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Construction and Transformation of *HVA1* Gene Expression Vector into Indonesian Elite Rice Varieties

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ABSTRAK

Konstruksi dan Transformasi Vektor Ekspresi Gen *HVA1* ke Varietas Padi Elit Indonesia. *Sri Koerniati, dan Hani Widhianata.* Transfer gen *osmoprotectant HVA1* merupakan satu upaya mengurangi pengaruh kekeringan pada tanaman padi. *HVA1* adalah satu dari kelompok protein *Late Embryogenesis Abundant* (LEA) yang berperan melindungi sel selama mengalami cekaman. Tujuan penelitian adalah untuk mengkonstruksi plasmid vektor ekspresi *HVA1* dan mentransformasikannya ke varietas padi elit Indonesia. Bahan yang digunakan adalah plasmid pBY520 (sumber *HVA1*); plasmid antara pRP9, dan plasmid pAY560326 (*backbone*); enzim restriksi *Bam*HI, *Hind*III, *Xho*I, dan *Spe*I; T4 DNA ligase, dan kit ekstraksi DNA dari gel. Metode yang digunakan mengikuti prosedur standar konstruksi plasmid vektor dan biologi molekuler. Tahap I: DNA pBY520 dan pRP9 dipotong dengan *Bam*HI dan *Hind*III, dan dielektroforesis dengan gel agarose 1%. Fragmen DNA gen *HVA1* dan pRP9 dipurifikasi, diligasi dengan T4 DNA ligase, dan ditransformasi ke *Escherichia coli* DH5-α dengan metode *heat shock*. *E. coli* ditanam di media LB padat (+kanamisin 100 mg/l). DNA plasmid baru diisolasi dari kultur koloni tunggal, dikonfirmasi dengan PCR dan primer spesifik untuk *HVA1*, dan hasilnya dinamakan pRP9_*HVA1*. Tahap II: DNA pRP9_*HVA1* dan pAY560326 dipotong dengan enzim *Xho*I dan *Spe*I; fragmen DNA *HVA1* dan pAY560326 dipurifikasi dan diligasi. Prosedur selanjutnya sama dengan pada tahap I. Plasmid yang dihasilkan dikonfirmasi dengan PCR dan dipotong dengan enzim *Xho*I dan *Spe*I; hasilnya dinamakan pAY_*HVA1*. Tahap III: pAY_*HVA1* ditransformasi ke sel *Agrobacterium* EHA-105. Kemudian pAY_*HVA1* ditransformasi ke padi varietas Ciherang dan Inpari 6 menggunakan metode infeksi awal *scutellum*. Hasil penelitian diperoleh sembilan nomor padi transgenik yang positif mengandung gen *HVA1*.

Kata kunci: Konstruksi vektor, *over-expression*, gen *osmoprotectant HVA1,* padi toleran kekeringan.

ABSTRACT

Construction and Transformation of *HVA1* Gene Expression Vector into Indonesian Elite Rice Varieties. *Sri Koerniati and Hani Widhianata.* Transfer of an osmoprotectant gene *HVA1* is an effort to reduce the effect of drought in rice. *HVA1* is one of the Late Embryogenesis Abundant (LEA) protein group that plays a role on cell protection during stresses. A study was done with an objective to construct a plasmid vector expressing *HVA1* and to transform it into Indonesian elite rice varieties. Materials used in the study were plasmid pBY520 (source of *HVA1*; intermediate plasmid pRP9; plasmid pAY560326 (backbone); restriction enzymes *Bam*HI, *Hind*III, *Xho*I, and *Spe*I; T4 DNA ligase, and gel DNA extraction kit. Methods used were standard procedure for plasmid vector construction and molecular biology. Step I: the pBY520 and pRP9 were cut with *Bam*HI and *Hind*III, and electrophorated with 1% agarose gel. DNA fragments of *HVA1* and pRP9 were purified, ligated with T4 DNA ligase, and transformed into *Escherichia coli* DH5-α by heat shock. *E. coli* were grown onto solid medium (+ kanamycin 100 mg/l). A new plasmid DNA was isolated from single colony culture of the bacteria, confirmed, and named pRP9_*HVA1*. Step II: DNA of pRP9_*HVA1* and pAY560326 were cut with *Xho*I dan *Spe*I enzymes, purified, and ligated. The next procedure was similar to step I, and the resulted plasmid was confirmed by PCR and digestion with *Xho*I dan *Spe*I enzymes, and named pAY_*HVA1*. Step III: pAY_*HVA1* was first transformed into *Agrobacterium* EHA-105 and then into rive varieties Ciherang and Inpari 6 using the early infection of scutellum transformation method. Nine transgenic rice lines that positively contain *HVA1* were obtained.

Keywords: Vector construction, over-expression, *osmoprotectant* gene *HVA1,* drought tolerant rice.

INTRODUCTION

The beginning of 21 century was marked by global scarcity of water resources, pollution, and increased salinity of soils and waters. Abiotic stresses have been major limiting factors in plant growth. In response to various environmental stresses, plants have developed different physiological and biochemical strategies to adapt or to tolerate the stresses. Recently, the osmoprotectants have been definitely proven to be among the most important factors to protect plants cells from dehydration (Yamaguchi-Shinozaki *et al*., 2002). They are non-toxic and accumulated to significant levels without disrupting plant metabolism when plant stresses.

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One of the osmoprotectants genes encodes for the Late Embryogenesis Abundant (LEA) proteins. Researches on LEA proteins have been on going for more than 20 years. The LEA proteins were first characterized in cotton as a set of proteins that are highly accumulated in the embryos at the late stage of seed development (Dure *et al*., 1989). Based on their common amino acid sequence domains, LEA proteins were classified into three groups. A homology region among group of 3 LEA proteins is composed of tandem repeats of an 11 amino acid motifs that form an amphiphilic α-helix structure. The LEA proteins have several possible functions. These include roles as antioxidants as well as membrane and protein stabilizers during water stress, either by a direct interaction or by acting as a molecular shield. Along with other hydrophilic proteins and compatible solutes, LEA proteins might also serve as "space fillers" to prevent cellular collapse at low water activities. These multifunctional capacities of LEA proteins are probably attributable in part to their structural plasticity, as they are largely lacking in secondary structure in the fully hydrated state, but can become more folded during water stress and/or through association with membrane surfaces. These proteins accumulate during the late stage of seed formation and in vegetative tissues under drought, heat, cold, and salt stresses conditions or with abscisic acid (ABA) application (Sivamani *et al*., 2000). LEA proteins may play a protective role in plant cells under various stresses conditions, and this protective role is very essential for the survival of the plant under extreme stress conditions (Baker *et al*., 1988; Dure *et al*., 1989).

HVA1, which is one of the group 3 of LEA proteins in barley (*Hordeum vulgare* L.), is highly induced by ABA and stress. The gene had been proven inducing a stable tolerance to osmotic stress in rice (*Oryza sativa* L.), oat, and wheat (*Triticum aestivum* L.) (Xu *et al*., 1996). A positive significant correlation between the increase growth of transgenic lines under stress over the controls, and the presence of *HVA1* transcript suggested more evidence that constitutive expression of *HVA1* gene in the transgenic plants can improve growth performances under salinity stress conditions (Oraby *et al*., 2005). The transgenic rice plants maintained higher growth rates than the non transformed (control) plants under the stress conditions, namely water deficit and salinity. The increased tolerance was reflected by both delaying the damage symptoms caused by the stress and by improving recovery upon removal of the stress conditions. The extent of the stress tolerance was correlated with the level of the *HVA1* protein accumulated in the transgenic rice plants (Xu *et al*., 1996).

One of approaches to create new plant variety is through genetic engineering. The technique can be used for inserting a gene from plants, bacteria or viruses into other plants. Hitherto, it can also be used for gene function analyses. In relation to the above mentioned *HVA1* gene, we tried to generate new rice variety by inserting the *HVA1* gene into the Indonesian elite rice verities Ciherang and Inpari 6. This effort was undertaken to elaborate drought and salinity stresses problems encountered in the Indonesian rice varieties.

This paper reports construction a plasmid vector containing *HVA1* gene, transformation the vector into *Agrobacterium tumefaciens,* and development rice transgenic lines using the *Agrobacterium* transformation method.

MATERIALS AND METHODS

The experiments were carried out in the Molecular Biology Laboratory of the Indonesian Center for Agricultural Biotechnology and Plant Genetic Resources Research and Development (ICABIOGRAD), Bogor, from September 2009 to March 2010. The plasmids used for the construction were pRP9 (provided by Dr. Kurniawan R. Trijatmiko, ICABIOGRAD) and pAY560326 (provided by Dr. Ouwerkerk, IBL, Leiden University), as intermediate and binary plasmids, respectively. Another plasmid (pBY520) that contains *HVA1* gene was obtained from Prof. Ray Wu (Cornell University, USA). The primers used for confirmation of the *HVA1* gene were 5'-TGGCCTCCAACCAGAACCAG-3' (forward) and 5'-ACGACTAAAGGAACGGAAAT-3' (reverse). The early infection of scutellum by *Agrobacterium* method developed by Toki *et al*. (2006) was used for the rice transformation. The tissue culture medium used were also prepared based on a protocol mentioned by Toki *et al*. (2006).

Construction of Plasmid Vector

Plasmid pBY520 as a source of the *HVA1* gene contained *Hind*III and *Bam*HI restriction enzymes. By using these two restriction enzymes, DNA of *HVA1* (1.0 kb) was cut off from pBY520 (Figure 1). Plasmid pAY560326 is a binary vector (backbone) that was used to express the *HVA1* gene. It has a 35S promoter that is needed for driving the gene. Plasmid pAY560326 has *Hind*III and *Bam*HI restriction sites, but these two sites are located before the 35S promoter. It has also, however, *Xho*I and *Spe*I restriction sites, which are

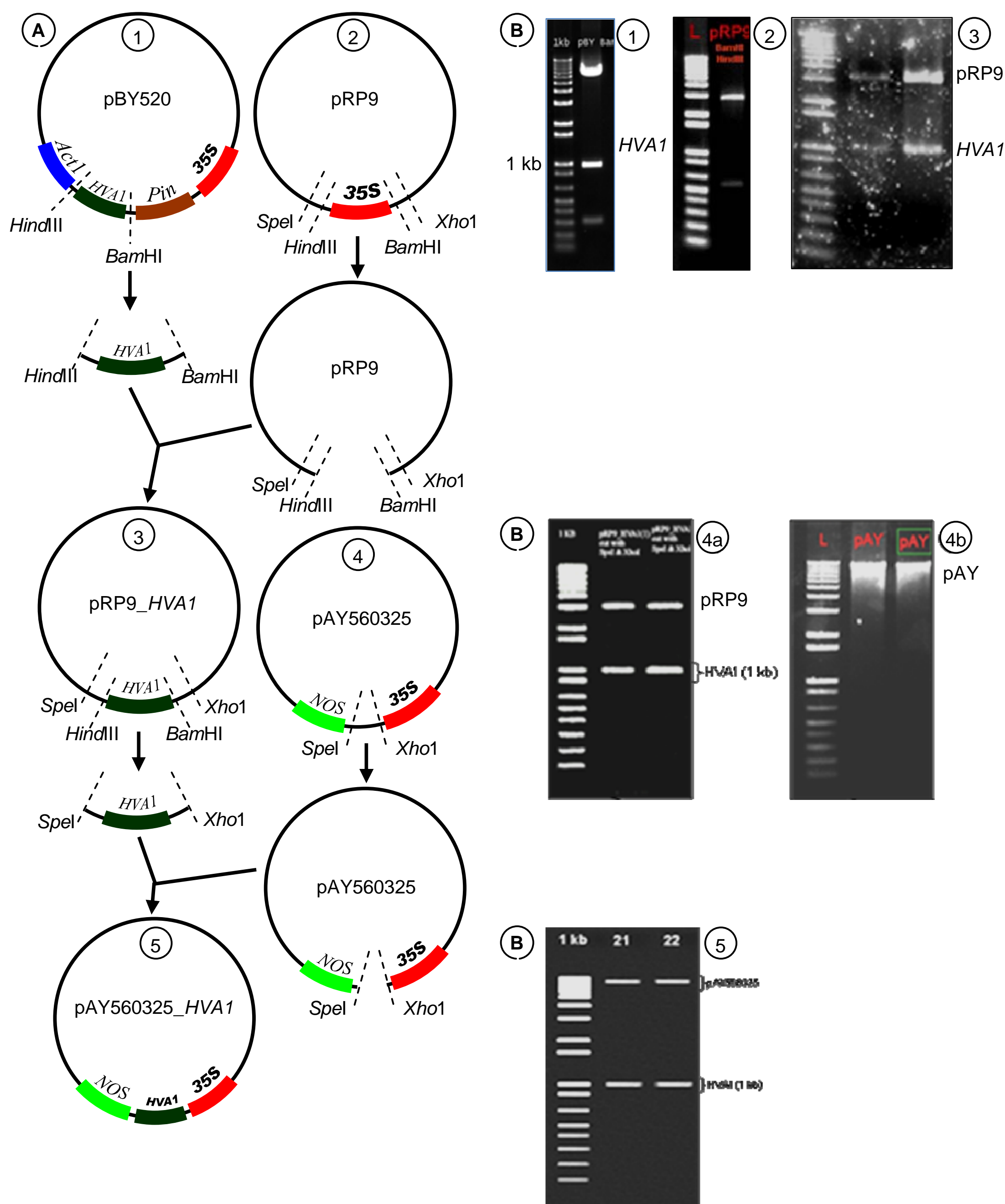

Figure 1. Construction strategy of *HVA1* binary expression vector. A = Schematic construction of *HVA1* binary expression vector: (1) *Hva1* gene was cut off from plasmid pBY520 using *Hind*III-*Bam*HI restriction enzymes; (2) Subcloning *Hva1* gene into plasmid pRP9, which was digested with the same enzymes to generate pRP9_*HVA1*; (3) *HVA1* gene was cut out of the pRP9.*HVA1* using *Spe*I-*Xho*I enzymes and inserted into 35S-Nos promoter-terminator in pAY560325; (4) pAY560325 was cut with the same enzymes to generate pAY560325_*HVA1* (5). B = gel electrophoresis for extractions of *HVA1* binary expression vector: (1) *HVA1* gene was cut out from plasmid pBY520 using *Hind*III-*Bam*HI. (2 + 3) The *HVA1* gene was subcloned into pRP9 plasmid, which was digested with the same enzymes, to generate pRP9_*HVA1*; (4a) *HVA1* was cut out from the pRP9-*HVA1* using *Spe*I-*Xho*I, and (4b) *HVA1* was inserted into 35S-Nos promoter-terminator in pAY560325, which was cut with the same enzymes to generate 5 pAY560326_HVA1.

located after the 35S promoter, and these can be used to insert the gene. Due to these reasons, pRP9, which has *Hind*III and *Bam*HI restriction sites, was used as an intermediate vector in the plasmid construction. Plasmid pRP9 also has *Xho*I and *Spe*I restriction sites that are needed to insert the *HVA1* fragment into plasmid pAY560326.

A ligation between the *HVA1* DNA fragment (insert) and the pRP9 (backbone) was carried out using T4 DNA ligase enzyme (NEB), after both fragments were purified using a gel extraction kit (Qiagen). The ligation reaction was then transformed into *Escherichia coli* (DH5-α) using the heat shock method. DH5-α competence cell were prepared according to standard procedure (Sambrook and Russell, 2001). The transformed cells were then plated on Luria Broth Agar (LBA) medium containing of ampicillin (100 mg/l), IPTG (0.1 mM/l) and X-gal (40 mg/l). The plate cultures were incubated in 37°C for 16 hours. White colonies were selected and grown in LB media containing 100 mg/l of ampicillin and shaked for 16 hours at 37°C. PCR of the colonies were carried out to confirm the existence of *HVA1* genes in the bacterial culture. One microliter of culture was used as a template for PCR. Confirmation was done by digesting the DNA using *Hind*III and *Bam*HI restriction enzymes. The pRP9_*HVA1* and pAY560326 were then digested using *Xho*I dan *Spe*I restriction enzymes and purified. A DNA fragment of *HVA1* (insert) and pAY560326 (backbone) were ligated and transformed into *E. coli* strain DH5-α. DNA of the new plasmid was isolated from single colony cultures of *E. coli*. Confirmation of the DNA was done both by cutting pAY560326_*HVA1* plasmid DNA using *Xho*I and *Spe*I enzymes and by PCR using specific primers for *HVA1* gene. The new plasmid obtained was named pAY560326_*HVA1*.

For the purpose of plant transformation, pAY560326_*HVA1* plasmid was then transformed into *A. tumefaciens* strain EHA-105 competent cells using the heat shock method. EHA-105 competent cells were prepared according to standard procedure (Sambrook and Russell, 2001). The transformed cells were then plated on solid YEP medium containing kanamycin (100 mg/l) and rifampicin (20 mg/l). The plates were incubated at 28°C for 48 hours. Selected single colonies were grown in YEP liquid medium containing the same antibiotics and shaked at 28°C for about 48 hours. PCR of the bacterial colonies were carried out to confirm the existence of *HVA1* in the bacterial culture.

Plant Transformation

The plasmid DNA construct was transformed into rice plant using the early infection of *A. tumefaciens* transformation method (Toki *et al*., 2006). Two Indonesian elite rice varieties (Ciherang and Inpari 6) were used. Steps of the transformation were as followed. Palea and lemma of rice flower were peeled off and surface sterilized in 70% ethanol for one minute, 20% sodium hypochlorite and 2 ml Tween for 30 minutes, and then washed five times using sterilized water and dried using sterile whatman papers. The seeds (scutellum part) were pre-cultured in N6D (N6 media + 2 mg/l 2,4-D + 300 mg/l casamino acid + 2878 mg/l L-prolin + 2 mg/l glycine + 7 g/L Agarose) for 5 days. Infection was carried by soaking the pre-cultured seeds in the infection media (AAM media + 300 mg/l casamino acid + 2878 mg/l L-prolin + 2 mg/l glycine + 100 μM acetosyringone) containing *A. tumefaciens* (OD = 0.1) for 1.5 minutes. Explants were then air dried, co-cultivated onto 2N6-AS medium (+100 μM acetosyringone) for 3 days in dark (co-cultivation). After co-cultivation, explants were washed three times with sterilized water, then once with sterilised water containing carbenicillin 500 mg/l. Then explants were planted onto N6D-ab selection medium (N6D media + 50 mg/l hygromycin + 400 mg/l cefotaxime + 100 mg/l vancomycine +7 g/l Agarose) for 2 times of 1 weeks, in dark. Regeneration of plantlets was carried out on the RE-III medium (MS media + 2000 mg/l casamino acid + 2 mg/l glycine + 2 mg/l kinetin + 0.002 mg/l NAA + 20 mg/l ygromycin + 100 mg/l cefotaxime + 100 mg/l vancomycine + 7 g/l agarose). To stimulate root formation, the planlets were transfered into the HF medium (MS media + 2 mg/l glycine + 20 mg/l hygromycin + 100 mg/l cefotaxime + 100 mg/l vancomycine +7 g/l Agarose).

PCR for Confirmation of Transgenic Lines

PCR using Taq polymerase and specific primers to *HVA1* were applied to check transgenic lines produced from transformation. Each PCR reaction had 1 nl of DNA (10 ng), and the PCR was carried in 30 cycles, each consisted of denaturation at 94°C for a minute, annealing at 50°C for 45 seconds, and extension at 72°C for a minute. Before running the cycles, the PCR was initiated with denaturation at 95°C for 2 minutes, terminated with extension at 72°C for 15 minutes and cooled down at 10°C for 15 minutes. PCR products were checked by electrophoresis using 1% agarose gel.

RESULTS AND DISCUSSIONS

A genetic engineering is an effort to manipulate genes of any organism through steps, such as gene isolation from any organism, preparing vehicle to transfer the gene, and transform it into another organism. When a plant has been genetically changed or a new gene has been by genetic engineering techniques, such plant is then called a transgenic plant.

Transformation using *A. tumefaciens* is one of methods that has been used for inserting of a foreign gene into dicots. Then, Hiei *et al*. (1994) found an efficient transformation method mediated by *A. tumefaciens* for monocots rice, using scutellum calli and super binary vector. Toki and Ellchi (1997) modified the method by reducing the tissue culture period. Recently, Toki *et al*. (2006) had successfully developed a transformation method using pre-cultured scutellum and reduced the tissue culture period.

A gene consists of DNA, which is a blueprint for making a specific component called enzymes. Enzymes are special types of proteins that responsive in making components such as drought tolerance. *HVA1* encoded protein, a member of LEA proteins that plays roles in plant tolerance to abiotic stresses, such as drought, cold, and salinity.

In the pBY520 plasmid, *HVA1* gene (about 1.0 kbin size) is located between *Actin1* promoter dan *pin* terminator. Cutting DNA with two enzymes restrictions in one reaction needs a buffer, which is compatible to both enzymes and amount 10% reaction volume. Restriction enzymes cut DNA specifically to become smaller DNA fragments and to isolate a small fragment that is required. The restriction enzymes cut DNA at specific nucleotide sequence called restriction sites. The restriction enzymes cut sugar-phosphate bonds from the DNA and at both DNA strings. The restriction enzymes also hydrolized deoxyribose and a phosphate group (P group) at 5' end and OH at 3' end of the DNA chains, hence it produces sticky, cohesive and blunt ends.

HVA1 fragment/gene was successfully isolated from pBY520 plasmid by cutting (double digestion) the plasmid using *Hind*III and *Bam*HI restriction enzymes (Figure 1.A.1) and incubated at 37°C for about 4 hours. This cutting produced three DNA fragments, and one kb fragment was DNA of *HVA1* gene (Figure 1.B.1). The DNA was then purified from the gel before it was ligated into pRP9 as an intermediate plasmid. Plasmid pRP9 had also been cut with the same enzymes to linearized the plasmid (Figure 1.A.2). Therefore, the ends of *HVA1* can be ligated to it, as both fragments were cut using similar endonuclease or endonucleases produced similar ends. The pRP9 plasmid produced two DNA fragments each with 3 kb size and about 600 bp of 35S promoter (Figure 1.B.2). A 35S fragment was replaced by the insert DNA that was the *HVA1* gene. A 3kb fragment was excised from the gel, purified, and ligated with an insert (DNA *HVA1*) using a T4-DNA ligase enzyme.

In this study, pRP9 plasmid that has *LacZ* gene at the multicloning site (MCS) was used as an intermediate vector, and white-blue *A. tumefaciens* colony selection was done to distinguished the right and wrong colonies. *A. tumefaciens* cells that have been introduced by the plasmid were plated onto LBA medium containing ampicillin 100 mg/l, IPTG dan X-gal. The plates were then incubated at 37°C for 16 hours. IPTG and X-gal were used for the white-blue selection of the bacterial colonies. White single colonies were chosen and cultured in 5 ml LB medium containing of 100 mg/l ampicillin, then incubated on a shaker (200 rpm/hour) at 37°C for 16 hours (Figure 2).

About 1 ml of the *E. coli* culture was boiled for about 5 minutes to destroy the cell membranes and then centrifuged. One microliter of it was used as a template for PCR reaction with specific primers of *HVA1* (forward 5'-TGGCCTCCAACCAGAACCAG-3' and reverse 5'-ACGACTAAAGGAACGGAAAT-3'). Three of 26 colonies, i.e., No. 24, 25, and 26 produced the right size of the *HVA1* fragment (Figure 3). DNA of plasmid pRP9_*HVA1* was then isolated from *E. coli* cultures No. 25 and No. 26 using the can lyses method. For reconfirmation, these plasmid DNA were double digested with *Hind*III and *Bam*HI restriction enzymes. The results showed that they had a 1.0 kb fragment that was the *HVA1* gene (Figure 1.B.3).

Double digestion of pRP9_*HVA1* plasmid with *Xho*I and *Spe*I restriction enzymes also resulted in a 1.0 kb fragment *HVA1* (Figure 1.B.4a). The same reaction was carried out to plasmid pAY560326 (Figure 1.B.4b).

Both purified DNA of *HVA1* and pAY560326 were ligated and transformed into *E. coli* DH5-α competent cells using the heat shock method. The cells were then grown on LBA plates containing 50 mg/l kanamycin, and the single colonies produced were grown in LB medium containing 50 mg/l. Among the 22 colony cultures, only two tubes, i.e., No. 21 and No. 22 produced a lot of cells that the DNA was isolated from. The double digestion of plasmid DNA using *Spe*I and

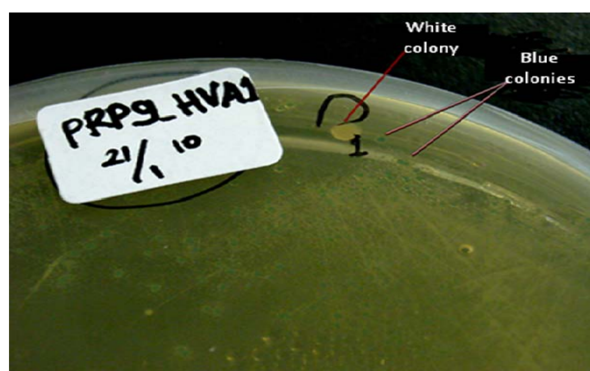

Figure 2. White and blue colonies on LBA medium containing 100 mg/l ampicillin, IPTG, and X-gal.

*Xho*I restriction enzymes that was carried out to confirm the presence of DNA was the right one. The results showed that the 1.0 kb DNA fragment was the *HVA1* gene and a backbone, which is pAY560325 plasmid (Figure 1.B.5). Based on this result, the plasmid vector expression of the *HVA1* gene was obtained and named pAY_*HVA1*.

The last step in the construction of *HVA1* expression vector was to transfer the pAY_*HVA1* plasmid DNA into *A. tumefaciens* strain EHA105 competent cells using the heat shock method. The transformed cells were then grown on LBA plates containing 75 mg/l rifampicin dan 100 mg/l kanamycin, and incubated at 28°C for 48 hours. The selected single colonies were then grown in LB medium containing 75 mg/l rifampicin. Two of 10 colonies grew in the medium. Plasmid DNA pAY_*HVA1* were isolated from both colony cultures and confirmation were carried out using PCR. The results showed that both cell cultures contained the right pAY_*HVA1* plasmid as they had the *HVA1* gene in them (about 600 bp DNA fragment) (Figure 4).

Plant Transformation

In order to test the pAY_*HVA1* construct, it was then transformed into rice varieties Ciherang and Inpari 6, two of the widely cultivated rice varieties in Indonesia. Eight transgenic plants of variety Ciherang were generated from 123 transformed explants (Table 1, Figure 5) and three transgenic plants of Inpari 6 were generated from 125 transformed explants (Table 1, Figure 6).

Table 1. Rice transformation (pAY_*HVA1*) efficiencies using the early infection of scutellum method.

Rice variety	Ciherang		Inpari 6	
Data observed	Total	Percentage	Total	Percentage
Transformed explants	123	-	125	-
Resistant hygromycin explants	47	38.2	25	20
Regenerated explants	13	27.7	6	24
Transgenic plants	8	6.5	3	2.4

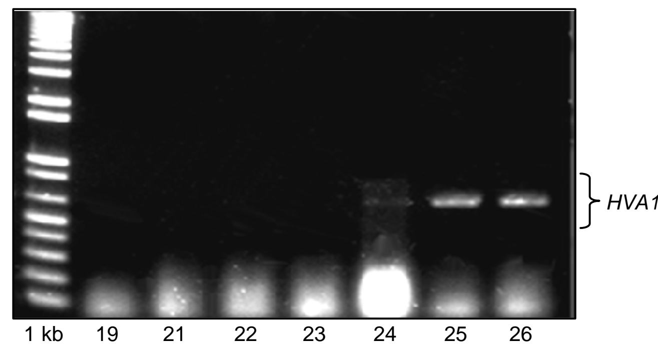

Figure 3. Gel electrophoresis of PCR products from DNA of *E. coli* colony cultures No. 19-26 using specific primers for *HVA1* gene. Colonies No. 19-24 negatives. Colonies No. 25 and 26 each produced a 600 bp DNA.fragment.

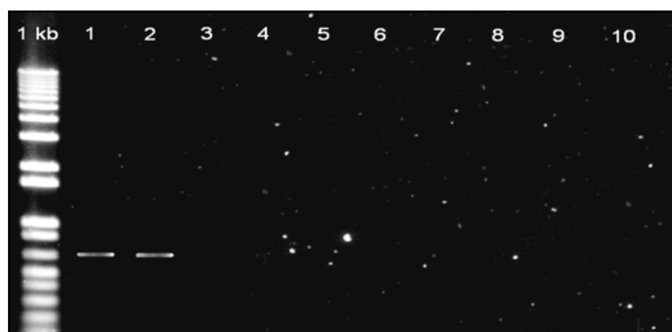

Figure 4. Gel electrophoresis of PCR products from DNA of *A. tumefaciens* colony cultures No. 1-10 using specific primers for *HVA1* gene. Colony cultures No. 1 and 2 produced positive results.

Confirmation of Transgenic Plants by PCR

Results of the transgenic confirmation of the *HVA1* gene by PCR using specific primers on rice varieties Ciherang and Inpari 6 are shown in Figure 7, a 600 bp DNA fragment was produced and this was similar to the fragment produced from PCR of DNA of *E. coli* and *A. tumefaciens* cell cultures containing of the pAY_*HVA1* plasmid. Nine of 11 transformed plants (Table 1) were confirmed putatively transgenic (Figure 7).

CONCLUSION AND SUGGESTION

Construction of vector for expression of a drought plasmid expression vector carrying *HVA1* was successfully constructed into pAY_*HVA1*. It consists of 35S promoter, 1 kb DNA sizes of *HVA1* gene and NOS terminator. The plasmid pAY_*HVA1* vector was also successfully transformed into EHA105 a high virulence strain of *A. tumefacies*. The efficiencies pAY_*HVA1* in to scutellum explants of Ciherang and Inpari 6 were 6.5 and 2.4, respectively. Of those 11 transgenic putative,

Figure 5. Transgenic plants of rice variety Ciherang generated from the transformation.

Figure 6. Transgenic plants of rice variety Inpari 6 generated from the transformation.

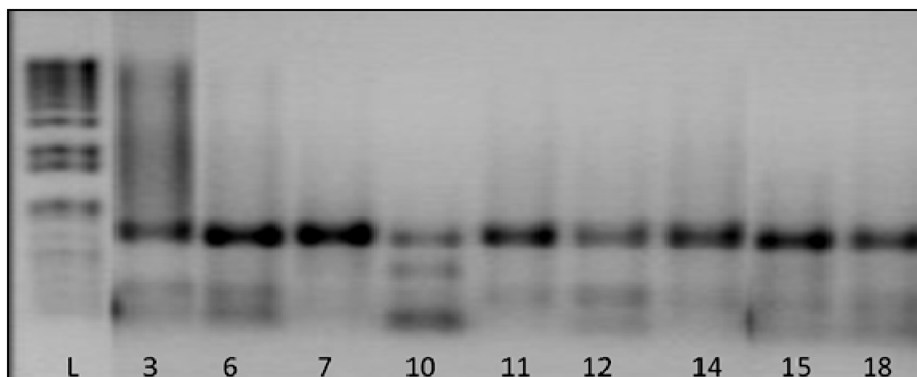

Figure 7. A 600 bp DNA fragment produced by PCR using specific primers for *HVA1* gene from transgenic plants of rice varieties Ciherang (nos. 3, 7, 10, 11, 12, and 15) and Inpari 6 (nos. 6, 14, and 18). L = 1 kb DNA adder (Invitrogen).

nine were confirmed transgenic by PCR using specific primers of *HVA1* gene.

These transgenic will be investigated further to find out how many copies of T-DNA within those nine lines and how T-DNA relates to their performances in drought or high salinity conditions.

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