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Cover Page Footnote

The authors wish to express their deepest gratitude and sincere appreciation to Prof. (R) Dr. M. Herman as the country coordinator of Agricultural Biotechnology Support Project II, which supported late blight resistance potato research in Indonesia

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Abstract

The genetically engineered potato cultivar Katahdin SP951 is resistant to late blight disease, which is caused by *Phytophthora infestans*. The biosafety and food safety of this cultivar should be assessed prior to its commercialization. Toxicity is one of the parameters tested in the food safety evaluation of transgenics. Toxicity testing includes the bioinformatics analysis of the homology of the RB protein of Katahdin SP951 with known proteins. Therefore, this study aimed to perform the bioinformatics analysis of Katahdin SP951 RB protein against homologous toxin proteins. Bioinformatics analysis was conducted by first translating the *RB* gene into an amino acid sequence by using the Emboss Transeq program from the EMBL-EBI website. The Shuffle Protein Program was then applied to obtain the randomized amino acid sequences of the RB protein. The Basic Local Alignment Search Tool (BLAST) Protein search program was utilized to identify proteins with high similarity and homology. Moreover, BLAST Suite-2 was used to analyze the homology between two or more sequence alignments. Results showed high homology between the sequences of the RB protein and those of known resistance (*R*) proteins with an E value of less than 0.22. This result may be attributed to the presence of numerous *R* proteins in plants. Further analysis indicated that the sequence of the RB protein had extremely nonsignificant homology with sequences of proteins known to be toxic in the Entrez protein database of National Center for Biotechnology Information. Therefore, the RB protein is nontoxic.

Keywords: bioinformatics, Katahdin SP951, Phytophthora infestans, toxicity

Introduction

Potato (*Solanum tuberosum* L.) is a starchy vegetable crop that is the fourth most important food crop after wheat, maize, and rice. In Indonesia, the potato is one of several strategic commodities necessary to support national food security [1].

Late blight disease, which is caused by *Phytophthora infestans*, is a major cause of reductions in potato yields [2]. It can result in 100% yield loss and is usually prevented through high-frequency fungicidal spraying. However, the intensive use of fungicidal spray not only results in environmental pollution but increases production costs. Therefore, several efforts have been made to minimize spraying. One of these approaches is the production of transgenic potato plants that are resistant to *P. infestans*.

The transgenic potato line SP951 was generated by inserting the *RB* gene from *Solanum bulbocastanum*, a wild diploid potato species, into the Katahdin potato variety. The *RB* gene is a resistance (*R*) gene and is thus

highly similar to other *R* genes. It is responsible for the high resistance of *S. bulbocastanum* to late blight [3]. Therefore, potato varieties transformed with the *RB* gene are expected to be resistant to late blight disease.

In Indonesia, the transgenic potato cultivar Katahdin SP951 has been conventionally crossed with the nontransgenic varieties Atlantic or Granola. Intensive field tests have been performed to verify the resistance of the progenies of crosses between these varieties and to select those with high levels of resistance [4]. The Indonesian government regulation *Peraturan Pemerintah no. 21 tahun 2005* states that the biosafety of a transgenic variety must be assessed prior to its release. Biosafety assessment includes subjecting the transgenic product to toxicity tests. A study on the oral toxicity of juice and powder from the transgenic potato cultivar Katahdin SP951 revealed that the nontransgenic variety Katahdin and its hybrid lines showed no effect on the clinical signs of mice [5].

In the present work, a bioinformatics analysis was conducted to determine the possibility that the amino

acid sequence of the RB protein shows significant homology with the sequences of proteins identified as toxins in the latest posting of the National Center for Biotechnology Information (NCBI) Entrez Protein Database (<https://www.ncbi.nlm.nih.gov/protein/>) and to support the results of toxicity tests. The database contains more than 5 millions amino acid sequences. The RB protein sequence was systematically compared with all publically available protein sequences in the database by using the Basic Local Alignment Search Tool Protein (BLASTP) search program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) [6]. Statistically significant amino acid sequence homologies were determined on the basis of the E value, which describes the number of hits expected to be seen by chance when a database of a certain size is searched. A high E value indicates a low match between two compared sequences, whereas a low E value indicates a high match between two compared sequences. An E value that is equal to or close to zero indicates that the query sequence and its result are likely related. The method of using E value to establish the amino acid sequence homology has been applied to transgenic products. The E value has been used to set a significant threshold for the assessment of the homologies of amino acid sequence in mEPSPS [7] and Vip3Aa20 [8]. In the present study, the RB protein of transgenic Katahdin SP951 was analyzed and compared with previously identified toxin proteins on the basis of amino acid sequence homology.

Materials and Methods

DNA and amino acid sequences. A 2,913-bp *RB* gene sequence (accession no. AY303171 [9]) was obtained from the leaves of the transgenic potato cultivar Katahdin SP951 [10] and was then translated into an amino acid sequence by using the Emboss Transeq program from EMBL-EBI

(http://www.ebi.ac.uk/Tools/st/emboss_transeq/) with standard parameters.

Shuffling of the amino acid sequence of the RB protein. The RB protein sequence was randomly shuffled (randomly rearranged) to produce five different shuffled amino acid sequences with the same amino acid composition as the initial RB protein. The online Shuffle Protein Program (http://www.bioinformatics.org/sms2/shuffle_protein.html) in the Sequence Manipulation Suite 2 [11] was utilized to shuffle the RB protein sequence.

Homology search for the RB protein. The E value was established by using the BLASTP search program from NCBI GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). The E value was used to search for RB protein homologs. The five shuffled amino acid sequences were input as the query sequences in the program with default parameters. The lowest E value was then used as the standard threshold in the homology search for the RB protein. The amino acid sequence of the RB protein was used as the query sequence in the BLASTP program. The parameters set for the search are shown in Table 1.

Results and Discussion

The *RB* gene (accession no. AY303171 [9]) obtained from transgenic potato Katahdin event SP951 has a length of 2,913 bp and codes for 971 amino acids (Figure 1). The amino acid sequence of the *RB* gene was then randomized by using the Shuffle Protein Program to obtain five shuffled amino acid sequences (data not shown). These sequences were designated as Shuffle 1, 2, 3, 4, and 5. The amino acid composition of the sequences is the same as that of the RB protein. Matches for the five sequences in the BLASTP program featured high E values (Table 2). The lowest E value obtained from the five shuffled amino acid sequences was 0.22.

```

MAEAFIQVLLDNLTSFLKGEVLVLLFGFQDEFQRLSSMFSTIQAVLEDAQEKLNNKPLEN
WLQKLNAAATYEVDDILDEYKTKATRFSSQSEYGRYHPKVI PFRHKVGRMDQVMKLLKAI A
EERKNFHLHEKIVERQAVRRETGSVLTEPQVYGRDKEKDEIVKILINNVSDAQHLSVLPI
LGMGGGLGKTTLAQMVFNDQRVTEHFHSKIWICVSEDFDEKRLIKAIVESIEGRPLLGEMD
LAPLQKKLQELLNGKRYLLVLDVWVEDQQKWANLRAVLKVGASGASVLT'TTRLEKVGSI
MGTLPQPYELSNLSQEDCWLLFMQRAFQGHQEEINPNLVAIGKEIVKKS GGVPPLAAKTGGI
LCFKREERAWEHVRDSP IWNLPQDESSILPALRLSYHQPLDLKQCFAYCAVFPKDAKMK
KEKLISLWMAHGFLLSKGNMELEDVGDEVWKELYLRSFFQEI EVKDGKTYFKMHDLIHDL
ATSLFSANTSSNIREINKHSYTHMMSIGFAEVVFFYTLPPLEKFI SLRVNLGdstFNK
LPSSIGDLVHLRYLNLYGSGMRS LPKQLCKLQNLQTLDLQYCTKLCCLPKETS KLGSLRN
LLLDGSQSLT CMPPRIGSLTCLKTLGQFVVGRRKKG YQLGELGNLNLYGSIKISHLERVKN
DMDAKEANLSAKGNLHLSMSWNNFGPHIYEESEVKVLEALKPHSNLTS LKIIYGFGRGIHL
PEWMNHSV LKNIVSILISNFRNCSCLPFFGDLPCLESLELHWGSADVEYVEEVDIDVHSG
FPTRIRFP SLRKLDIWDFGSLKGLLKEGEEQFPVLEEMI IHECPFLTSSNLRALTS LR
ICYNKVATSFPEEMFKNLANLKYLTISRCNNLKE LPTSLASLNALKSLKIQLCCALES LP
EEGLEGLSSLTELFVEHCNMLKCLPEGLQHLTTLTSLKIRGCPQLIKRCEKGI GEDWHKI
SHIPVNIYI*

```

Figure 1. Amino Acid Sequence of the RB Protein from the Transgenic Potato Katahdin Event SP951. Translation was Performed by Using the Emboss Transeq Program

Table 1. Parameters Used for the Identification of RB Protein Homologs. *) E Value Obtained After BLASTP by Using Five Shuffled Amino Acid Sequences from the RB Protein

| Parameter | Entry |
|-------------------------------|-------------------------------------|
| Query sequence | RB protein sequence |
| Database | Nonredundant protein sequences (nr) |
| Program selection (algorithm) | BLASTP (protein-protein BLAST) |
| Maximum target sequences | Max (20000) |
| Expect threshold (E value) | 0.22 * |
| Word size | 3 |
| Max match in query range | 0 |
| Matrix | Blosum62 |
| Gap costs | Existence: 11 extension: 1 |
| Compositional adjustment | Conditional |

Table 2. E values Obtained from the BLASTP Program Using Five Shuffled Amino Acid Sequences (Shuffle 1, 2, 3, 4, and 5)

| Shuffled amino acid sequence | E value |
|------------------------------|----------|
| Shuffle 1 | 2.1–8.2 |
| Shuffle 2 | 0.69–8.9 |
| Shuffle 3 | 4.6–9.0 |
| Shuffle 4 | 0.22–9.6 |
| Shuffle 5 | 1.6–8.1 |

The homology search for the RB protein was conducted by using the BLASTP program with the parameters described in Table 1. The search yielded 26,537 proteins with E values of less than 0.22 (data not shown). The presence of conserved domains, such as the NB-ARC and LRR domains, in the RB protein sequence increased the number of matches. The homology search yielded 248 proteins with E values of 0.0 from 32 species. Those proteins included 194 RB proteins, R proteins, and domains of R proteins, as well as 64 entries for hypothetical and unnamed proteins (data not shown).

Shuffle 1, 2, 3, 4, and 5 provided high E values with their matching proteins in the BLASTP program (Table 2). A high E value indicates that the matches are likely nonsignificant and may be attributed to the fact that the shuffle amino acid sequences do not occur naturally but only occur as the result of randomization by the Shuffle Protein Program. The lowest E value obtained for the five shuffled amino acid sequences was 0.22. This value was consequently considered as the threshold for significant matches for homologous RB proteins.

An E value of 0.22 is considered to be high, and therefore, this value would likely to identify a high number of protein from GenBank as homologs to RB proteins. Indeed, the results of homology search using the BLASTP program with the parameters described in Table 1 yielded 26,537 proteins. The homologs of the RB proteins are

present in *Solanum* and other plant species. For example, RGA1, RGA2, RGA3, RGA 4, and RGA 5 are blight-resistant proteins that are highly conserved in several species [12,13]. In addition, the presence of conserved domains, such as the NB-ARC and LRR domains, in the RB protein sequence increased the number of matches.

Although 248 proteins from 32 plant species have an E value of 0.0, only 25 proteins share more than 70% identity with the RB protein (Table 3). In other words, there are 25 short protein sequences matches with 70% identity to RB proteins. These short sequences are possibly conserved regions present in the RB protein.

Given that EYU29791.1 is a hypothetical protein, its identity as an actual protein and its existence remain unconfirmed in the absence of real experimental data [14]. This hypothetical protein was derived from *Erythranthe guttata* and *Mimulus guttatus*. It contains a region that was identified as Toxin 10, which is an insecticidal crystal toxin found in *Bacillus* strains. The region of Toxin 10 spans from amino acid 4 to amino acid 120 [15]. BLASTP results showed that EYU29791.1 and the RB protein share a homology of only 23% (Table 4). The Toxin 10 region of EYU29791.1 was aligned against the RB protein sequence by using the BLAST Suite-2 sequence program to emphasize these results as explained below.

Table 3. Search Results for RB Protein Obtained by Using the BLASTP Program with an E value of 0.0 and Identity of More Than 70%

| No. | Protein Description | Ident | Assession |
|-----|---|-------|----------------|
| 1 | RecName: Full = Disease resistance protein RGA2; AltName: Full = Blight resistance protein RPI; AltName: Full = RGA2-blb [<i>Solanum bulbocastanum</i>] | 99% | Q7XBQ9.1 |
| 2 | Late blight resistance protein Rpi-sto1 [<i>Solanum stoloniferum</i>] | 99% | ACI25288.1 |
| 3 | Late blight resistance protein Rpi-pt1 [<i>Solanum stoloniferum</i>] | 99% | ACI25289.1 |
| 4 | Disease resistance protein RGA2, putative [<i>Solanum bulbocastanum</i>] | 97% | AAP45188.2 |
| 5 | Disease resistance protein RGA2, putative [<i>Solanum bulbocastanum</i>] | 95% | AAP45164.2 |
| 6 | PREDICTED: disease resistance protein RGA2-like [<i>Solanum tuberosum</i>] | 81% | XP_006352943.1 |
| 7 | Blight resistance protein RGA3 [<i>Solanum bulbocastanum</i>] | 80% | AAR29071.1 |
| 8 | RecName: Full = Putative disease resistance protein RGA1; AltName: Full = RGA3-blb [<i>Solanum bulbocastanum</i>] | 79% | Q7XA42.2 |
| 9 | PREDICTED: putative disease resistance protein RGA1-like [<i>Solanum tuberosum</i>] | 79% | XP_006364463.1 |
| 10 | Blight resistance protein SH10 [<i>Solanum tuberosum</i>] | 81% | AAR29074.1 |
| 11 | RB [<i>Solanum verrucosum</i>] | 80% | ABO28718.1 |
| 12 | RB [<i>Solanum verrucosum</i>] | 80% | ABO28722.1 |
| 13 | Blight resistance protein T118 [<i>Solanum tarijense</i>] | 80% | AAR29076.1 |
| 14 | Blight resistance protein SH20 [<i>Solanum tuberosum</i>] | 79% | AAR29075.1 |
| 15 | PREDICTED: putative disease resistance protein RGA1-like [<i>Solanum tuberosum</i>] | 75% | XP_006366885.1 |
| 16 | NBS-LRR resistance protein [<i>Solanum bulbocastanum</i>] | 76% | ACI16480.1 |
| 17 | RecName: Full = Putative disease resistance protein RGA3; AltName: Full = Blight resistance protein B149; AltName: Full = RGA1-blb [<i>Solanum bulbocastanum</i>] | 74% | Q7XA40.2 |
| 18 | Disease resistance protein RGA2, putative [<i>Solanum bulbocastanum</i>] | 76% | AAP45185.2 |
| 19 | PREDICTED: LOW QUALITY PROTEIN: putative disease resistance protein RGA3 [<i>Solanum lycopersicum</i>] | 74% | XP_010325692.1 |
| 20 | Blight resistance protein [<i>Capsicum annuum</i>] | 72% | ADB43255.1 |
| 21 | Blight resistance protein B149 [<i>Solanum bulbocastanum</i>] | 73% | AAR29073.1 |
| 22 | PREDICTED: putative disease resistance protein RGA4-like [<i>Solanum tuberosum</i>] | 71% | XP_006352941.1 |
| 23 | Disease resistant protein rga3, putative [<i>Solanum bulbocastanum</i>] | 72% | AAP45181.2 |
| 24 | PREDICTED: putative disease resistance protein RGA1-like [<i>Solanum tuberosum</i>] | 73% | XP_006352944.1 |
| 25 | Blight resistance protein RGA5 [<i>Capsicum annuum</i>] | 75% | AFU51535.1 |

Table 4. Search Results for RB Protein Homologs Obtained by Using the BLASTP Program and Assessment of Toxic Properties

| Description | E value | Ident | Accession | Protein function | Toxicity | References |
|--|---------|-------|----------------------------|--|----------|------------|
| rp3-like disease resistance protein [<i>Sorghum bicolor</i>] | 2e-88 | 29% | ACE86402.1 | Controlling reaction against pathogenic fungi and its toxin | Nontoxic | [16] |
| rp3-like disease resistance protein [<i>Sorghum bicolor</i>] | 4e-88 | 27% | ACE86400.1 | Controlling reaction against pathogenic fungi and its toxin | Nontoxic | [16] |
| rp3-like disease resistance protein [<i>Sorghum bicolor</i>] | 4e-87 | 29% | ACE86396.1 | Controlling reaction against pathogenic fungi and its toxin | Nontoxic | [16] |
| Tsn1 [<i>Aegilops speltoides</i>] | 2e-64 | 30% | ADG84876.1 | Converting sensitivity against pathogenic fungal toxin | Nontoxic | [17] |
| Tsn1 [<i>Aegilops speltoides</i>] | 4e-64 | 30% | ADG84875.1 | Converting sensitivity against pathogenic fungal toxin | Nontoxic | [17] |
| Tsn1 [<i>Triticum turgidum</i> subsp. durum] [<i>Triticum durum</i>] | 1e-62 | 30% | ADG84878.1 | Converting sensitivity against pathogenic fungal toxin | Nontoxic | [17] |
| RecName: Full = Disease susceptibility protein LOV1; AltName: Full = Disease resistance protein RPP8-like protein 1; AltName: Full = Protein LONG VEGETATIVE PHASE1 [<i>Arabidopsis thaliana</i>] | 5e-55 | 28% | A7XGN8.1 | Converting sensitivity against pathogenic fungal toxin | Nontoxic | [18] |
| RecName: Full = Inactive disease susceptibility protein LOV1; AltName: Full = Disease resistance protein RPP8-like protein 1; AltName: Full = Protein LONG VEGETATIVE PHASE1 [<i>Arabidopsis thaliana</i>] | 2e-53 | 28% | A9QGV6.1 | Converting sensitivity against pathogenic fungal toxin | Nontoxic | [19] |
| hypothetical protein MIMGU_mgv1a017786 mg, partial [<i>Erythranthe guttata</i>] [<i>Mimulus guttatus</i>] | 1e-21 | 23% | EYU29791.1 | hypothetical protein containing Bacillus Toxin 10 | Nontoxic | [15] |
| PREDICTED: receptor-like protein 12 [<i>Malus domestica</i>] | 4e-05 | 40% | XP_008356243.1 | Receptor-like protein containing Alpha toxin that is not present in RB protein | Nontoxic | [20] |

Table 5. Alignment between the Toxin 10 Region (Amino Acid 4 to Amino Acid 120) from Protein EYU29791.1 and the RB Protein. Alignment was Performed by Using Sequences Generated by BLAST Suite-2

Unnamed protein product

Sequence ID:|cl|Query_58977 Length: 970 Number of Matches: 3

Range 1: 31 to 125

| Score | Expect | Method | Identities | Positives | Gaps |
|---------------|--------|---|------------|------------|----------|
| 25.0 bits(53) | 0.001 | Composition-based stats. | 19/98(19%) | 51/98(52%) | 7/98(7%) |
| Query | 5 | FEELSYLQSF LKAI---SPPQSSRNKRVD AFERQIKDAVHKLEDLID-FHISDQFLSASD | | | 60 |
| | | F+LS+S++A+ ++ NK+++++ A++++D++D+ S S+ | | | |
| Sbjct | 31 | FQRLSSMFSTIQAVLEDAQE KQLNNKPLENWLQKLNAATYEVD DILDEYKTKATRF SQSE | | | 90 |
| Query | 61 | DGLTFVFSQQLLEFRQEVSSFTNKILKTKKEEYDINRQN | | 98 | |
| | | G + +++FR +V ++++K + R+N | | | |
| Sbjct | 91 | YGR---YHPKVIPFRHKV GKRMDQVMK LKAI A EERKN | | 125 | |

Range 2: 55 to 91

| Score | Expect | Method | Identities | Positives | Gaps |
|---------------|--------|---|------------|------------|----------|
| 14.2 bits(25) | 4.5 | Composition-based stats. | 7/37(19%) | 19/37(51%) | 0/37(0%) |
| Query | 68 | SQQLLEFRQEVSSFTNKILKTKKEEYDINRQNQQQSDF | | 104 | |
| | | ++L +Q++++ T++ +EY QS++ | | | |
| Sbjct | 55 | NKPLENWLQKLNAATYEVD DILDEYKTKATRF SQSEY | | 91 | |

Range 3: 116 to 136

| Score | Expect | Method | Identities | Positives | Gaps |
|---------------|--------|--------------------------|------------|------------|----------|
| 13.5 bits(23) | 8.1 | Composition-based stats. | 7/21(33%) | 11/21(52%) | 0/21(0%) |
| Query | 15 | LKAISPPQSSRNKRVD AFERQ | 35 | | |
| | | LKAI+ +++ ERQ | | | |
| Sbjct | 116 | LKAIAEERKNFHLHEKIVERQ | 136 | | |

Table 6. Homology Search between XP_008356243.1 and the RB Protein Using the BLASTP Program. The Homologous Sequence Occured Outside the Alpha Toxin Region (Amino Acid 22 to 87) of XP_008356243.1

PREDICTED: receptor-like protein 12 [Malus domestica]

Sequence ID:ref|XP_008356243.1| Length: 810 Number of Matches: 1

Range 1: 182 to 256

| Score | Expect | Method | Identities | Positives | Gaps |
|----------------|--------|--|------------|------------|----------|
| 50.8 bits(120) | 4E-05 | Compositional matrix adjust. | 30/75(40%) | 43/75(57%) | 4/75(5%) |
| Query | 528 | LRVLNLGDSTF---NKL PSSIGDLVHLRYLNLYGSGMRS-LPKQLCKLQNLQTL DLQYCT | | | 583 |
| | | L+ LNL D++F +++PSSIG L +LRYLNL SG +P ++ +L L LD+ C | | | |
| Sbjct | 182 | LQSLNLADNSFVN XSRIPSSIGKLTNLR YLNLSDSGYSGKIPIEISRLTXLXVLDISLCK | | | 241 |
| Query | 584 | KLCCLPKETSKLGS L | 598 | | |
| | | C E+ L L | | | |
| Sbjct | 242 | HQCATKLESPXLSML | 256 | | |

The alignment results showed that homology between the Toxin 10 region of EYU29791.1 and the RB protein was 19%, which was lower than that between EYU29791.1 and the RB protein. In addition, the E value of the alignment drastically increased to 0.0001 (Table 5). These results indicate that the Toxin 10 region of EYU29791.1 and the RB protein have very low homology. Therefore, in contrast to Toxin 10, the RB protein lacks a toxic property.

XP_008356243.1 is a receptor-like protein from apple (*Malus domestica*). The region encompassing amino acid 22 to amino acid 87 of this protein contains an Alpha toxin.

Conclusion

The NCBI Entrez Protein Database revealed that the sequence of the RB protein from the transgenic potato cultivar Katahdin SP951 has extremely nonsignificant homology with that of any proteins known to be toxic to humans. Therefore, the RB protein is nontoxic.

Acknowledgments

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