



## STAY GREEN GENES FRAGMENT HOMOLOGY ANALYSIS OF INDONESIAN SORGHUM

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### SUMMARY

Stay-green (SGR) is an essential trait in sorghum associated with grain yield under drought and high-temperature stress conditions. The study sought to analyze the homology of the stay-green gene fragment in Indonesian sorghum cultivars, comparing it with the *SGR* gene sequences in the GenBank database. Two primer pairs, designated as *SGR\_1* and *SGR\_2*, got designed from the *SGR* gene of *Sorghum bicolor* and used to amplify seven sorghum genotypes. The DNA fragments of 300 bp and 1000 bp produced by *SGR\_1* and *SGR\_2*, respectively, underwent sequencing. Amino acid analysis of the seven sorghum genotypes resulted in high homology with senescence-inducible chloroplast *SGR*-protein 1 from *Zea mays* and *SGR*-chloroplastic of *Setaria italica*. An observation on a conservative region with the *SGR* domain noticed *SGR* genes, derived from sorghum genotypes, clustered separately with those from other *SGR* genes available in the GenBank database. The first group consisted of a sorghum genotype (Samurai 2), the second group consisted of Super 2, Numbu, and Kawali, while the third group consisted of PI-150-20-A, Pahat, and B69 with coefficient similarity of 10%, 14%, and 30%, respectively. Although Indonesian sorghum has a different group in GenBank, it has similar nucleotide and amino acid sequences, with identity values of 95%-100% and 51%-100%, respectively. The amino acid diversity of the DNA fragments of the *SGR* gene is highly potential to develop molecular markers, especially the stay green character. The finding will support a sorghum breeding efficiently and precisely, especially for yield improvement under drought-stress conditions.

**Keywords:** Drought stress, phylogenetic analysis, sequence identity, *SGR-like* (*SGRL*)

**Key findings:** The *SGR* gene sequence contains a conservative region and has high homology with the senescence-inducible chloroplast stay-green protein 1 and stay-green chloroplast proteins. These results prove that the *SGR* gene family in Indonesian sorghum cultivars is *SGR-like* (*SGRL*).

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### INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is a drought-tolerant crop that has the potential to be developed in areas with limited rainfall to

support food and energy diversification. Although sorghum is drought tolerant compared with maize and wheat, drought stress, during flowering and grain-filling, can significantly reduce grain yield (Besufekad and

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Bantte, 2013; Menezes *et al.*, 2015). The impact of drought stress on plants depends on its intensity and duration and the stage of plant development (Emendack *et al.*, 2018; Liang *et al.*, 2019; Rifka *et al.*, 2020). According to Assefa *et al.* (2010) and Batista *et al.* (2019), drought stress decreased sorghum yield to 36% and 55% in the vegetative and generative stages, respectively.

The stay-green trait is an important character related to drought tolerance in sorghum (Borrell *et al.*, 2014a). Identification of potential genes, such as, stay-green can be used as a material for developing molecular markers to support sorghum breeding programs (Rini *et al.*, 2017; Singh and Singh, 2018). Understanding the physiological and genetic basis of stay-green relating to drought-tolerance mechanisms proves fundamental for unraveling the molecular mechanism of the stay-green trait to develop new cultivars adapted to dry conditions by incorporating the functional stay-green trait into the sorghum breeding materials for yield improvement. Identification of homologous gene sequences responsible to such a drought-tolerance trait can be considered as a promising strategy to understand molecular mechanism of the stay-green trait in sorghum. The use of stay-green not only provides an understanding of the molecular mechanism of leaf senescence but also in the practical approach to increasing crop yields, especially on plants under biotic and abiotic stress (Kusaba *et al.*, 2013). However, the role of the *SGR* gene underlying the leaf-senescence mechanism is not clearly understood (Teng *et al.*, 2016).

The stay-green gene (*SGR*) encodes a target protein in chloroplasts, which is conserved in higher plants, and generally induced during the aging process of plants (Park *et al.*, 2007). Two subfamilies of *SGR* proteins, known as *SGR* and *SGR*-like (*SGRL*), found in monocot and dicot plants, have been reported (Barry, 2008). The difference between *SGR* and *SGRL* proteins lies in the cysteine motif present in the C-terminal section. The structure of the *SGR* protein consists of three domains, including the highly-conserved *SGR* domain, chloroplast transit peptide, and the variable C-terminal region (Aubry *et al.*, 2008; Jiao *et al.*, 2020). Other studies reported that *SGR* or *NON-YELLOWING 1 (NYE1)*, *SGR2 (NYE2)*, and *SGRL* exhibited as key regulators of chlorophyll degradation in Arabidopsis and rice plants, causing loss of green leaf pigment (senescence) (Jiang *et al.*, 2007; Park *et al.*, 2007; Rong *et al.*, 2013; Wu *et al.*, 2015). Many plant species, including Arabidopsis, have

stay-green phenotypes as a result of mutations in *SGR* orthologs (termed *NONYELLOWING [NYE]*) (Ren *et al.*, 2007). In addition, Armstead *et al.* (2007) identified a gene-controlling Mendelian green cotyledon traits, namely *STAYGREEN1* (also termed *STAYGREEN1; SGR1*) or *NONYELLOWING1 (NYE1)* in Arabidopsis (Cha *et al.*, 2002; Park *et al.*, 2007; Ren *et al.*, 2007). It indicates that *SGR* and *NYE* are of the same gene family or homologs and play a role as regulators of chlorophyll degradation.

In various plant species, studies reported the stay-green play a role in maintaining leaf greenness and photosynthetic activity under stress conditions (Spano *et al.*, 2003; Hörtensteiner, 2009; Kusaba *et al.*, 2013). Faye *et al.* (2021) reported that the stay-green character of sorghum plays a role in adapting to drought stress and correlates positively with the inhibition of chlorophyll catabolism (Gregersen *et al.*, 2013; Thomas and Ougham, 2014). Morphological characters associated with stay-green include an increase in grain yield (Luche *et al.*, 2015), increased resistance to stem rot (stem lodging) and spot blotch (Joshi *et al.*, 2007; Adeyanju *et al.*, 2016), and efficiency in water use (Christopher *et al.*, 2016). Several researchers reported that the overexpression of the *SGR* gene could be a regulator of chlorophyll degradation in plants (Armstead *et al.*, 2007; Ren *et al.*, 2007; Sakuraba, 2015) and resulted in the generation of reactive oxygen species (ROS), causing cell death in rice plants at the germination phase (Jiang *et al.*, 2011). Based on previous studies, *SGR2/NYE2* acts as a positive regulator of chlorophyll degradation in Arabidopsis plants (Delmas *et al.*, 2013; Wu *et al.*, 2015). Sakuraba *et al.* (2014a) reported otherwise, that *SGR2/NYE2* acts as a negative regulator. The difference in the two cases points to the presence of the *SGR* gene and differences in the genetic background of the plant, resulting in differences in gene function in chlorophyll metabolism (Nakano *et al.*, 2014; Sakuraba *et al.*, 2015).

The stay-green (*sgr*) mutant in several plant species showed the ability to retain chlorophyll (Alós *et al.*, 2008; Sato *et al.*, 2009). Based on their ability to retain green pigment and photosynthetic activity, stay-green mutants can be classified into two categories, namely, functional stay-green and non-functional stay-green (Hörtensteiner, 2009). Functional stay-green reveals a valuable trait for improving yield, increasing tolerance to drought and high temperatures stresses, while the non-functional stay-green

causes a decrease in the rate of photosynthesis (Thomas and Howarth, 2000). Reports on the phenomenon of stay-green mutations displayed in rice (Jiang *et al.*, 2007), sorghum (Kassahun *et al.*, 2010), Arabidopsis (Sakuraba *et al.*, 2012), maize (Kosgey *et al.*, 2013), and soybean (Nakano *et al.*, 2014; Fang *et al.*, 2014). The stay-green character mutant in Arabidopsis plants caused damage to the chlorophyll-degrading Mg<sup>++</sup> dechelatease enzyme so that it can retain chlorophyll (Gregersen *et al.*, 2013; Kuai *et al.*, 2018). In contrast, the stay-green mutant in rice (*ossgr*) displayed during photosynthetic activity inhibition at the grain filling period gave no increase in rice production compared with its wild type, reflecting that the stay-green mutant in rice was a part of the non-functional stay-green category (Park *et al.*, 2007; Jiang *et al.*, 2007). Knowledge of the genetic basis of stay-green is very complex and varies in plant species and therefore, the exploration of genes and molecular mechanisms underlying the stay-green trait in sorghum needs serious undertaking.

Sorghum genotypes possessing stay-green properties show a potential source of germplasm for the genetic improvement of sorghum. The study aims to analyze the homology of the stay-green gene fragment in Indonesian sorghum cultivars and compare it with the *SGR* gene sequences in other plants in the GenBank database. Information on *SGR* gene sequences from Indonesian sorghum cultivars can serve to develop a gene-based marker for the stay-green in sorghum. It can further be applied as a marker-assisted selection in producing high-yielding sorghum cultivars with better adaptation to drought-stress conditions.

## MATERIALS AND METHODS

### Genetic materials and DNA isolation

The recent study used a total of seven sorghum genotypes, which consisted of B69 (breeding line), Kawali, Numbu, Samurai 2, Pahat, and Super 2 (national cultivars), and PI-150-20-A (an introduced cultivar). DNA isolation took place at the Plant Molecular Biology laboratory of IPB University, using leaf samples from the shoots of young leaves six weeks after planting (6 WAP).

The leaf surface got cleaned from possible contaminants using alcohol and tissue paper. The DNA extraction procedure started

with the lysis process, DNA washing, and dissolving of the DNA following a modified Cetyl Trimethyl Ammonium Bromide (CTAB) protocol. The total genomic DNA of each sorghum genotype got isolated according to the DNA extraction method (Doyle and Doyle, 1987). Healthy leaves of each sample underwent grinding into a fine powder with a mortar and pestle. A total of 1 g of the crushed leaf sample gained transfer to a 1.5 ml microtube containing extraction buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCL, pH 8.0, and 2% v/v mercaptoethanol), followed by an incubation step at 65°C for 30 min. The solution received additional 500 µL of *chloroform: isoamyl alcohol* (24:1) and then centrifuged at 10 000 rpm for 5 min.

The transfer of the supernatant to a new microtube followed. DNA precipitation proceeded by adding 95% ethanol (temperature -10°C) and then centrifuged at 10000 rpm for 5 min. The washing of DNA pellets used 70% ethanol, then air-dried, followed by dissolving the DNA pellet in 100 µL TE (tris-EDTA). The sorghum genomic DNA got stored in a freezer at -20°C for PCR preparation.

### Primer design and PCR amplification

Two primer pairs, designated as SGR\_1 (forward 5' CCATCCAGATCAAAGTGTTC 3' and reverse 5' CGTGGAAACACGTGTAGTAA 3') and SGR\_2 (forward 5' CCTTGC GGAAGATGTAGTAG 3' and reverse 5' TCACGCACTCATATAACGAC 3'), resulted from the *SGR* gene of *Sorghum bicolor* (AY850140.1) and used to amplify seven sorghum genotypes. PCR amplification proceeded with a total volume of 25 µl containing 2 µL forward primer 1 µM, 2 µL reverse primer 1 µM, 0.5 µL 1U/µl Taq DNA polymerase (MyTaq HS red mix), 4 µL DNA 40 ng/ µL, 5 µL 1x dNTPs, and 11.5 µL 2x PCR buffer. DNA amplification took place with 35 cycles under the following steps: initial denaturation for 5 min at 94°C, then denaturation for 5 sec at 94°C, annealing for 30 sec at 48°C, 1 min elongation at 72°C, and final elongation for 10 min at 72°C. The PCR products obtained separation by electrophoresis technique on 1% agarose gel, stained with ethidium bromide solution (0.01%), and visualized using a UV light transilluminator AlphaImager® Mini, AlphaInnotech, Avantor™ (Cell Bioscience Inc., USA).

### BLAST and phylogenetic analysis

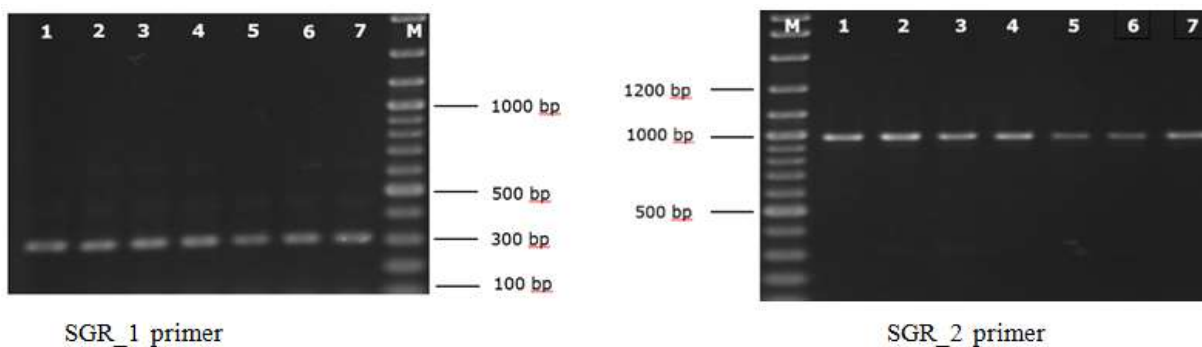
The DNA sequencing and purification of PCR products preceded using the method of 1<sup>st</sup> BASE Asia company, Singapore (<https://base-asia.com/>). The analysis of the forward and reverse fragment sequences of each genotype used the Geneious Prime version 2020.2.4 application. Both the sequence results from SGR\_1 and SGR\_2 primers underwent editing and evaluation using the Geneious software to obtain the *SGR* gene full length nucleotide sequences of each sorghum genotype. The sequence of nucleotide bases derived from seven sorghum genotypes got compared with those derived from the *SGR* gene in the NCBI GenBank database using the BLASTn and BLASTp algorithms (<http://blast.ncbi.nlm>). The nucleotide sequence then got translated into amino acids using the Geneious program version 2020.2.4. To identify the differences in the *SGR* gene function observed in sorghum with that in other plants, the protein of the seven genotypes of several plants' homology with sequencing results was aligned using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The phylogenetic analysis of the *SGR* gene resulted from comparing the amino acid sequences of the *SGR* gene derived from seven sorghum genotypes with those derived from several plant species deposited in the GenBank database. Constructing the phylogenetic tree ensued from the neighbor-joining method of

1000 bootstrap with *Molecular Evolutionary Genetic Analysis* (MEGA) software (Tamura *et al.*, 2011).

### RESULTS

#### PCR amplification of *SGR* genomic fragment

The PCR product amplified from the total genomic DNA of each of the sorghum genotypes resulted in a single band with a fragment size of about 300 and 985 base pairs (bp) for the SGR\_1 primer and the SGR\_2 primer, respectively (Figure 1). The PCR conditions used in the study enabled to amplify seven sorghum genotypes with high-quality nucleotide sequences produced by fragment sequencing. The total DNA concentration of the sorghum genome of 40 ng/ $\mu$ L and 1  $\mu$ M, respectively, showed the forward and reverse primers were suitable because they could produce clear DNA bands when visualized in 1% agarose gel. This result indicates that the newly developed primer, designed in the study, complements the binding site of the *SGR* gene, although the primary fragment size of SGR\_1 resulted in a shorter length product size (300 bp) than that in the predicted primary target of SGR\_1 (estimated PCR product size 940 bp). DNA fragments of PCR amplification products from two *SGR* primers got sequenced to determine the nucleotide base.



**Figure 1.** Visualization of PCR-agarose products from seven sorghum cultivars genotyping with SGR\_1 primer and SGR\_2 primer, (1) Numbu, (2) Samurai 2, (3) Kawali, (4) B69, (5) Pahat, (6) Super 2, (7) PI-150-20-A, (M) DNA marker size (100 bp ladder).

### BLAST parameter analysis

The BLASTn analysis determined the similarity of the nucleotide sequences of the seven sorghum genotypes with the accession

nucleotide sequences in the GenBank database. The results of the BLASTn analysis showed the identity of the amplified fragment of the putative *SGR* gene of the seven Indonesian sorghum genotypes (Table 1).

**Table 1.** Sequence identity of nucleotide sequences derived from *SGR* gene fragments with the accession deposited in the GenBank database.

Accession number	Accession information	Query (%)	Identity (%)	E-value
XM_002462673.2	<i>Sorghum bicolor</i> protein STAY GREEN, chloroplastic	85	100	0
AY850140.1	<i>Sorghum bicolor</i> senescence-inducible chloroplast stay-green protein (SGR)	85	100	0
XM_039940182.1	<i>Panicum virgatum</i> protein STAY-GREEN, chloroplastic-like	42	95	1.00E-88
XM_039940181.1	<i>Panicum virgatum</i> protein STAY-GREEN, chloroplastic-like	42	95	1.00E-88
XM_039940180.1	<i>Panicum virgatum</i> protein STAY-GREEN, chloroplastic-like	41	96	2.00E-86
XM_039940179.1	<i>Panicum virgatum</i> protein STAY-GREEN, chloroplastic-like	42	95	1.00E-88
XR_003226548.1	<i>Panicum hallii</i> polygalacturonate 4-alpha galacturonosyl transferase-like	9	95	2.00E-27
XM_034724514.1	<i>Setaria viridis</i> protein STAY-GREEN, chloroplastic-like	30	95	2.00E-92
XM_004957392.3	<i>Setaria italica</i> protein STAY-GREEN, chloroplastic	30	95	2.00E-92
AY850139.1	<i>Zea mays</i> senescence-inducible chloroplast stay-green protein 2	39	95	1.00E-84
NM_001112301.2	<i>Zea mays</i> senescence-inducible chloroplast stay-green protein 2	39	95	1.00E-84
EU955357.1	<i>Zea mays</i> clone 1529261 senescence-inducible chloroplast stay-green protein 1	39	95	1.00E-84

**Table 2.** Sequence identity between translated amino acid of *SGR* gene fragments identified from seven sorghum genotypes with that in accessions in the GenBank database.

Accession number	Accession information	Query (%)	Identity (%)	E-value
ACG39204.1	senescence-inducible chloroplast stay-green protein 1 [ <i>Zea mays</i> ]	18	100	2.00E-31
XP_035820927.1	senescence-inducible chloroplast stay-green protein 1 isoform X1 [ <i>Zea mays</i> ]	18	100	1.00E-30
NP_001105771.2	senescence-inducible chloroplast stay-green protein 2 [ <i>Zea mays</i> ]	19	98	3.00E-32
PWZ13232.1	Polygalacturonate 4-alpha-galacturonosyltransferase [ <i>Zea mays</i> ]	14	98	3.00E-20
TVU10153.1	hypothetical protein EJB05_43664 [ <i>Eragrostis curvula</i> ]	15	100	3.00E-23
XP_004957449.1	protein STAY-GREEN, chloroplastic [ <i>Setaria italica</i> ]	18	100	8.00E-31
OEL20820.1	Protein STAY-GREEN, chloroplastic [ <i>Dichantheium oligosanthes</i> ]	20	97	3.00E-34
XP_039796113.1	protein STAY-GREEN, chloroplastic-like [ <i>Panicum virgatum</i> ]	19	97	2.00E-32
KAF8700981.1	hypothetical protein HU200_033872 [ <i>Digitaria exilis</i> ]	19	98	1.00E-33
AKA59741.1	stay-green protein [ <i>Zoysia japonica</i> ]	15	98	4.00E-22
ADK56113.1	stay-green protein [ <i>Bambusa emeiensis</i> ]	19	95	1.00E-30
AAW82958.1	senescence-inducible chloroplast stay-green protein [ <i>Sorghum bicolor</i> ]	64	51	2.00E-19
CAB3457689.1	unnamed protein product [ <i>Digitaria exilis</i> ]	15	98	1.00E-22
CAD6224105.1	unnamed protein product [ <i>Miscanthus lutarioriparius</i> ]	69	51	1.00E-23

Based on the sequence alignment results, nucleotide sequences of SGR's Indonesian sorghum genotypes have a high homology (identity) (95%–100%) with that available in the reference sequences of the SGR gene deposited in GenBank. In detail, the SGR genes observed from seven Indonesian sorghum genotypes had a high level of identity against accessions of *Sorghum bicolor* (100% identity), *Panicum virgatum* (95%–96%), *Panicum hallii* (95%), *Setaria viridis*, *Setaria italica*, and *Zea mays* (95% each). This finding indicated that the amplification product of the seven sorghum genomes used in this study proved to be part of the SGR gene fragment. The SGR gene from the sorghum genotype has a Query cover value (9%–85%) with accessions in GenBank, such as *Sorghum bicolor* (85%), *Panicum* sp. (9%–42%), *Setaria* sp. (30%), and *Zea mays* (39%), while the range is E-value (0–2). Query cover value shows the nucleotide length presentation aligned with the database on BLAST.

The results of the BLASTp analysis showed that the SGR gene sequences of the seven sorghum genotypes had a homology sequence (identity) of 51%–100% with accessions deposited in the NCBI database and had a Query coverage value of 15%–69% (Table 2). Of these, 14 predicted candidate genes showed to be related to the SGR gene and two protein products produced from *Digitaria exilis* CAB3457689.1 and *Miscanthus lutarioriparius* CAD6224105.1 whose function remains unknown. The amino acid prediction analysis showed that the SGR genes from seven Indonesian sorghum genotypes had homology with *senescence-inducible chloroplast stay-green protein 1* from *Zea mays* (100% identity), *Sorghum bicolor* (51%), and *STAY-GREEN and chloroplastic* (identity 97% and 100%) on *Dichantheium oligosanthes* (OEL20820.1) and *Setaria italica* (XP\_004957449.1) plants. Partial sequences of the SGR gene PCR amplification products from seven Indonesian sorghum genotypes have been registered with GenBank with access numbers AY850140.1 and NC\_012871.2.

### Amino acid sequence and phylogenetic analysis

Based on the results of the alignment and BLASTp, amino acids from the putative SGR genes of seven Indonesian sorghum cultivars with SGR genes from other crops, such as, *Oryza sativa* OsSGR (AY850134.1), *Zea mays* ZmSGR2 (AY850139.1), *Sorghum bicolor* SbSGR (AY850140.1), *Panicum virgatum*

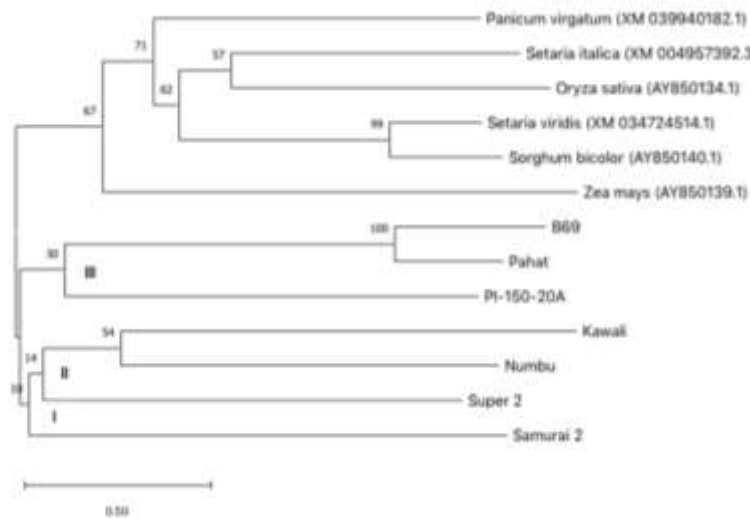
PvSGR (XM\_039940182.1), *Setaria italica* SiSGR (XM\_004957392.3), and *Setaria viridis* SvSGR (XM\_034724514.1) showed conserved amino acid residues from the SGR gene in other plants' amino acid residues (Figure 2). The conserved amino acid motif in stay-green genes, such as, MAAATA and STMSL, is similar to the amino acid sequence of the Indonesian sorghum SGR gene. In addition, key amino acid residues that determine the function of the SGR gene existed, namely, glycine (Q), Alanine (A), and Tryptophan (W) at positions 22, 27, and 124 of the SbSGR protein family, respectively. Based on the translated amino acid residues, it indicated a high diversity of SGR genes between Indonesian sorghum genotypes.

In the conserved area of the SGR gene from Indonesian sorghum, the insertion of one amino acid residue, Alanine (A), in the Super 2 cultivar (amino acid position 119) occurred. In addition, one substitution mutation that causes a non-synonymous single nucleotide polymorphism (SNP) happened so that there are differences in the encoded amino acid residues, such as, in the Kawali and Numbu cultivars, namely, the amino acid residues of Valine (V) and Leucine (L), respectively, at position amino acids 137 and 126 (Figure 2). Further to the presence of amino acid insertions and substitutions, conserved motifs with alleles mutant sequences from rice, SGR Y84R, and SGR V99M ensued (Jiang *et al.*, 2007; Park *et al.*, 2007), namely, the LPR motif on cultivars B69, Kawali, Numbu, Samurai 2, Pahat, and Super 2, but LTLAVS motifs did not show in Kawali and Numbu cultivars. Mutant gene sequences from other plants, such as, the *chlorophyll retainer* gene (CL W144R) from pepper and the *green-flesh* gene (GF R134S) from tomato (Barry *et al.*, 2008) also showed conserved sequences in the Pahat and Super 2 cultivars, with AEWK and DLIAGLRYIF motifs, on the Kawali cultivar, respectively (Figure 2). This indicates that the candidate protein from the Indonesian sorghum cultivar may have the same function as the SGR gene protein in other plants in the GenBank database.

The phylogenetic analysis constructed using the neighbor-joining method showed that the predicted amino acid of SGR genes elucidated from the seven sorghum genotypes separated into three major groups as compared with the reference SGR gene sequence groups in the GenBank database (Figure 3). The first group consisted of one sorghum genotype (Samurai 2), the second group consisted of Super 2, Numbu, and



**Figure 2.** Sequence alignment of conserved SGR genes from seven sorghum genotypes and six accessions from the NCBI GenBank protein database. The identical amino acid residues were indicated with the black background, while the conserved protein of about 60% is marked in gray.



**Figure 3.** Phylogenetic of the sequence variation of the predicted amino acid of the SGR gene from seven sorghum genotypes and homologs. The numbers in the figure indicated the output values of the bootstrap test using 1000 iterations. The phylogenetic tree was conducted in MEGA X.

Kawali sorghum genotypes, and the third group consisted of PI-150-20-A, Pahat, and B69, with coefficient similarity of 10%, 14%, and 30% respectively. Meanwhile, the reference SGR proteins formed four groups consisting of 1) *Zea mays*, 2) *Sorghum bicolor* and *Setaria viridis*, 3) *Oryza sativa* and *Setaria italica*, and 4) *Panicum virgatum*. *Sorghum bicolor* and *Setaria viridis* have the highest coefficient of similarity (99%) compared with SGR protein sequences from other monocot plants.

The phylogenetic analysis showed that the seven Indonesian sorghum genotypes do not occur in the same branch with reference SGR gene sequences from *Setaria italica* (SiSGR XM\_004957392.3), *Oryza sativa* (OsSGR AY850134.1), *Setaria viridis* (SvSGR XM\_034724514.1), *Sorghum bicolor* (SbSGR AY850140.1), *Panicum virgatum* (PvSGR XM\_039940182.1), and *Zea mays* (ZmSGR2 AY850139.1). The clustering of Pahat and B69 showed closer with *Zea mays* accessions compared with *Sorghum bicolor* (AY850140.1) in the GenBank database. Although the Indonesian sorghum has a different group from other plants in GenBank, they have similar nucleotide and amino acid sequences, with 95%–100% and 51%–100% identity values, respectively (Tables 1 and 2). In addition, the SGR protein identified in seven Indonesian sorghum genotypes showed homology with STAY-GREEN, a chloroplastic protein from the reference family, Poaceae.

## DISCUSSION

The two newly-designed primers, the SGR\_1, and SGR\_2, successfully amplified the target DNA fragments of the seven sorghum genotypes, which produced a good clear banding pattern. Based on estimated product sizes, the SGR\_2 primer produced a DNA fragment of about 985 bp for each sorghum genotype, following its primary target. On the other hand, the SGR\_1 primer produced a DNA fragment of 300 bp in size, demonstrating a shorter fragment size for each sorghum genotype obtained compared with the expected target size of the primer (940 bp). The study has not identified the factors that cause the discrepancy between the size of the PCR fragments and the size of the primary target fragments. But this may be caused by interactions between identical primer base sequences making the DNA polymerase bind to identical parts and cause a decrease in amplification efficiency during the PCR process.

Hence, the primary product amplification of SGR\_1 did not match the primary target. Another possibility may be the annealing temperature used is not specific for the SGR\_1 primer (Potapov and Ong, 2017), therefore a need to identify the cause of the discrepancy in the size of the PCR fragments and the previously-designed primer targets. Additionally, the primary size, melting temperature, the composition of guanine and cytosine, and the concentration of the primer used also affect the ability of the primer to amplify the target nucleotide (Ozturk and Can, 2017).

The SGR gene sequences derived from the seven sorghum genotypes in this study revealed high-nucleotide sequence similarities with those in other plants deposited in the GenBank database with identity values of >90% (Table 1). Similarly, their amino acid sequences also displayed high similarity with the amino acid sequences of the reference SGR gene, except for *Miscanthus lutarioriparius* (CAD6224105.1) and *Sorghum bicolor* (AAW82958.1), with an identity value of 50% for each accession (Table 2). The high similarity value identity indicated that the protein encoded by the SGR gene from the seven sorghum genotypes may have the same function as the predicted amino acid SGR gene available in the GenBank database.

The study also found *STAY-GREEN chloroplastic* (XP\_004957449.1) protein products and *STAY-GREEN chloroplastic-like* (XP\_039796113.1) showed identities of 100% and 97%, respectively (Table 2). Based on previous studies, *STAY-GREEN chloroplastic* can trigger chlorophyll degradation when it interacts with the light-harvesting complex photosystem II (LHCII) subunit (Hörtensteiner, 2009; Park *et al.*, 2007). In contrast, *STAY-GREEN chloroplastic-like* (SGRL) expression rapidly down-regulates senescing in leaves, suggesting that SGRL activity restricts to pre-senescing leaves (Sakuraba *et al.*, 2014a), while *STAY-GREEN chloroplastic-like* (SGRL) at the protein level has similarities/homologous to *SGR1* and *SGR2* (Sakuraba *et al.*, 2014a). Physiological and molecular mechanisms of *STAY-GREEN chloroplastic* and *STAY-GREEN chloroplastic-like* (SGRL) play a role in chlorophyll metabolism, and homologous SGRs are not always positive regulators of chlorophyll degradation depending on the evolutionary process of each plant species (Sakuraba *et al.*, 2015).

Based on the alignment results of amino acid sequences derived from seven sorghum genotypes and those from other



plants, conserved and non-conserved amino acid residues occurred (Figure 2). The conserved amino acid sequence refers to an amino acid that is identical to the *SGR* gene in other species and has not been changed during the evolutionary process. The conserved amino acid residues found in the seven genotypes of sorghum designated as Tyrosine (Y), Tryptophan (W), Arginine (R), Valine (V), and Isoleucine (I) associated with important functions of *SGR* protein. These amino acid residues cause mutations in the *stay-green* (*SGR*) gene sequences in rice, *green-flesh* (GF) in tomato, and *Chl retainer* (CL) in pepper and produce a stay-green phenotype (Jiang *et al.*, 2007; Park *et al.*, 2007; Barry *et al.*, 2008) which causes differences in alleles with wild-type (Borovsky and Paran, 2008). The stay-green phenotype gets controlled by single-recessive genes in tomato, pepper, and *Brassica campestris* (Akhtar *et al.*, 1999; Efrati *et al.*, 2005;). Moreover, the amino acids, Tyrosine, Valine, Tryptophan, and Arginine correlated with the *SGRL* protein (Rong *et al.*, 2013). Based on this, the *SGR* gene family identified from the seven Indonesian sorghum genotypes proved *SGR*-like (*SGRL*).

The amino acid sequences identified from the Pahat and Super 2 genotypes revealed a total of 18 and 12 amino acid residues, which conserved with the *SGR* Y84R and *SGR* V99M proteins (Jiang *et al.*, 2007; Park *et al.*, 2007). Similarly, Pahat and Super 2 identified four conserved amino acid residues with CL W144R protein, and only the Kawali genotype exhibited the conserved amino acid sequences with predicted GF R134S protein (Barry *et al.*, 2008). The *SGR*, CL, and GF proteins are those associated with the stay-green phenotype (Barry *et al.*, 2008). The conserved amino acid domain between *SGR* and *SGRL* probably has the same protein function. The differences in the amino acid sequences observed in the seven sorghum genotypes with those in the *SGR* gene need further analysis and thus provide a great opportunity to obtain new genes that may only be available in Indonesian sorghum cultivars. Moreover, the expression analysis of the *SGR* gene derived from Indonesian sorghum collections would be prospective and challenging, particularly to determine the relationship between these genes and the phenotype of sorghum cultivars in the field.

The cysteine (C) amino acid residue serves as one of the amino acids that act as an enzyme catalytic site. In this study, the amino acid residue of cysteine at 301 and 314 bp showed and have known to be conserved in

the Samurai 2 and Pahat genotypes, respectively. The amino acid cysteine plays a crucial role in the formation of crosslinking or intramolecular regulation during the aging process. It also causes the formation of ROS, which leads to the formation of dimers or polymers of the *SGR* gene resulting in the acceleration of chlorophyll degradation and detoxification during the aging process (Jiao *et al.*, 2020). Jiang *et al.* (2011) reported that overexpression of *SGR* (*Ov-SGR*) resulted in the generation of singlet oxygen and ROS and caused cell death in rice sprouts. Likewise, the *Ov-SGR* causes damage to the thylakoid membrane resulting in a reduction of photochemical efficiency in photosystem II (Mur *et al.*, 2010). Overexpression of the *SGR* and *SGRL* genes are catalyzed by different reactions. *SGR* catalyzes chlorophyll a to pheophytin a, whereas *SGRL* catalyzes the reaction of chlorophyll a and chlorophyllide a to pheophytin a and pheophorbide a, respectively; the two gene families do not use chlorophyll b as a substrate (Tang *et al.*, 2011). Although different reactions catalyze the *SGR* and *SGRL*, the presence of conserved amino acid domains in *SGRL* and *SGR* consider the gene families have similar biochemical functions (Jiang *et al.*, 2011).

The phenotype associated with delayed senescence in plants illustrates one strategy to increase crop yields (Borrell *et al.*, 2014b). The mechanism of senescence in plants is complex and influenced by many factors, including stay-green genes, transcription factors, stress-related genes, and other genes related to senescence in plants (Christ and Hörtensteiner, 2014). Plants that can maintain green (*non-yellowing*) leaves for a longer period after the seed maturation phase are considered the stay-green genotypes. Various factors influenced the stay-green phenotype, such as, chlorophyll catabolism (Hörtensteiner and Kräutler, 2011), chlorophyll synthesis (Kusaba *et al.*, 2013), nutrient remobilization (Gregersen, 2013), and hormones (Zwack and Rashotte, 2013) therefore, the mechanism that controls the stay-green character in plants is very complex. Vadez *et al.* (2011) reported a similar thing on sorghum, which revealed that the selection for stay-green characters proved very complex because of the interaction between the genotype and the environment. Therefore, the changes in gene expression in plants, at different stages of growth, development, and environmental and genetic background require much investigation.

The homologous *SGRs* have been identified in various plant species, including

rice (Sato *et al.*, 2009; Park *et al.*, 2007; Jiang *et al.*, 2011; Rong *et al.*, 2013), Arabidopsis (Ren *et al.*, 2007; Aubry *et al.*, 2008; Mecey *et al.*, 2011; Sakuraba *et al.*, 2014a), and alfalfa (Zhou *et al.*, 2011). Amino acid sequence analysis showed that the SGR protein of seven sorghum genotypes contained 320–334 amino acid residues. Differences in plant species resulted in variations in the number of homologous SGRs (Barry *et al.*, 2008; Sakuraba *et al.*, 2014b). A previous study reported that three homologous SGRs came out in Arabidopsis plants (Sakuraba *et al.*, 2014a), while in rice and soybean plants, each possessed one and two homologous SGRs homologs (Jiang *et al.*, 2011; Nakano *et al.*, 2014; Sakuraba *et al.*, 2015).

Overexpression of SGR1 and SGRL in Arabidopsis caused the leaves to turn yellow more quickly, while *sgr1/nye1-1* and *sgrl* mutants showed a stay-green phenotype in abiotic stress conditions, including salinity stress, osmotic stress (mannitol), and drought stress (Sakuraba *et al.*, 2014a; Rong *et al.*, 2013). The difference between SGR and SGRL lies in the C-Terminal; SGR has a cysteine-rich motif (CRM, P-X3-C-X3C-X-C2-FP-X5-P) which plants need for the Mg-dechelatase process (Xie *et al.*, 2019) while the SGRL does not have said motif. Furthermore, SGRL expression increased in young leaves, seeds, and flower organs, then decreased in old tissue, whereas SGR expression shows the opposite (Li *et al.*, 2017). SGRL catalyzes from chlorophyll a to pheophytin a and chlorophyll b to pheophorbide a, while SGR catalyzes only from chlorophyll a to pheophytin a (Shimoda *et al.*, 2016). In this study, no notable amino acid residues occurred in the C-Terminal region implying that the predicted protein identified from the amino acid sequences of the seven sorghum genotypes is SGRL type.

Additionally, the amino acid sequences of the seven sorghum genotypes showed no amino acid residues in the C-Terminal region, indicating that they belong to the SGRL type and the grouping does not belong to the SGR gene class from other species. This phenomenon agrees with previous studies reported by Rong *et al.* (2013) and Jiao *et al.* (2020), which stated that the family I (SGR) and family II (SGRL) genes formed different groups. The group differences between Indonesian sorghum genotypes and reference plants in the GenBank database relate to the sorghum genotype used in this study as a result of gamma-ray mutations. The study suspects that the Indonesian sorghum genotype experienced changes in the protein

structure of the SGR gene. The finding supports previous research that gamma-ray is one of the mutagens causing changes in the structure and composition of genetic material, often permanent and passed on to the next generation, and affecting gene expression (Wi *et al.*, 2005; Belfield *et al.*, 2012; Kim *et al.*, 2014).

All SGR gene homologs, ranging from algae to higher plants, are predicted to be located in chloroplasts (Xie *et al.*, 2019), including the prediction of the SGR gene for Indonesian sorghum cultivars. In general, the SGR gene not only plays an essential role in the degradation of chlorophyll during the leaf aging process but also plays a role in the growth and development of other plant organs (Ramkumar *et al.*, 2019). This study did not analyze the function of the SGR gene from the Indonesian sorghum cultivar, but only the nucleotide and amino acid sequence alignment of the stay-green gene. Based on the identified amino acid sequences, results revealed that the SGR protein of the Indonesian sorghum cultivar contained a conserved domain, which also existed in the SGR protein of other plants. The conserved domain is vital to the function of stay-green proteins (Jiang *et al.*, 2011). The stay-green character serves as one of the significant characteristics that can increase photosynthetic activity in drought-stress conditions (Tian *et al.*, 2013). Understanding the physiological and genetic basis of stay-green drought tolerance is the core for developing high-yielding and drought-tolerant sorghum cultivars. Therefore, more studies are needed to identify and characterize potential genes, such as, the SGR gene associated with a functional stay-green phenotype in sorghum, to support a drought-tolerant sorghum breeding program.

## CONCLUSIONS

The amplification of the SGR gene in seven cultivars of Indonesian sorghum resulted in a 300 bp – 985 bp amplicon, which encodes 320–334 amino acids. The identity level of identified SGR genes reaches 51%–100% with several SGR genes in the GenBank database. The conserved amino acid residues occurred in the seven genotypes of sorghum, designated as Tyrosine (Y), Tryptophan (W), Arginine (R), Valine (V), and Isoleucine (I), associated with important functions of the SGR protein. Based on the alignment of amino acid sequences, the seven genotypes of sorghum formed three distinct and separate groups from the grouping

of *SGR* sequences of other species in the GenBank database. Although not in the same group as the reference *SGR* gene, the *SGR* protein of the Indonesian sorghum cultivar has the same biochemical function as the *SGR* gene in other plants.

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