During our evaluation, using the B73 RefGen_v4 sequence in maizegdb.org, we noted that upstream and downstream sequences that bordered the site of insertion mapped to a region on chromosome (b) (4) However, approximately half of the corn genome nucleotide sequence provide in Appendix 1 did not map to the above-mentioned region on chromosome (b) (4) Please provide an explanation for this discrepancy.

Agrivida's response:

The sequence presented in Appendix 1 is comprised of the right border flank (or "left side") genomic sequence, the T-DNA insertion sequences, and the left border flank (or "right side") genomic sequence. The sequences of the T-DNAs themselves do not align with the sequence of the B73 reference genome. However, as described previously, we determined that the right border flank aligns with nucleotides (b) (4) of maize chromosome (b) (4), and that the left border flank aligns with nucleotides (b) (4) of maize chromosome (b) (4).

To repeat this analysis, we used BLASTN to search the B73 RefGen_v4 sequence in maizegdb.org on February 6, 2020, with both the right border flank sequence and the left border flank sequence. The top hits returned by these searches (shown below) again corresponded to those same regions of maize chromosome (b) (4).

BLASTN sequence alignment of the right border flank showed 100% sequence identity to nucleotides (b) (4) of B73 maize chromosome (b) (4) for the entire sequence length ((b) (4) bp) with (b) (4) nucleotide mismatches at the 3' end.

BLASTN sequence alignment of the left border flank showed 99.88% sequence identity to nucleotides (b) (4) of B73 maize chromosome (b) (4) for the entire sequence length ((b) (4) bp) with (b) (4) nucleotide mismatches. These results confirm that the flanking sequences are derived from maize chromosome (b) (4).

To illustrate these alignments, we present below

1. The sequence of the right border flank (RBF_PY1203)
2. The top hit ("query") returned from maizegdb.org when the right border flank was used in a BLASTN search of B73 RefGen_v4. This sequence corresponds to maize chromosome (b) (4) from nt (b) (4).
3. The alignment of sequences 1 and 2, above, using the publicly using available online tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)
4. The sequence of the left border flank (LBF_PY1203)
5. The top hit ("query") returned from maizegdb.org when the left border flank was used in a BLASTN search of B73 RefGen_v4. This sequence corresponds to maize chromosome (b) (4) from nt (b) (4). Note

maizegdb.org introduced dashes into the sequence of the top hit to indicate single-nucleotide gaps in the alignment relative to the subject sequence (LBF_PY1203)

6. The alignment of sequences (b) (4) and (b) (4), above, using Clustal Omega (with mismatches highlighted)

>RBF_PY1203

(b) (4)

(b) (4)

query (b) (4)

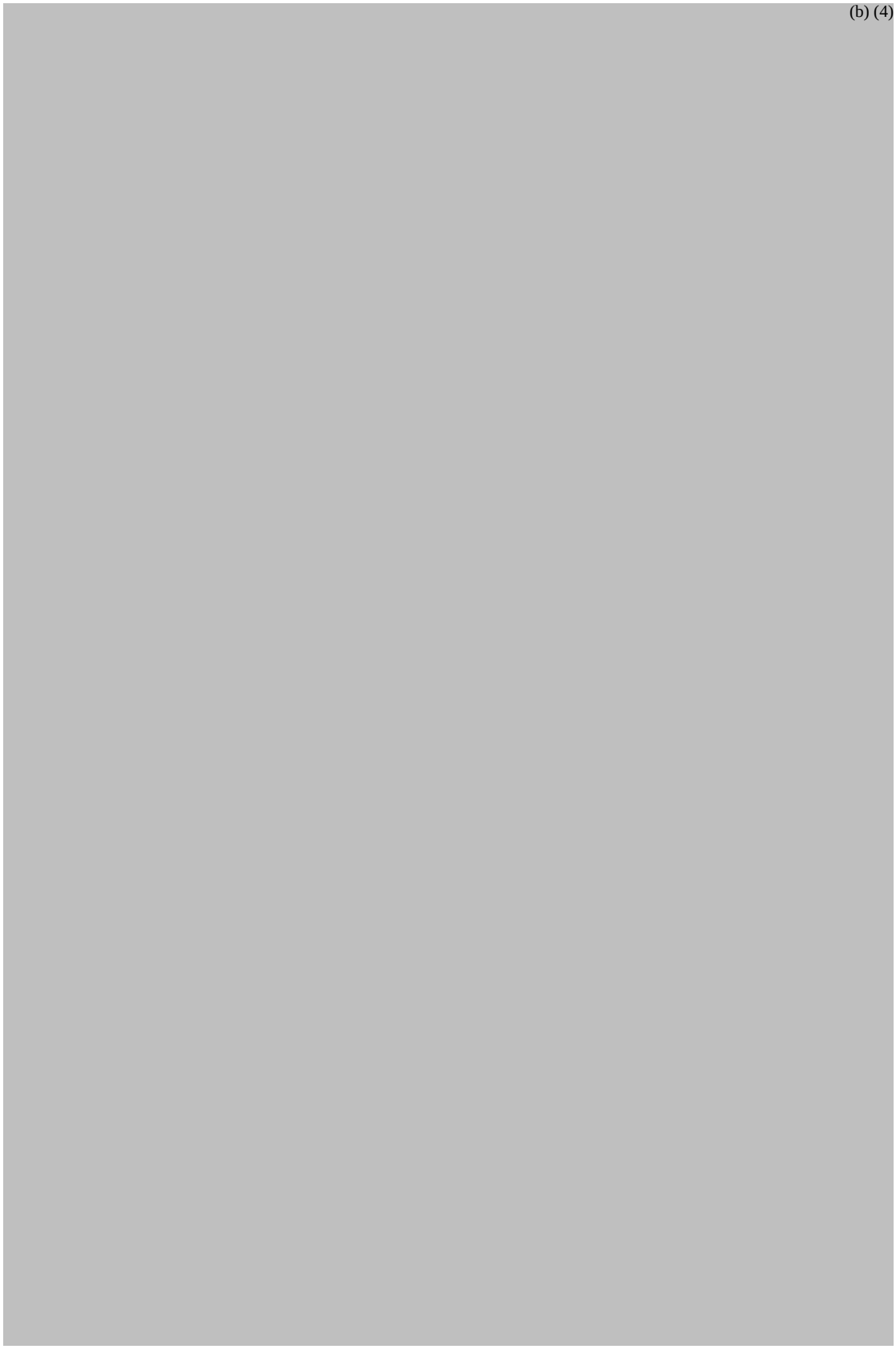
(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)



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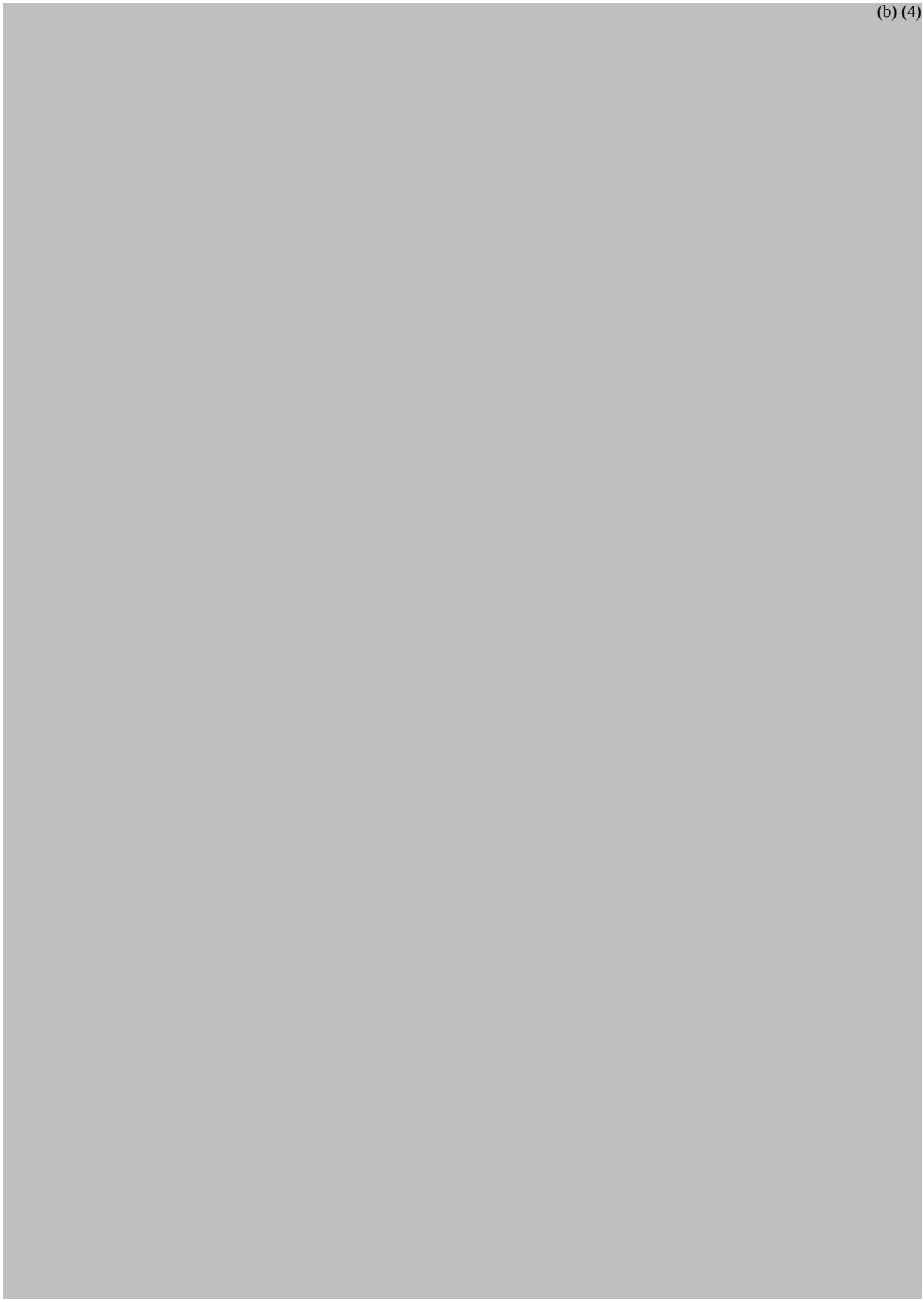

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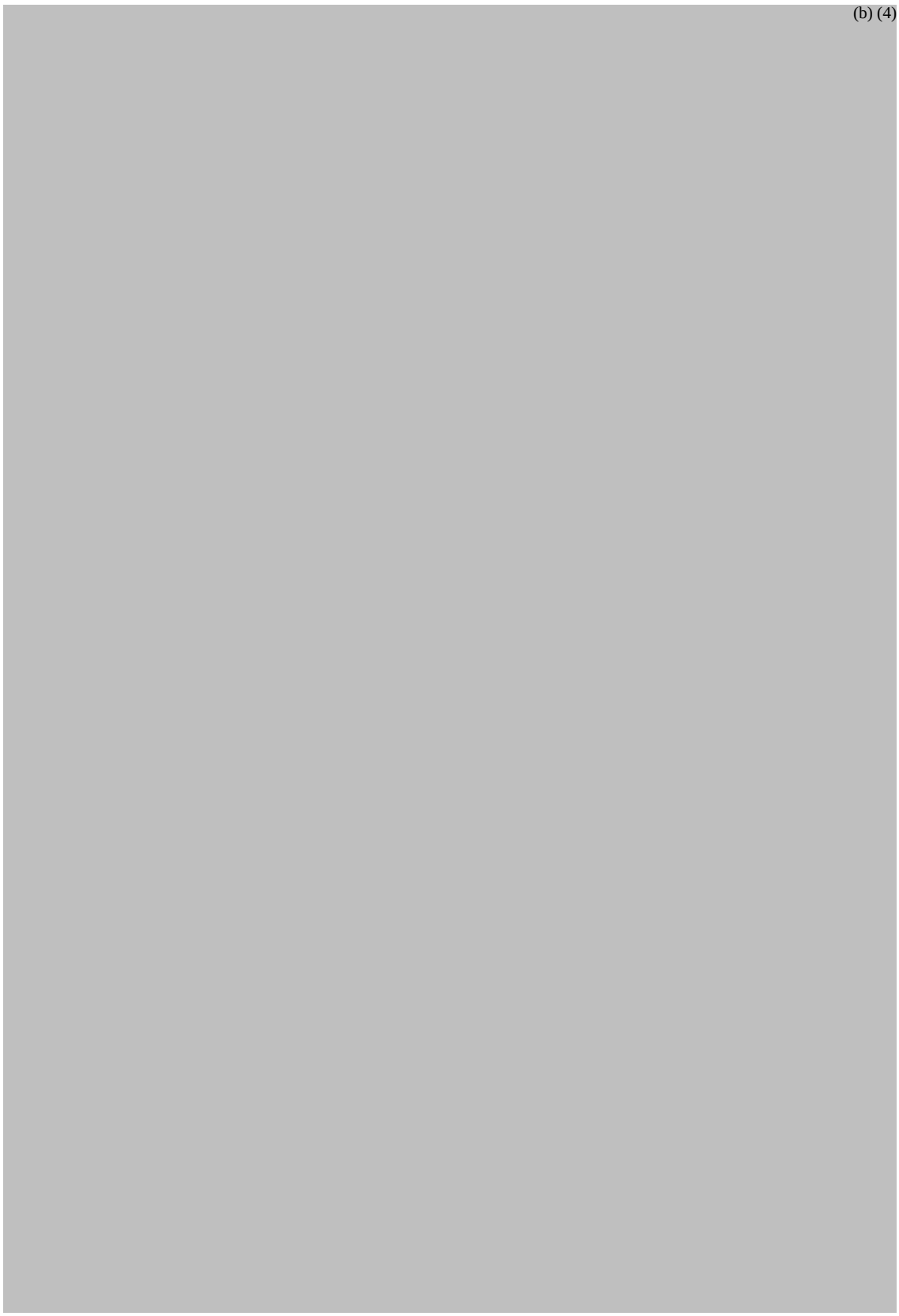

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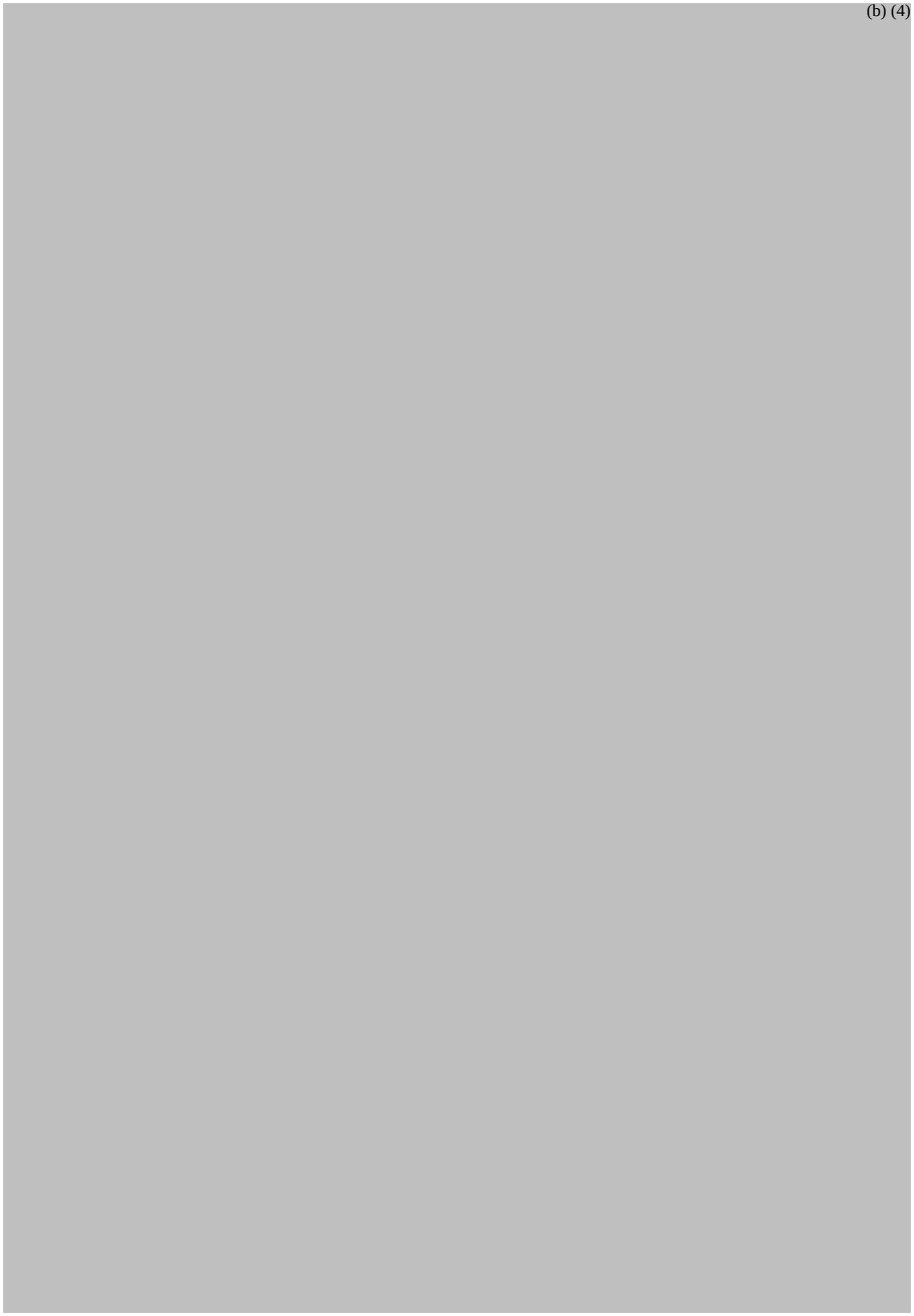

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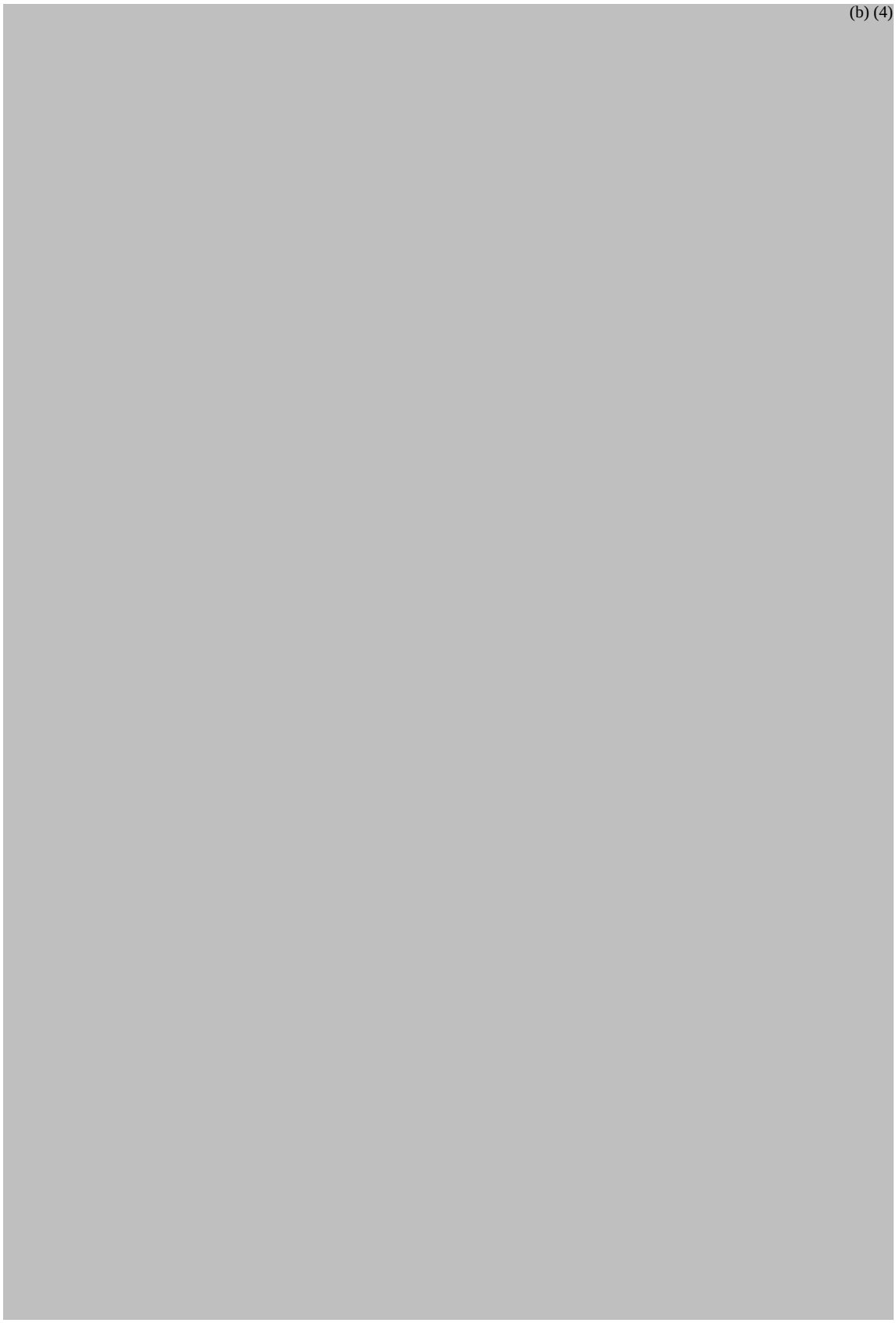

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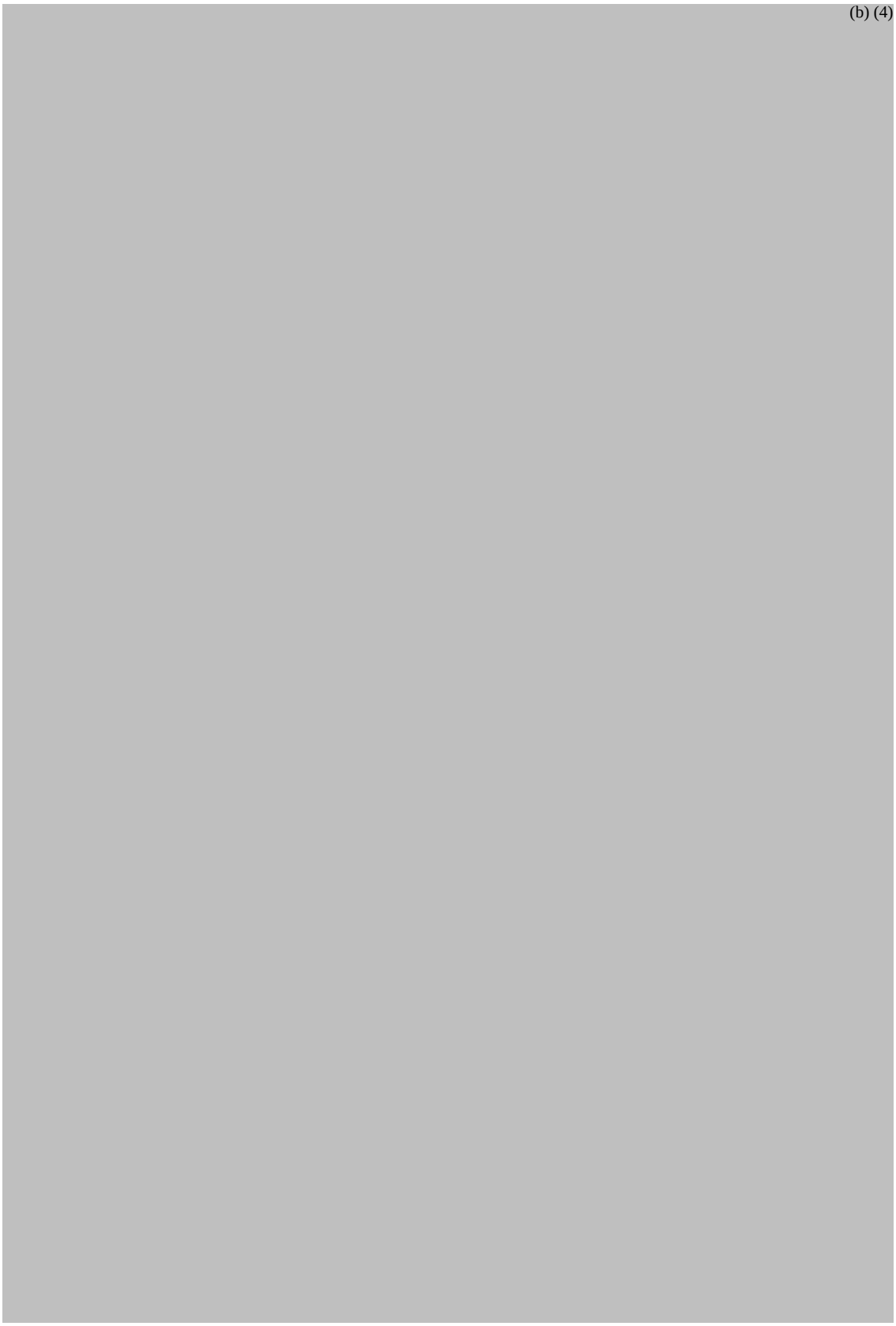

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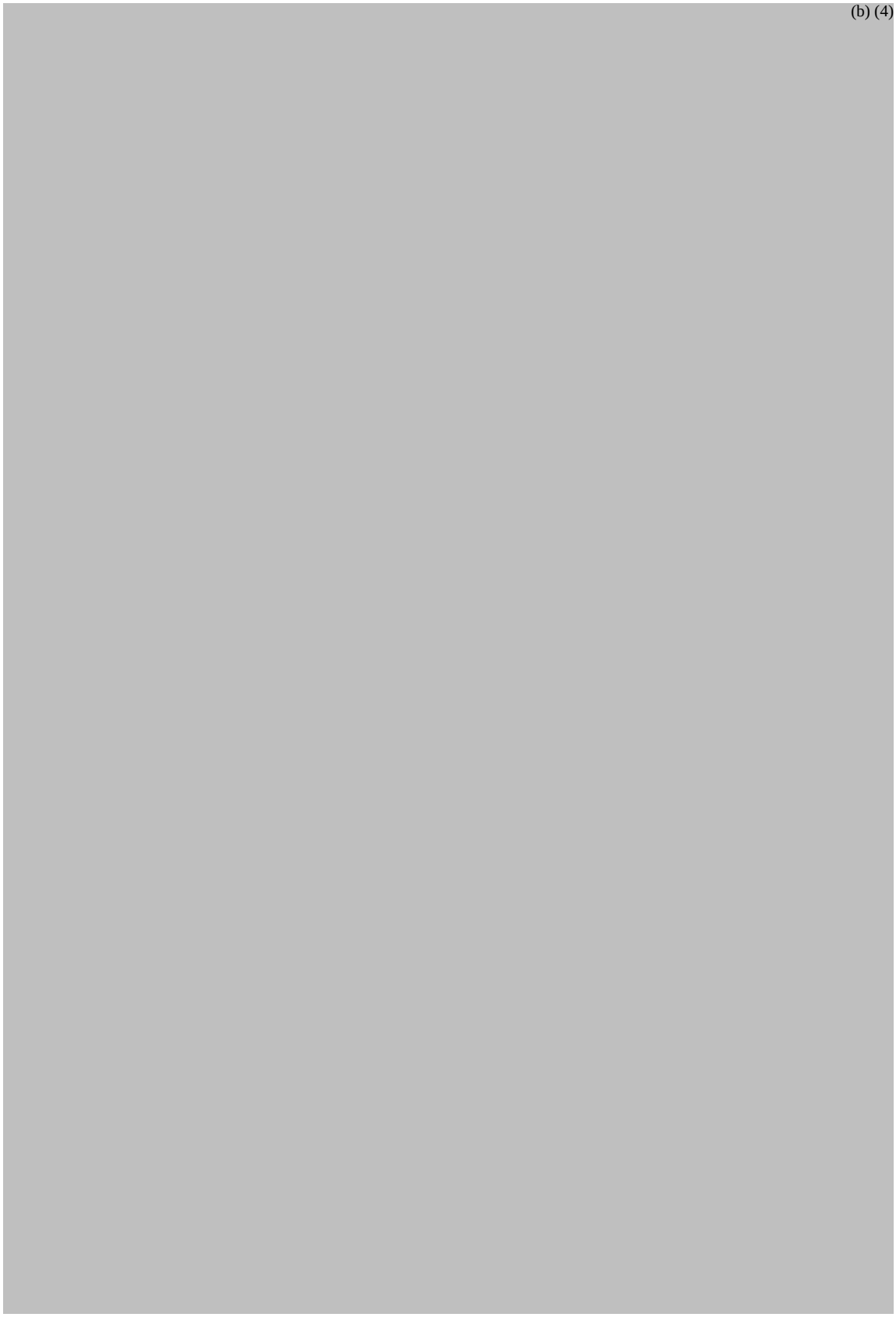
(b) (4)

(b) (4)

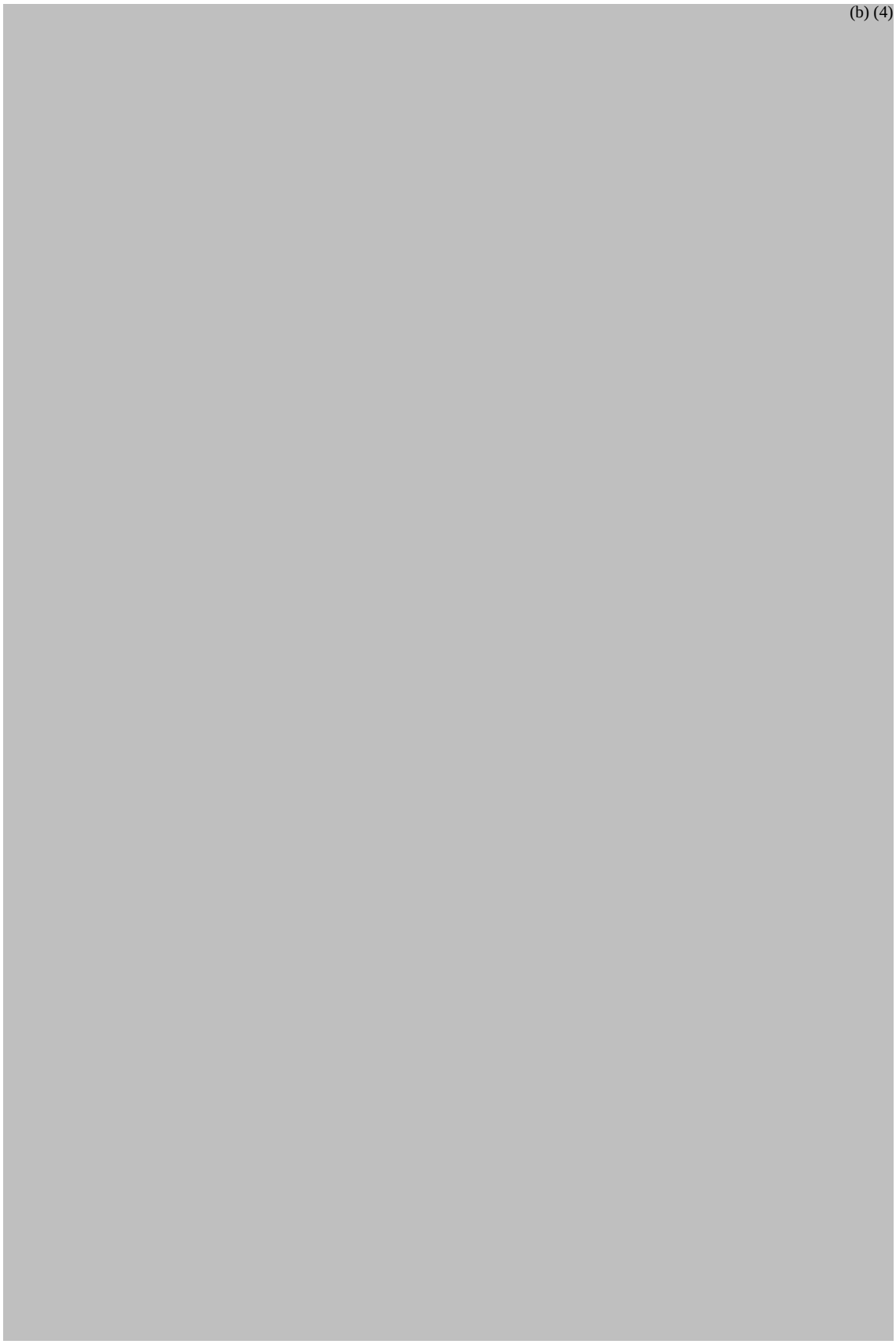
(b) (4)



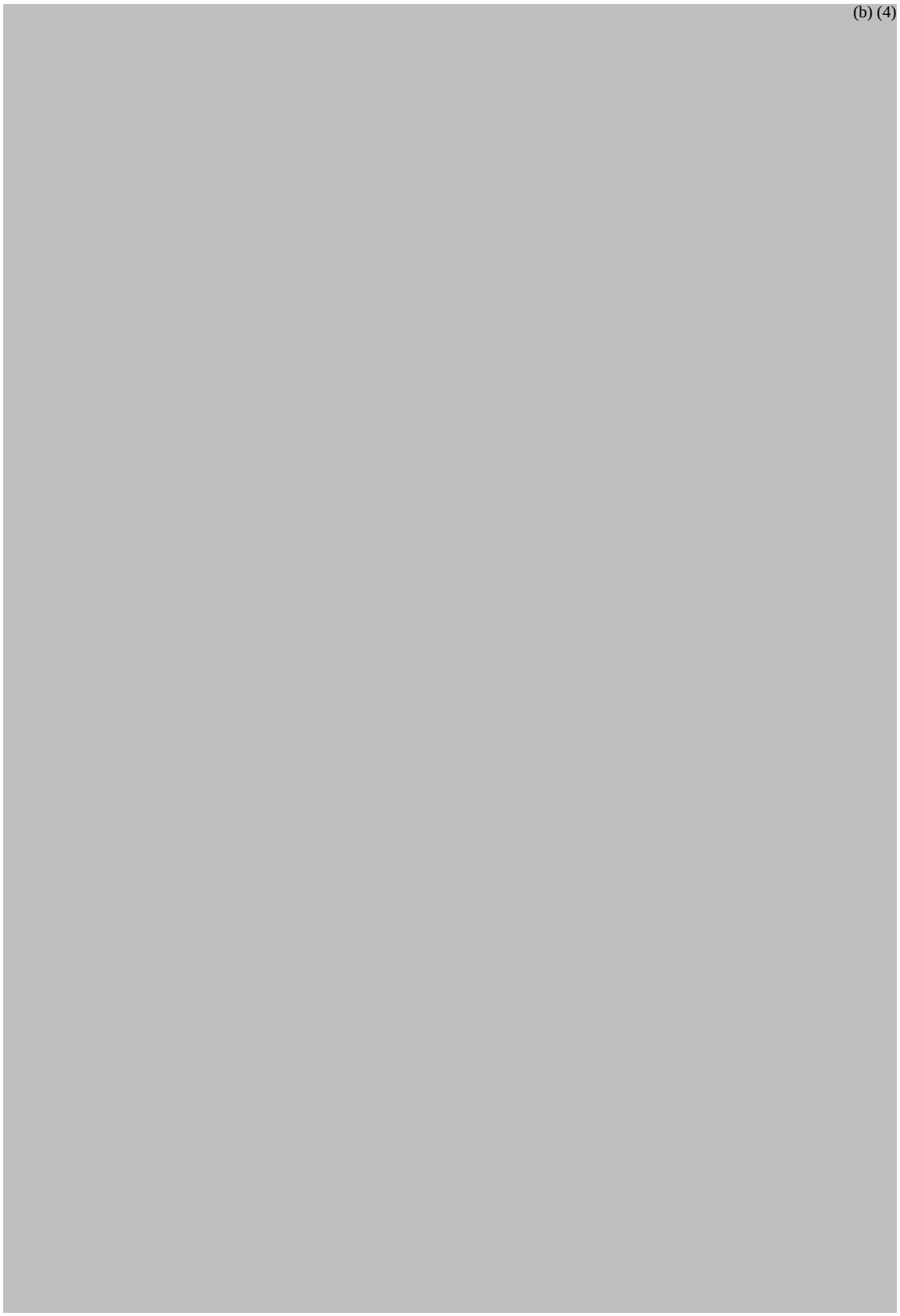
(b) (4)

(b) (4)

(b) (4)



(b) (4)



(b) (4)

[redacted]

Additional comment from Agrivida, Inc.

While conducting the above analysis, we noted an error in the formatting of the sequence presented in Appendix 1 of AGRN-032. Approximately (b) (4) nucleotides (nt (b) (4)) at the junction between the right border flanking genomic sequence and the T-DNA were mistakenly indicated in uppercase. These nucleotides should have been indicated in lowercase, to denote that they are part of the sequence that was derived from maize chromosome (b) (4) and are not part of the T-DNA. An amended version of Appendix 1 is shown below with the revised formatting of the text (note: no changes have been made to the nucleotides in this sequence other than formatting).

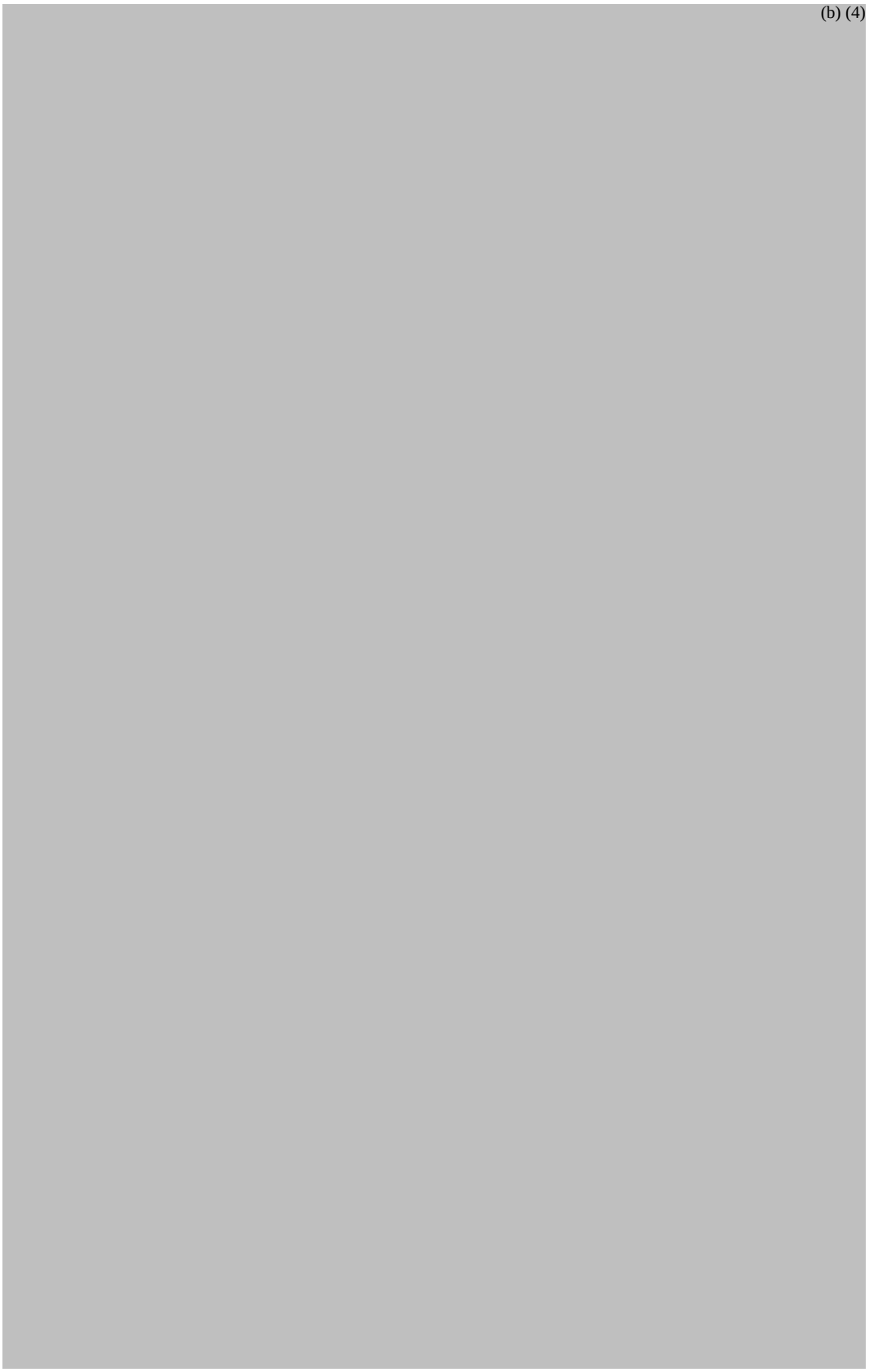
Appendix 1

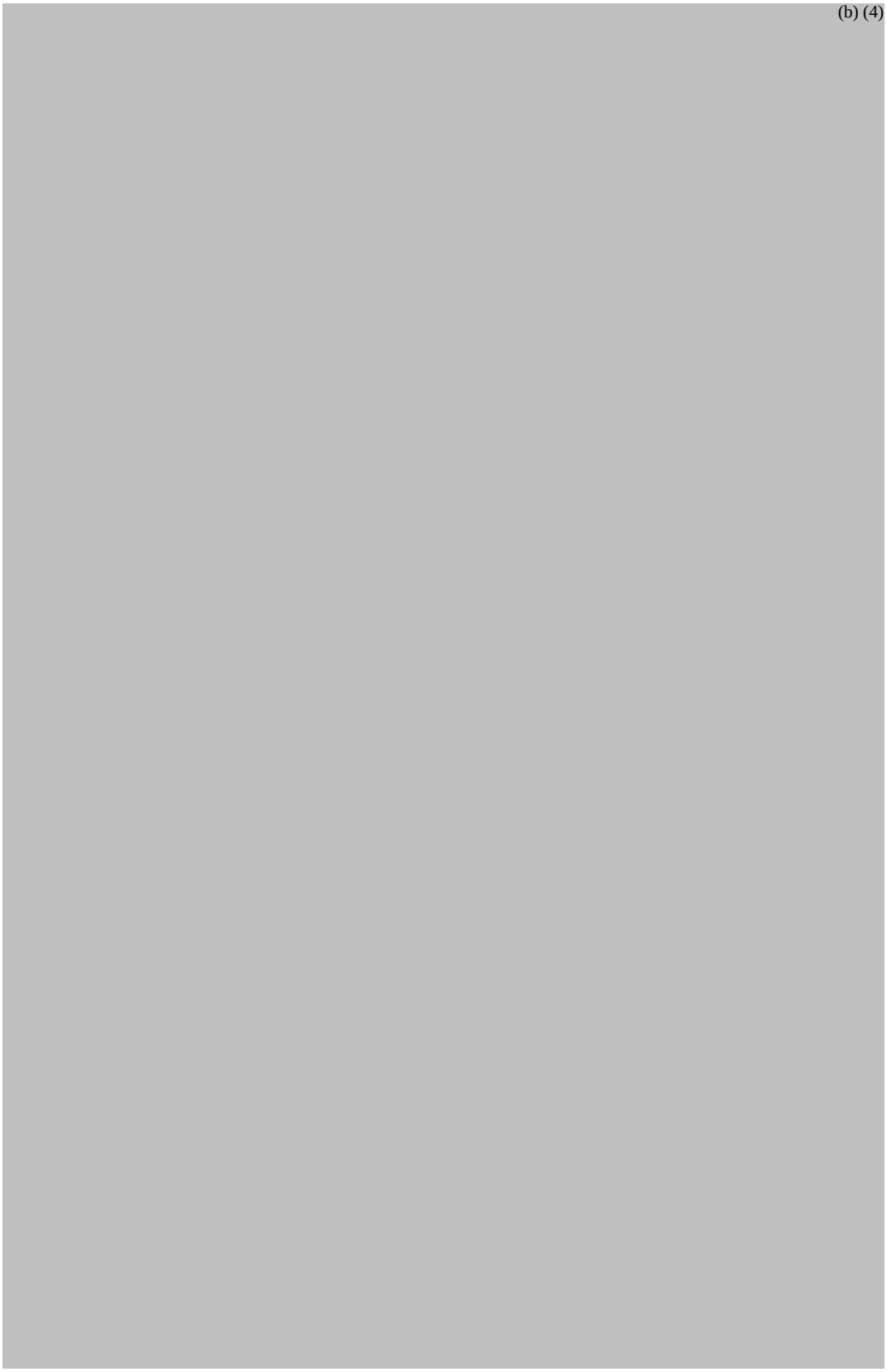
Nucleotide sequence of the locus of T-DNA insertion and the flanking maize genomic DNA in Event PY1203. Maize genomic DNA sequence is presented in lower case letters while the sequence of the T-DNA insert is presented in upper case. Table 3 indicates the positions of key elements with respect to the locus sequence.

(b) (4)

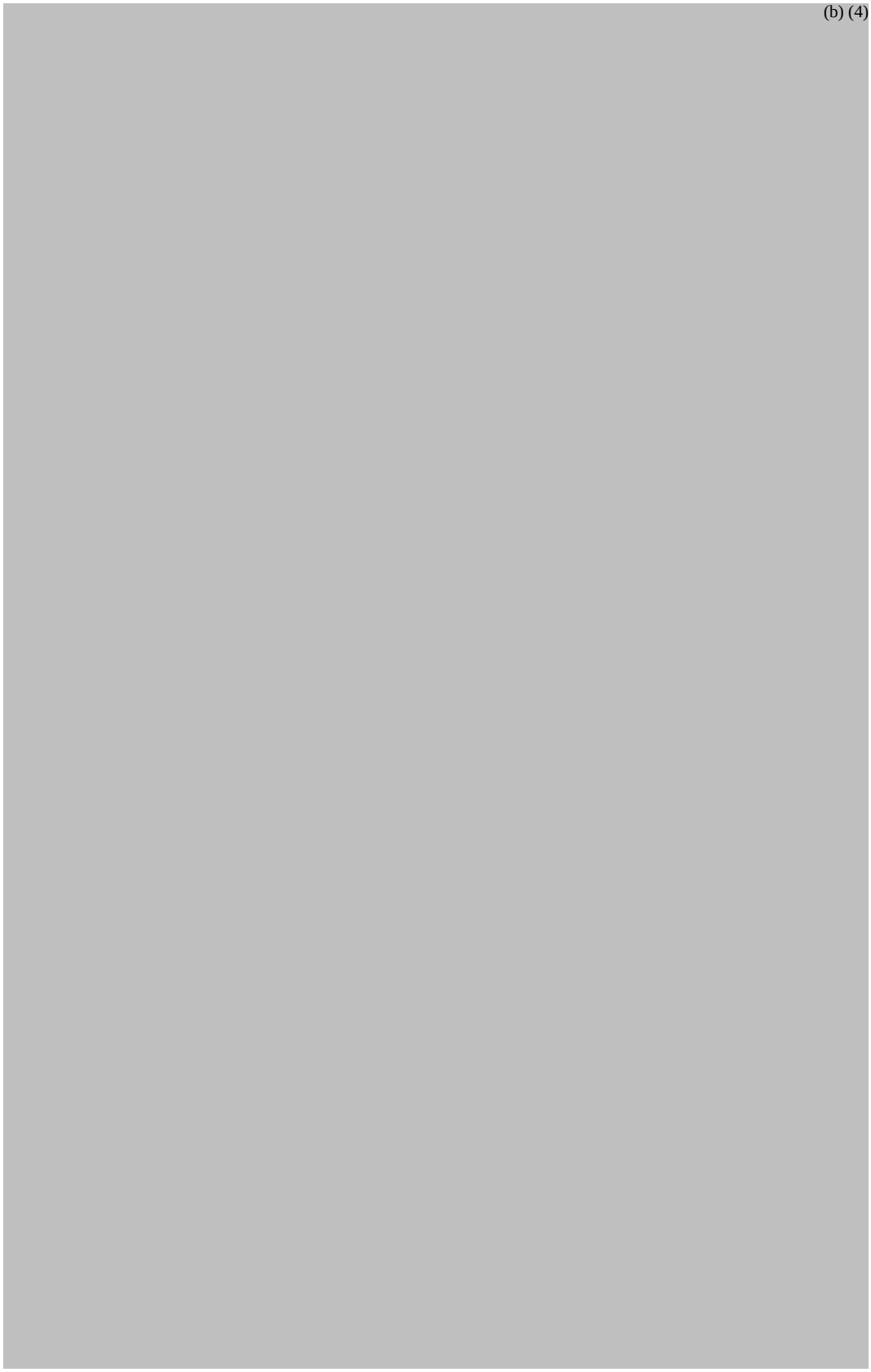
[redacted]

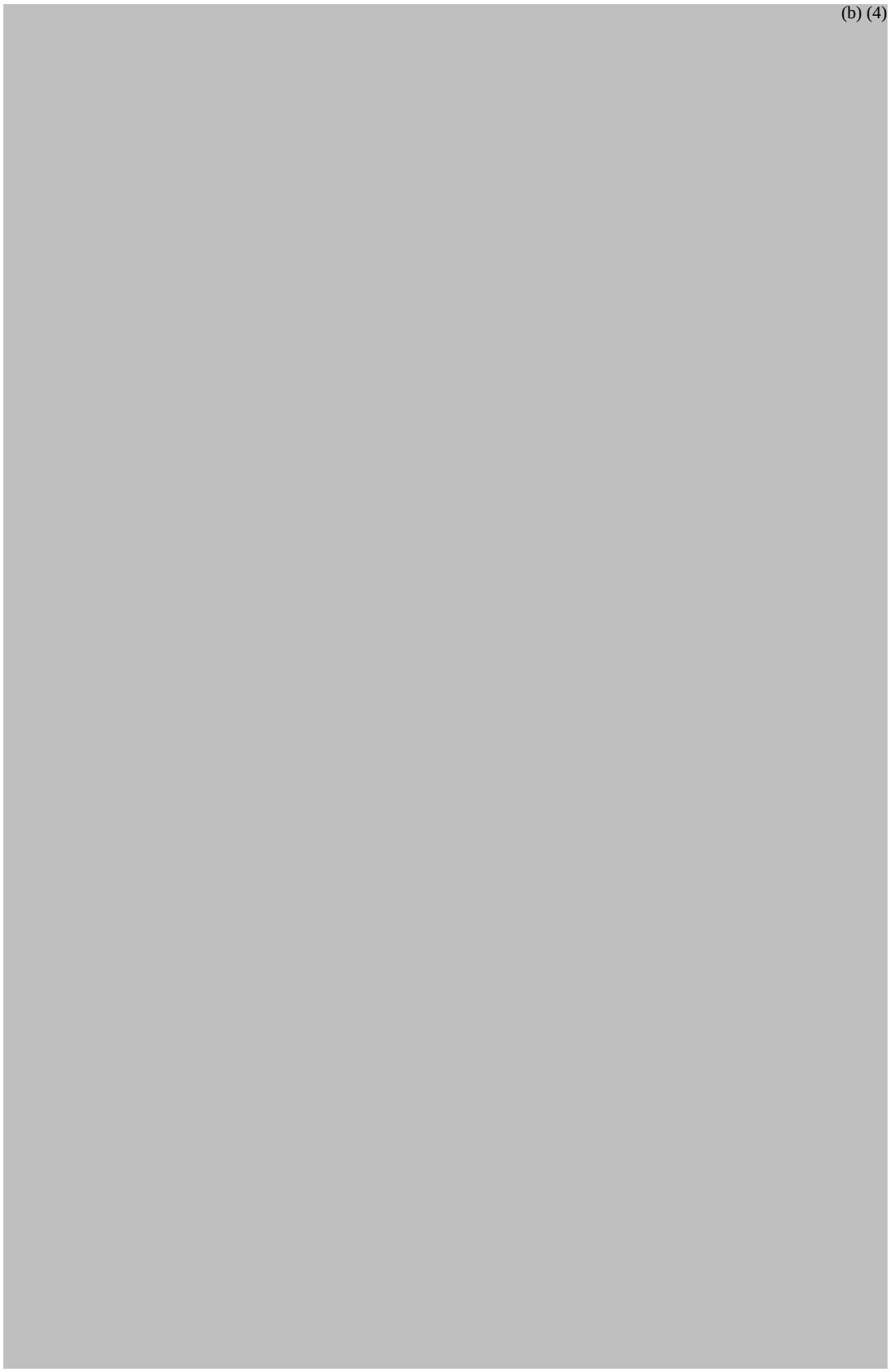
(b) (4)



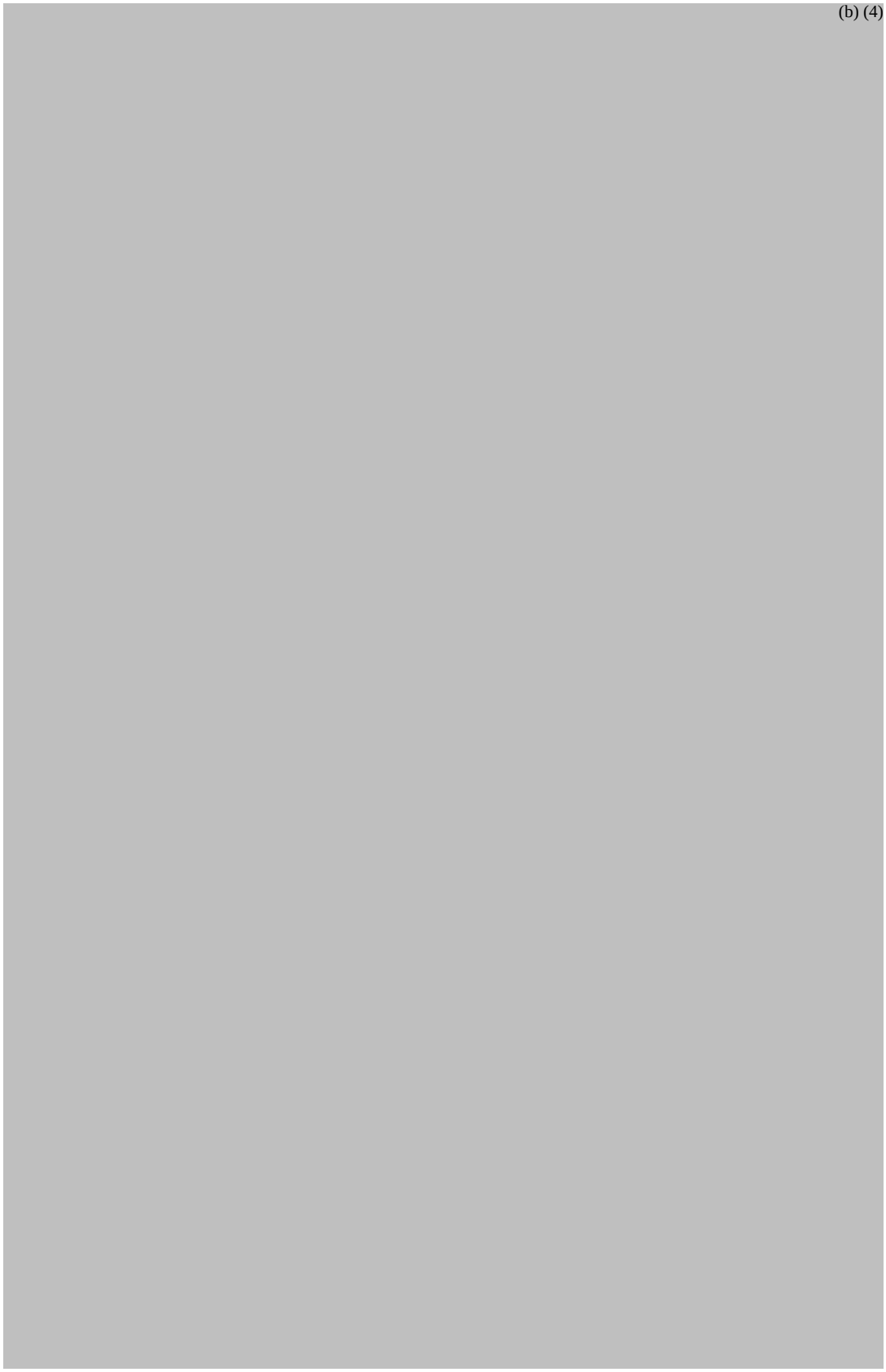

(b) (4)

(b) (4)




(b) (4)

(b) (4)



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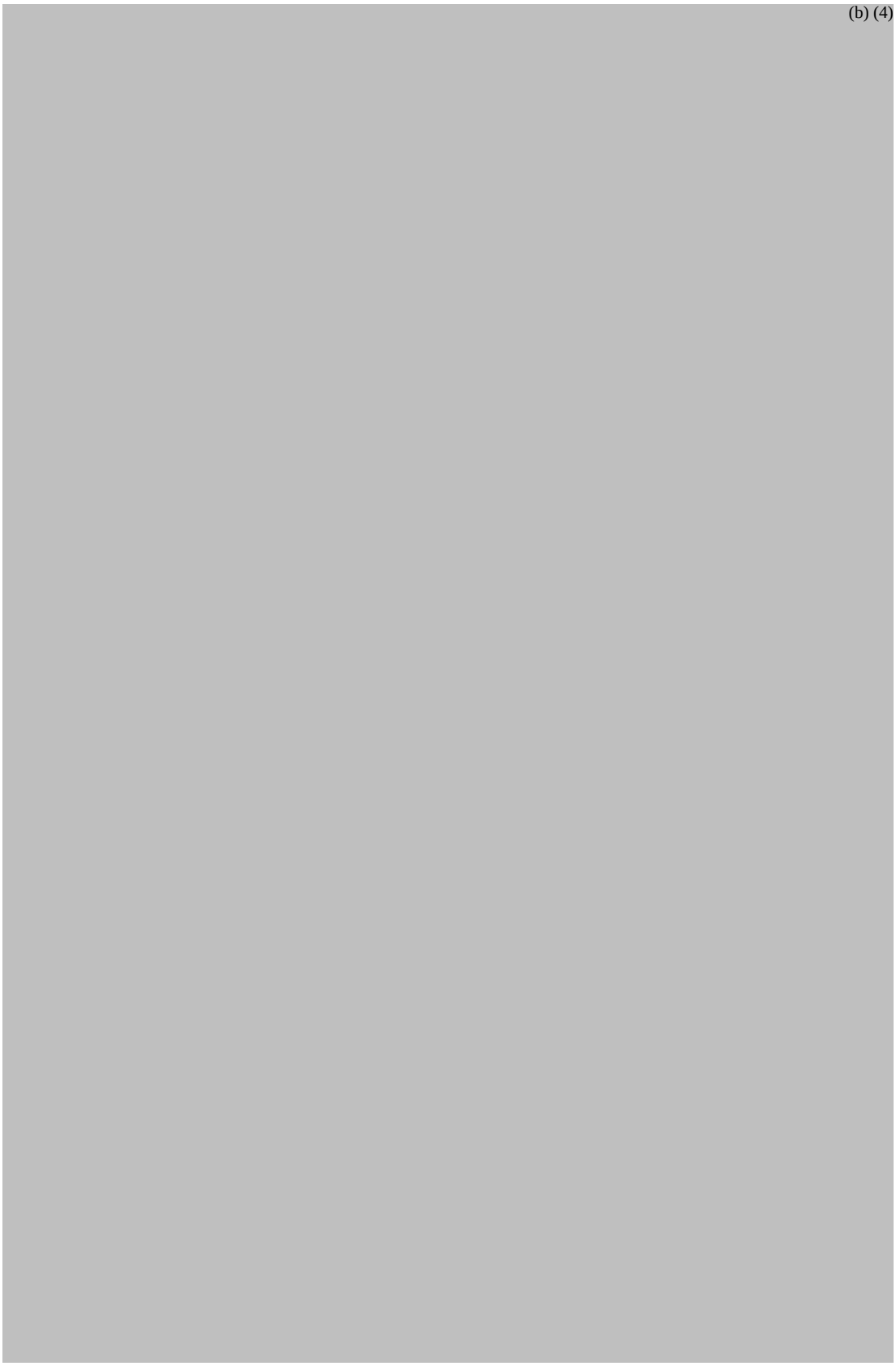

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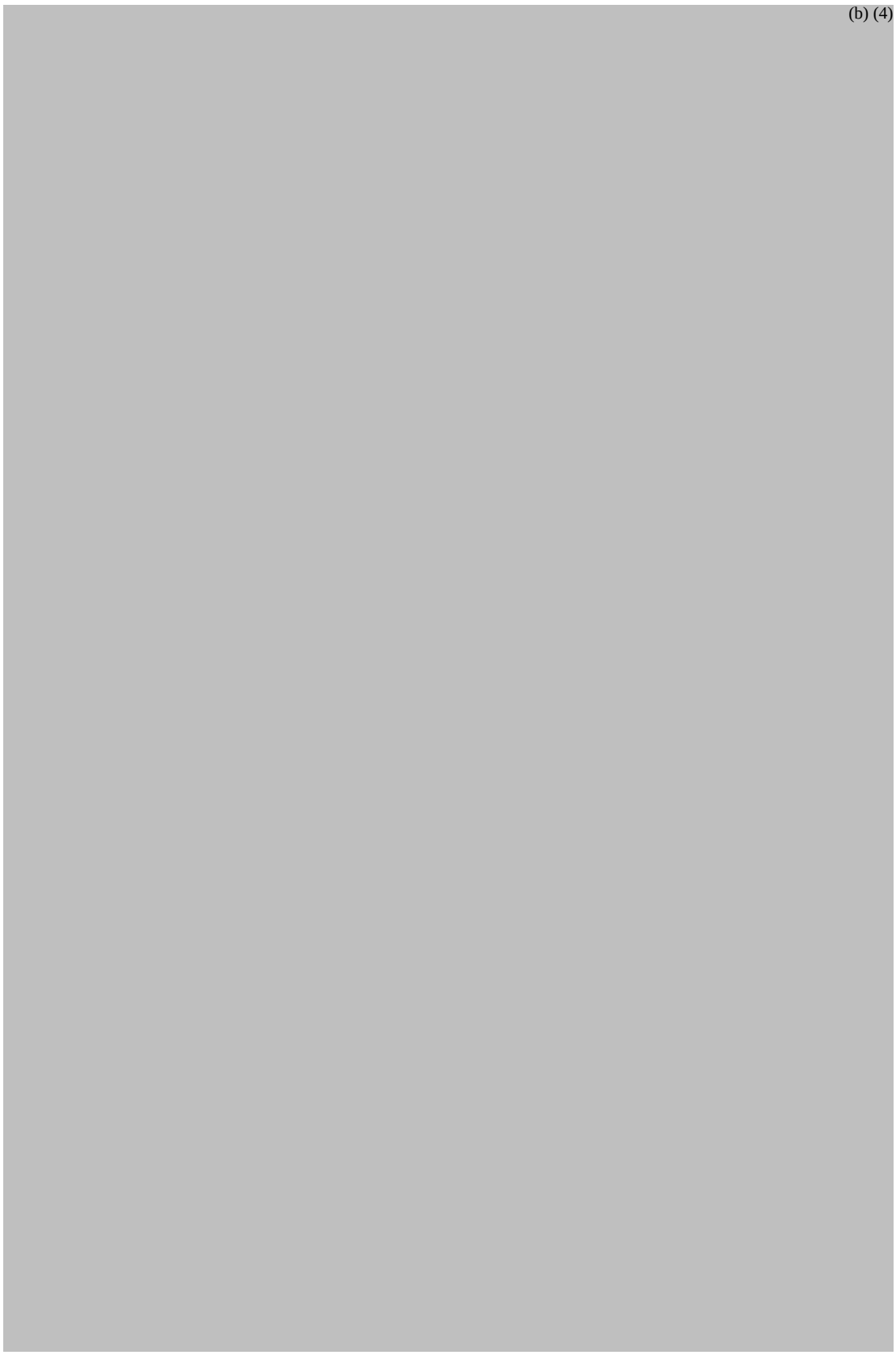

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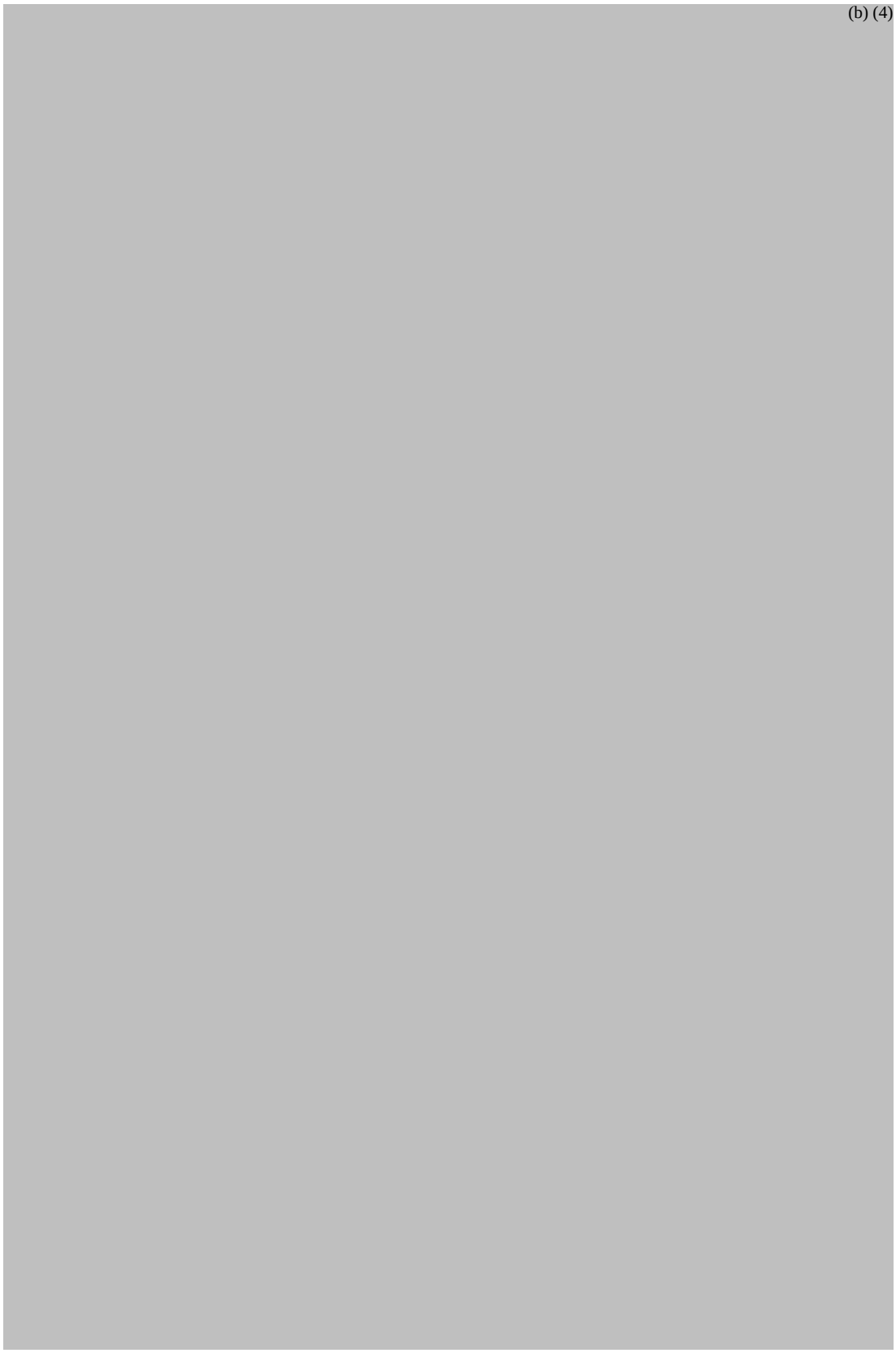

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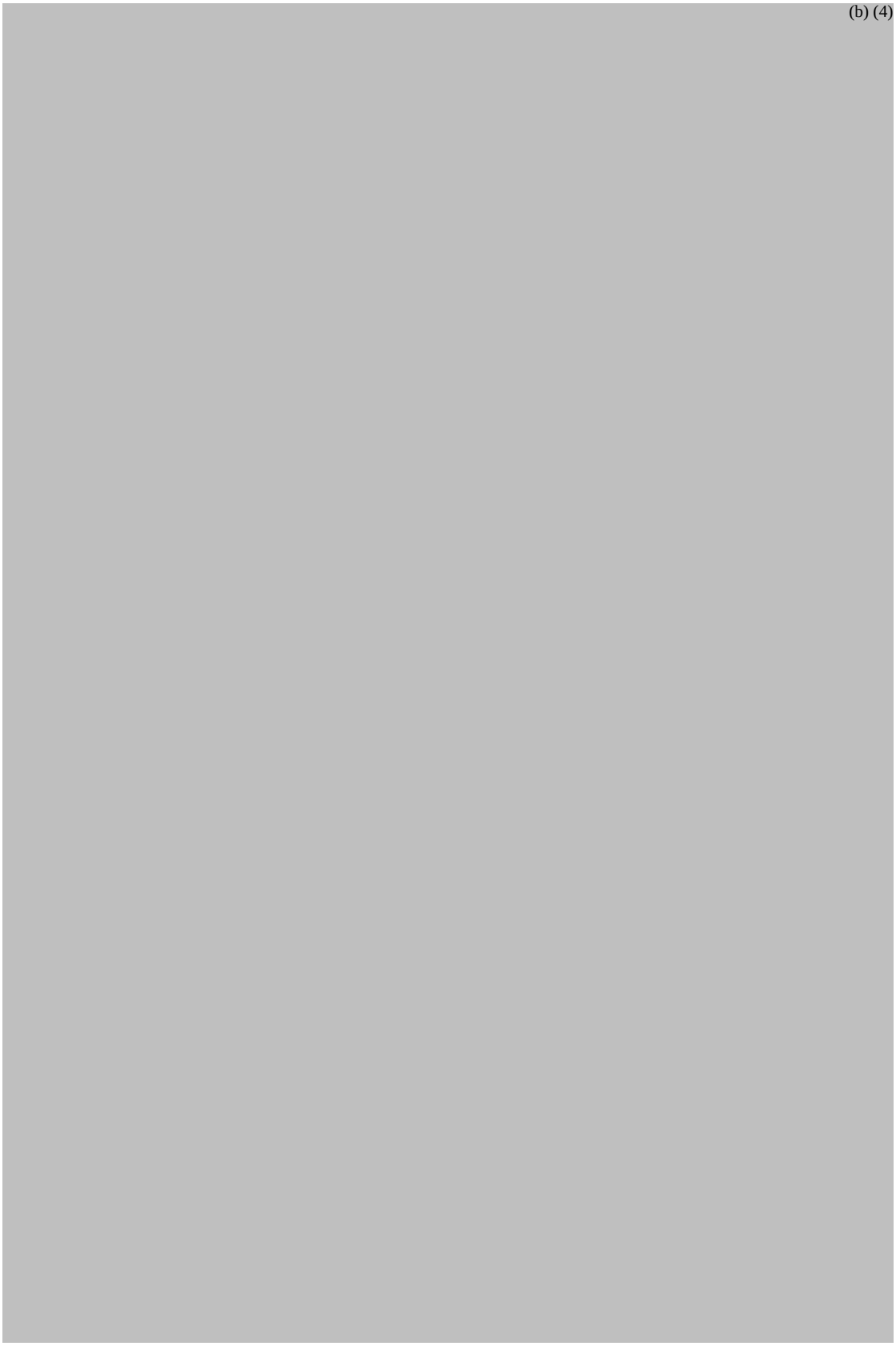

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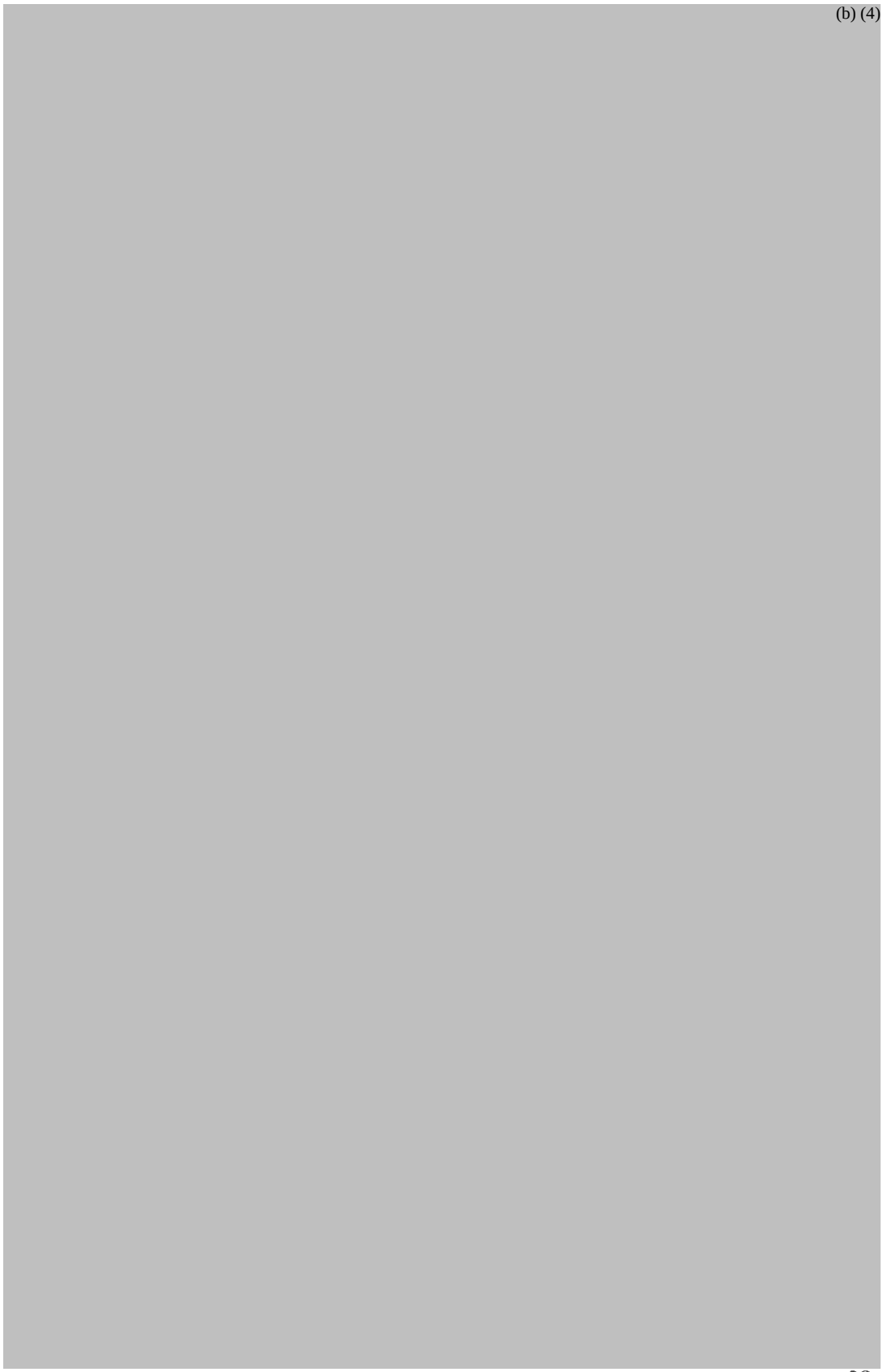
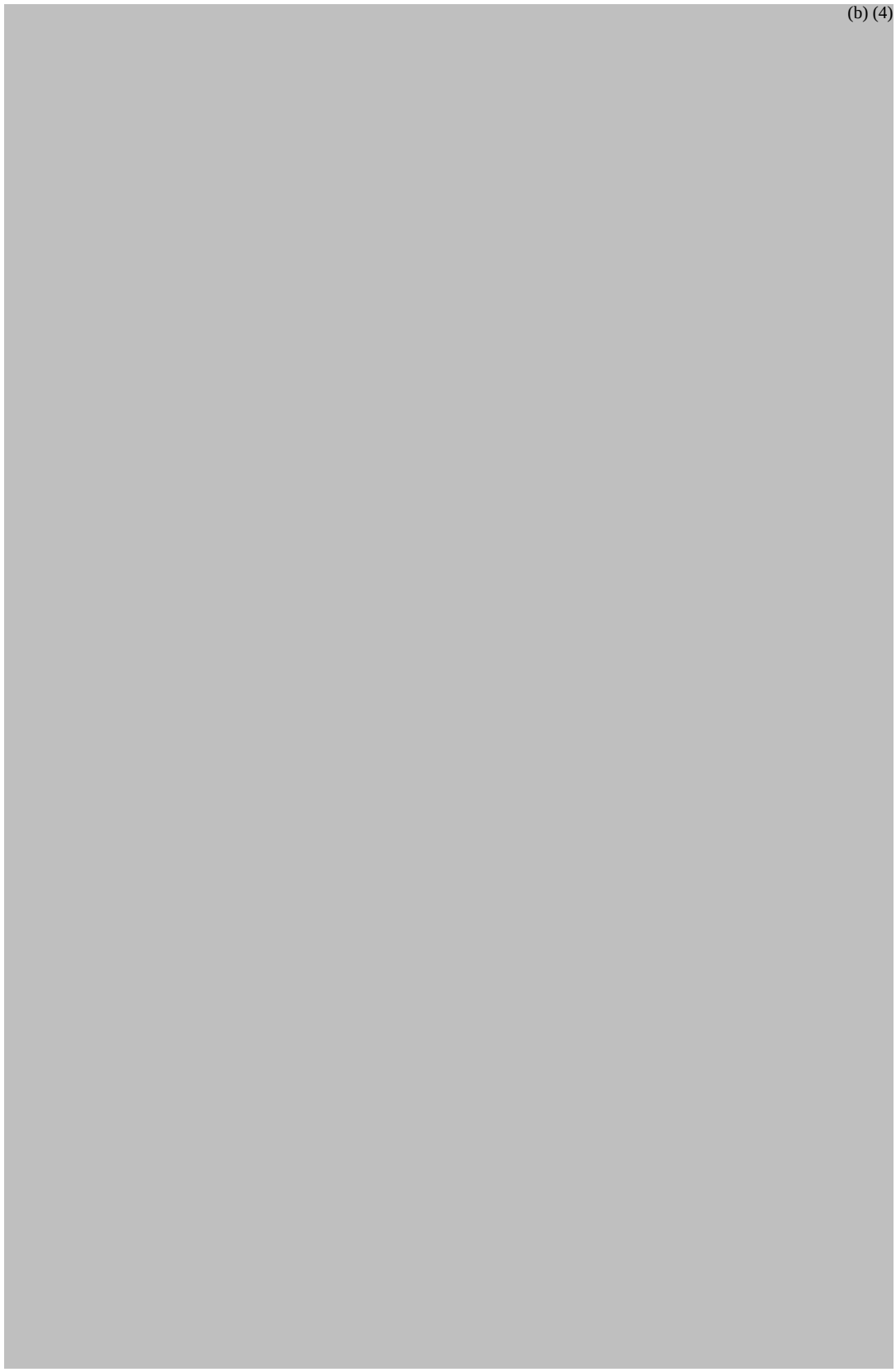
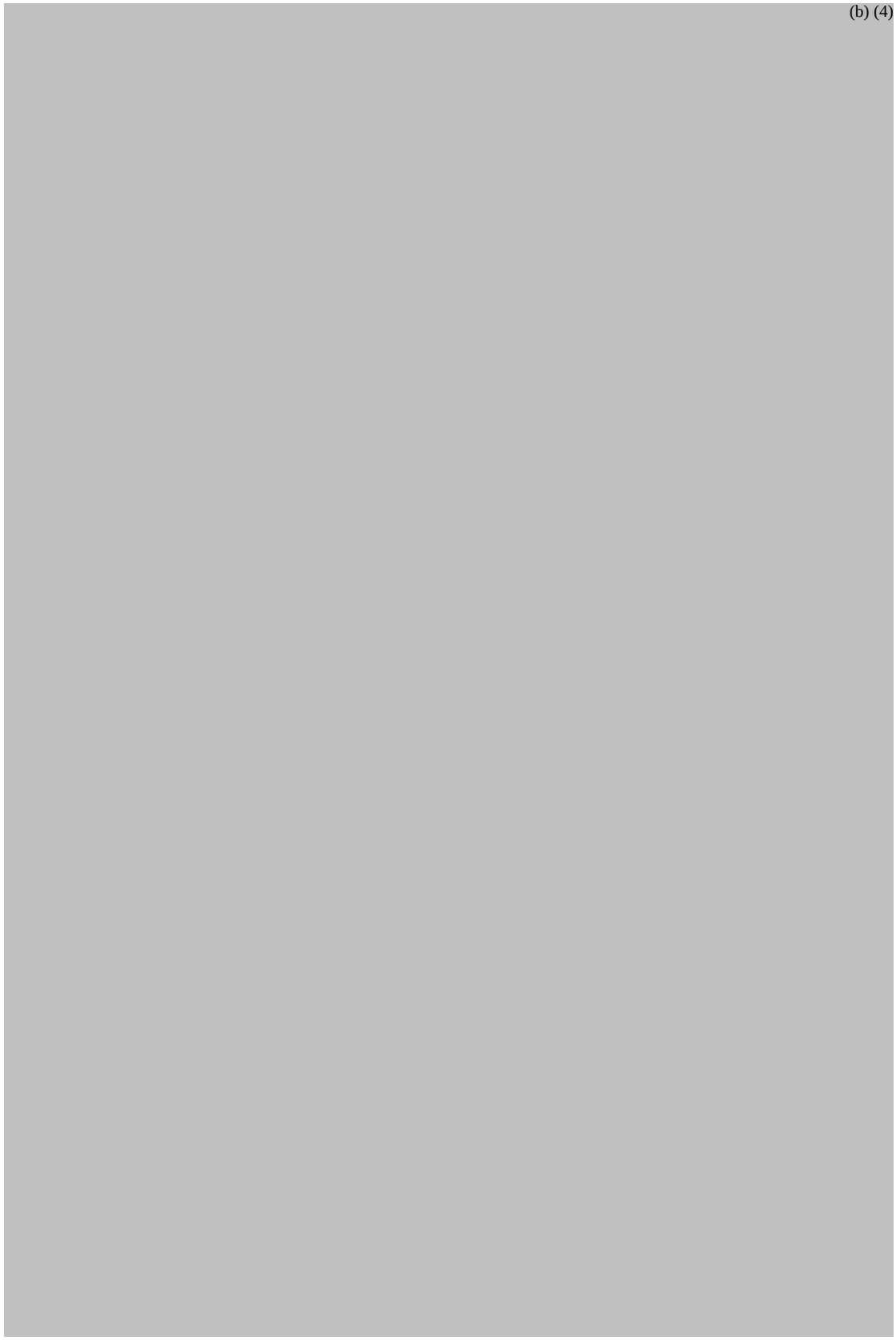



(b) (4)

(b) (4)



(b) (4)