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REVIEW ARTICLE

Development of *Bt* rice potential for yellow stem borer control

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Abstract
Rice (*Oryza sativa* L.) production is always threatened by biotic and abiotic stresses. Both stresses can reduce rice productivity and quality. Yellow stem borer (YSB; *Scirpophaga incertulas* Walker) is one of the biotic stresses and is reported as the most destructive pest of tropical rice insects. The application of pesticides is less effective since the insect larvae live and feed inside the stem, thus inhibiting pesticides to reach the larvae. Planting YSB-resistant cultivars is a good strategy besides environmentally friendly. However, no sufficient level of resistance to YSB has been identified among rice germplasm collection, making the use of conventional breeding methods is difficult. *Bacillus thuringiensis* (*Bt*) is a Gram-positive bacterium produces insecticidal proteins, Cry toxins that are toxic to YSB. The introduction of *cry* genes from *Bt* into rice plants by using genetic engineering has been widely and successfully carried the out. A number of strategies have been conducted to increase the expression level and to prolong the effectiveness of *Bt* toxins in transgenic rice, including plant codon usage-optimized genes, the use of strong promoters and wound-inducible promoters and gene stacking. Insect bioassays under laboratory, greenhouse and field conditions showed that transgenic rice plants harboring and expressing *cry* genes are highly resistant to YSB compared to the original cultivars from which transgenic plants were developed. Thus, the development of transgenic rice plants harboring and expressing *cry* genes from *Bt* is a good strategy to build plant resistance against YSB.

Keywords *Bacillus thuringiensis* · *cry* · Resistance · Rice · Yellow stem borer

Introduction

Rice (*Oryza sativa* L.) is an important food crop for more than half of the world's population with globally providing approximately 20% of the calorie intake. Most of the population growth occurs in Asia where people make rice as their staple food (Kubo and Purevdorj 2004; Chatterjee and Mondal 2014). The International Rice Research Institute predicted that 800 million tons of rice will be required in 2025 (Purevdorj and Kubo 2005). Thus, increasing human population has to be in line with rice production to meet food security. Rice supply and demand must be balanced. However, rice production faces many problems that come from biotic and abiotic stresses. Among biotic stresses, insect pests contribute to the decline in rice productivity and quality, among which is yellow stem borer (*Scirpophaga incertulas* Walker).

Yellow stem borer (YSB) which is one of Lepidopteran, is a dominant pest in Asia (Renuka et al. 2017). YSB is a pest of deepwater rice and is reported as the most destructive tropical insect pest in paddy fields, since their chronic pattern of infestation and widespread distribution (Cohen et al. 2008; Pathak and Khan 1994; Ghareyazie et al. 1997; Deka and Barthakur 2010). YSB is a monophagous pest and there are no reports that YSB has successfully completed its life cycle in any other plants outside the *Oryza* species (Renuka et al. 2017).

YSB attacks the rice plant from seedling to grain filling stage, resulting in different symptoms (Devasena et al. 2018). The symptoms of deadhearts are produced when insect attacks at vegetative stage, meanwhile the symptoms called whiteheads occur when insect attacks at generative stage of rice plant growth (Cohen et al. 2008; Deka and Barthakur 2010; Ho et al. 2013). The production losses also vary. Every 1% of deadhearts or whiteheads cause production losses of 2.5% and 4%, respectively (Renuka

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et al. 2017), even when no chemical control is applied, YSB attacks can destroy the whole crop (Breitler et al. 2004).

The application of pesticides that commonly used to control insect pests is ineffective since larvae of YSB burrow deep into the rice stem and feed inside the stem thus inhibiting pesticides to reach the larvae (Manikandan et al. 2016; Kumar et al. 2010). Moreover, the excessive use of pesticides, beside increasing the production costs also cause deleterious effects to the environment and human health and lead to the emergence of pesticide-resistant insects (Nayak et al. 1997; Breitler et al. 2004; Ho et al. 2006).

Planting cultivars with built-in resistance to YSB is a good strategy (Ho et al. 2006; Hoa and Nhu 2011). One of the important requirements for conducting a plant breeding program is the availability of resistant genes in the plant. However, The International Rice Research Institute (IRRI) has screened more than 30,000 germplasm collection of rice and no sufficient level of resistance to YSB among germplasm has been identified, making the option to use the conventional breeding methods is ineffective. Therefore, another strategies need to be considered (Wu et al. 1997; Chen et al. 2005; Kumar et al. 2010; Yong-Mei et al. 2014; Breitler et al. 2000; Hoa and Nhu 2011).

Bacillus thuringiensis (*Bt*) has been identified as a reservoir of resistant genes against YSB. *Bt* is a Gram-positive bacterium produces proteins with entomopathogenic properties, known as Cry toxins (Bravo et al. 2007; Bravo and Soberón 2008). The development of insect-resistant transgenic rice lines by introducing *cry* genes from *Bt* with different versions has been widely carried out (Ye et al. 2001; Cheng et al. 1998; Gao et al. 2011; Manikandan et al. 2016). Bioassays against YSB showed that the transgenic rice plants expressing *Bt* insecticidal proteins exhibited resistance against YSB under laboratory, greenhouse and field conditions (Tu et al. 2000; Datta et al. 2002; Kumar et al. 2010; Chakraborty et al. 2016). In this article the overview of YSB, mode of action of Cry toxins and the development of YSB -resistant transgenic rice lines expressing Cry toxins will be reported.

Yellow stem borer and its impact on rice plants

The moths lay eggs in masses and egg masses are covered with brownish hairs. The newly emerged larvae crawl upward to the tip of the leaf to stay for only short periods. Some larvae form threads and swing to land on other plants or fall on the water (Fig. 1). The larvae that remaining on the tip will climb down to the base and crawl between the leaf sheath and stem. The larvae feed on the leaf sheath tissues for about 1 week and then bore into the stem, staying in the pith and feed on the inner surface of the stem walls, severance the apical parts of the plant from the base. This causes the central leaf does not open, the color changes to brown and dries out and easy to pull out although the

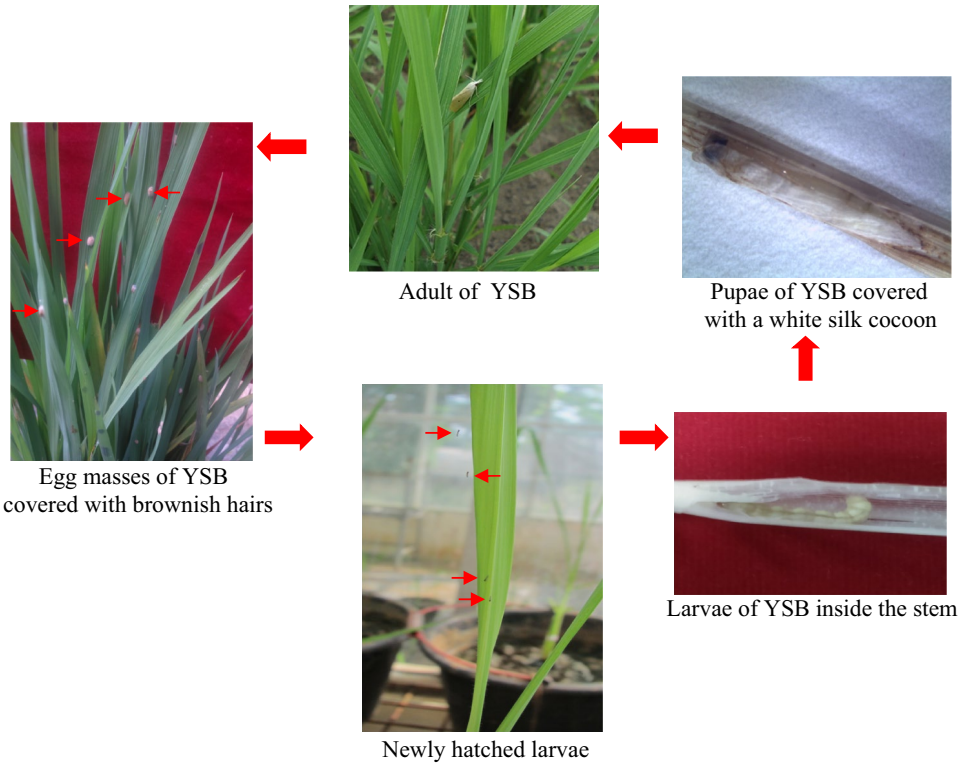

Fig. 1 The life cycle of yellow stem borer

remain leaves still green and healthy. The symptoms called deadhearts (Fig. 2a). If the larvae of YSB feeding above the primordia and no further damage occurs, the dead central leaves are pushed out by the new growth, so the plants can compensate for the damage caused by YSB by producing new shoot (Pathak and Khan 1994).

Infestation of YSB during the reproductive stage or after panicle initiation causes the severance of growing plant parts from the base dries the panicles and panicles failed to emerge completely. However, when panicles have emerged, the damage blocks the transport of nutrients from the stem to the grains results in the production of panicles without grains. The affected panicles are being whitish and remain straight. The symptoms are known as whiteheads (Fig. 2b) (Ghareyazie et al. 1997; Pathak and Khan 1994; Deka and Barthakur 2010).

Since the insect larvae live and feed inside the stem and remain there for the rest of their lives as larvae and pupae, thus protecting them completely from the application of chemical pesticides (Nayak et al. 1997; Ho et al. 2006).

Mode of action of Cry toxins in Lepidopteran

Bacillus thuringiensis (*Bt*) is a Gram-positive soil bacterium produces crystalline inclusions during sporulation (Höfte and Whiteley 1989; Bravo et al. 2018). The crystal inclusions consist of δ-endotoxins, that are encoded by a number of *cry* genes (Tu et al. 2000; Riudavets et al. 2006; Bravo et al. 2007, 2012, 2018; Sharma et al. 2000). The various δ-endotoxins have been classified into classes (Cry 1, 2, 3, 4, etc.) based on amino acids similarities. These classes are then composed into subclasses (Cry1A, Cry1B, Cry1C, etc.) and each subclass is divided into subfamilies or variants (Cry1Aa, Cry1Ab, Cry1Ac, etc.) (Sanchis and Bourguet 2008).

Cry proteins with entomopathogenic properties are grouped into five types based on the specificity of the target insect. Cry1 proteins are specific to Lepidopteran larvae, Cry2 proteins are specific to Lepidopteran and Dipteran larvae, Cry3 proteins are specific to Coleopteran larvae, Cry4 proteins are specific to Dipteran larvae and Cry5 proteins are specific to Lepidopteran and Coleopteran larvae (Sharma et al. 2000; Bravo et al. 2007).

The main target of *Bt* toxins is insect midgut. When susceptible larvae ingest the crystalline inclusions, the crystals are solubilized under alkaline condition (high pH) which is typical of Lepidopteran insect midgut, releasing proteins called δ-endotoxins. The δ-endotoxins are in fact prototoxins. Further, they are cleaved by midgut proteases to produce mature toxins which exhibit a highly specific insecticidal activity. The region within the N-terminal toxin is composed of at least 6 α-helices which play an important role in penetrating the peritrophic membrane (Sharma et al. 2000; Pardo-López et al. 2013; Palma et al. 2014; Bravo et al. 2015, 2018).

The activated toxins bind to the specific receptors, on the brush border membrane of the midgut epithelial cells of susceptible larvae before penetrating into the target cell membrane. Further, the α-helices penetrate the membrane irreversibly, forming pores. The formation of toxin-induced pores in the membrane, destroying the cells and lead to the death of the susceptible insect larvae (Tu et al. 2000; Bravo et al. 2018; Kumar et al. 2008; Sharma et al. 2000; Schünemann et al. 2014).

Cry proteins are highly specific to a limited number of target insect species and they are harmless to non-target insects including natural enemies, vertebrates, plants and humans (Riudavets et al. 2006; Pardo-López et al. 2013; Bravo et al. 2012, 2018).

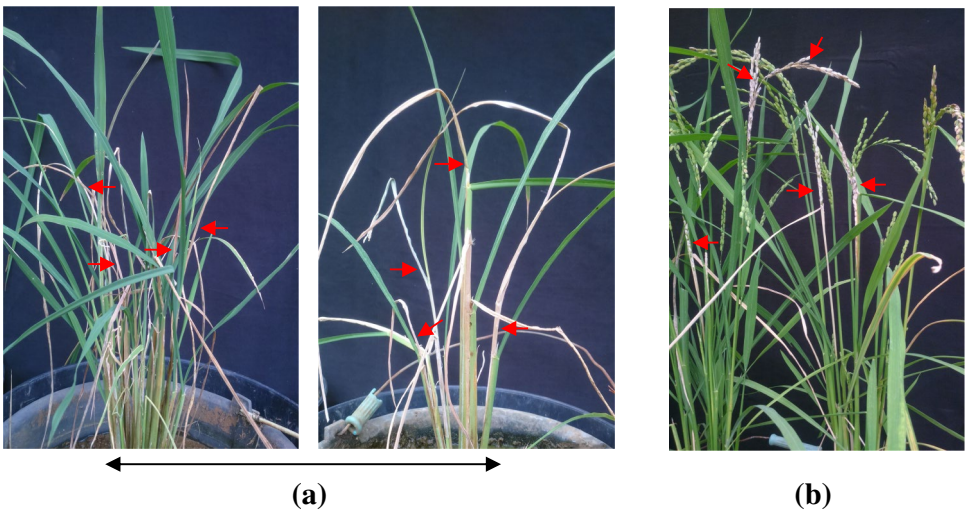

Fig. 2 The symptoms caused by yellow stem borer in infected rice at different stages of rice plant growth are indicated by red arrows. **a** Deadhearts at the vegetative stage; **b** whiteheads at generative stage

The improvement of rice plant resistance to YSB by introducing the *cry* genes

Since no sufficient level of resistance to YSB has been identified among rice germplasm collection, the development of YSB-resistant cultivars through conventional breeding methods is difficult (Kumar et al. 2010; Ho et al. 2006). Therefore, an alternative strategy is to employ genetic engineering that allows breeders to introduce desirable genes across taxa. Genetic engineering can overcome the crossing by producing new shoot barriers between unrelated organisms and this technology complements traditional methods in crop improvement (Nayak et al. 1997; Ramesh et al. 2004; Ho et al. 2006).

Insecticidal proteins from *Bt*, also known as Cry proteins have been reported can control the major Lepidopteran pests of rice including YSB (Frutos et al. 1999; Tabashnik et al. 2015; Zhou et al. 2014; Breitler et al. 2000). By using genetic engineering, it is possible to introduce *cry* gene from *Bt* into rice plant to build resistance toward YSB. Several gene transformation techniques can be used to transfer the desirable genes into plants and the most widely used is *Agrobacterium*-mediated transformation (Deka and Barthakur 2010). Transformation of *cry* genes into rice genome has been succesfully conducted and since 1995 *Bt* crops have been grown increasingly over the years (Breitler et al. 2004; Liu et al. 2016).

The expression of single *cry* gene in transgenic rice plants to confer full rice plant protection from YSB has been reported in both Japonica and Indica rice. Transgenic Indica Basmati rice (B-370) expressing *cry1Ac* and *cry2A* genes separately and each driven by the maize ubiquitin promoter and CaMV35S promoter respectively, exhibited a high level of resistance to YSB under field studies for two consecutive years. Five transgenic rice lines of B-370 showed resistance against YSB with the percentage of deadhearts and whiteheads ranged from 16.2 to 36.6% and 13.3 to 18.1%, respectively, compared to 100% of deadhearts and whiteheads in untransformed B-370 control. Nevertheless, the level of resistance of transgenic rice harboring *cry2A* gene was lower compared to transgenic rice containing *cry1Ac*. These data might be correlated with the protein accumulation in the plants. The content of Cry1Ac in four transgenic lines within the range of 0.97–1.13% of total leaf soluble protein. Meanwhile, the content of Cry 2A in one transgenic line was lower and calculated within the range of 0.12–0.95% of total leaf soluble protein. The low expression of Cry2A may be due to the gene was driven by the CaMV35S promoter, thus the use of strong promoters such as ubiquitin promoter should be considered (Bashir et al. 2004). The maize ubiquitin promoter is a constitutive promoter that can enhance the expression of foreign genes in monocots and is more active than CaMV 35S promoter in transgenic rice plants (Zhou et al. 2013; Breitler et al. 2004; Bashir et al. 2005).

There are many approaches to increase the expression of *cry* gene in transgenic rice plants, in addition to the use of strong promoters. Most of published studies reported that plant codon usage-optimized genes and the use of green tissue-specific promoters are the options (Bano-Maqbool et al. 1998; Wu et al. 1997; Breitler et al. 2004; Chakraborty et al. 2016).

The plant codon-optimized *cry1C** gene with GC content of 44.65% and shares 84% nucleotide sequence homology with the original *cry1Ca5* gene and a modified *cry2A** gene with GC content of 59.04% and shares 69.45% homology in nucleotide sequence with the original *cry2Aa*, and each gene was driven by a maize ubiquitin promoter had been introduced into Indica rice cv. Minghui 63, separately (Tang et al. 2006; Chen et al. 2005). Evaluation of resistance under field conditions in one homozygous transgenic line, T1c-19 line harboring *cry1C** with the highest Cry1C* protein content (1.38 μg g^{-1} fresh leaf) showed that no deadhearts and whiteheads symptoms were observed in T1c-19 (0%), whereas the percentage of deadhearts and whiteheads in Minghui 63 as a control of susceptible cultivar was 17.3% and 5.6%, respectively (Tang et al. 2006). Moreover, no pesticides for YSB were applied during the experiment.

Similar expression of *cry2A** gene in transgenic rice lines against YSB was obtained. Laboratory assay to determine the efficacy of *cry2A** gene showed that the Cry2A* content in four homozygous transgenic lines within the range of 9.65 -12.11 μg g^{-1} fresh leaf was able to kill 100% larvae of YSB within 5 days after infestation. On the contrary, in original susceptible Minghui 63, the percentage of dead larvae was only 10.1% and the remaining larvae grew normally. Plant resistance performance in the field showed that the percentage of deadhearts and whiteheads of four homozygous transgenic lines ranged from 0.98 to 4.18% and 0.00 to 0.50%, respectively, whereas in original susceptible Minghui 63 reached 17.24% and 5.57% (Chen et al. 2005). These data explained that transgenic rice lines with codon optimized, *cry1C** and *cry2A** genes under the control of a maize ubiquitin promoter were highly significantly resistant against YSB compared to the original susceptible Minghui 63 under laboratory and field conditions.

Breitler et al. (2000) reported that a novel monocot codon-optimized *cry1B* synthetic gene with a GC content of 58% and driven by the maize ubiquitin promoter had been transferred into highly susceptible cultivar to Striped Stem Borer (SSB; *Chilo suppressalis* Walker), Ariete cultivar and confers full protection in one homozygous transgenic event (A64) against SSB under phytotron conditions. The accumulation of Cry1B at 0.4% of the total soluble protein was sufficient to protect transgenic plants from SSB attacks and this was demonstrated by 100% insect mortality within

7 days after infestation. On the contrary, no dead larvae (0%) were found in original susceptible Ariete cultivar. Similar to YSB, SSB is a rice stem borer and belongs to the Lepidopteran group. SSB is most abundant in temperate countries and is reported as an important pest in Asia and Europe and no sufficient level of resistance to SSB in rice germplasm has been identified (Breitler et al. 2000, 2004).

The use of a green tissue-specific promoter of *rbc*S gene to target protein to the chloroplast is another alternative. The use of a *Chrysanthemum rbc*S1 promoter to drive a chimeric *cry2AX1* to the chloroplast, resulted in higher expression and better stability of Cry2AX1 in green tissues. A chimeric *cry2AX1* with a total of 633 amino acids composed of the N-terminal residues, 1–585 are from Cry2Aa and the C-terminal residues, 586–633 are corresponded to 576–623 of Cry2Ac (Manikandan et al. 2016; Chakraborty et al. 2016). A chimeric *cry2AX1* was then introduced into Indica rice line, JK1044R. The highest expression level of Cry2AX1 among five homozygous transgenic lines was detected in BtE15 line. The level of Cry2AX1 in the leaf of BtE15 line was 0.68 µg g^{-1} fresh leaf at vegetative stage and 1.18 µg g^{-1} fresh leaf at the generative stage, whereas in leaf-sheath reached 0.81 and 1.34 µg g^{-1}, respectively. Evaluation of transgenic BtE15 line against YSB had been conducted using *in vitro* and *in planta* bioassays. *In vitro* bioassay on BtE15 line showed that the percentage of dead larvae was 88% at vegetative stage and 93% at the generative stage, meanwhile in non-transgenic control plants were 7% and 8%, respectively. *In planta* bioassay on BtE15 line showed that the percentage of deadhearts and whiteheads were 1.74% and 0%, respectively, and in non-transgenic control plants were 22.08% and 15.74%. These data indicated that the accumulation of Cry2AX1 in green tissues of BtE15 line exhibited significantly resistant against YSB (Chakraborty et al. 2016). In addition to have a better stability and expression of Cry2AX1, the use of *rbc*S1 promoter can avoid the accumulation of Cry in rice seed endosperm (Chakraborty et al. 2016; Ye et al. 2009).

The continuous exposure of transgenic crops expressing a single *Bt* toxin may lead to the breakdown of plant resistance to YSB in the field. This is due to the pests have the potential to evolve to breakdown the effectiveness of *Bt* toxins. Many strategies to prevent or to delay the emergence of pest resistance in the stem borer populations to *Bt* toxins have been proposed (Chen et al. 2005; Kumar et al. 2010; Manikandan et al. 2016). The concept of gene stacking by combining two or more *cry* genes with different modes of action in one plant has been recommended as an efective strategy (Chen et al. 2005; Bravo and Soberón 2008; Kumar et al. 2010; Manikandan et al. 2016). This strategy based on the assumption that the emergence of insect resistant to a *Bt* toxin is due to a mutation in one of the insect's genes and single mutation does not confer simultaneous resistance to other toxins. Multiple mutations are required to break the effectiveness of two or more *Bt* toxins. Thus, the combination of two *cry* genes should have different modes of action, otherwise a single mutation of insect may confer a cross resistance to both Cry toxins (Bashir et al. 2004, 2005; Chen et al. 2005; Tang et al. 2006).

The translational fusion gene *cry1B-cry1Ab* driven by the maize ubiquitin promoter had been introduced into two popular cultivars in Vietnam i.e. Nang Huong Cho Dao (NHCD) and Mot Bui (MB). The *cry1B* and *cry1Ab* genes were fused at 28th codon downstream from Cry1B activation-site codons and 29th codon upstream from Cry1Ab activation-site codons. This fusion gene encodes a single Cry1B-Cry1Ab fusion protein with molecular weight of 66-kDa. Western blot analysis showed that the presence of a single band of 66-kDa in two transgenic rice lines, Nab4 and Mab2 indicated the expression of *cry1B-1Ab* with the level of protein ranged from 0.8 to 1.3% of the total soluble protein. Insect bioassay under laboratory conditions using the cut-stem method showed that the percentage of dead larvae in transgenic rice lines tested reached 100% at 4 days after infestation. Meanwhile, the percentage of dead larvae in untransformed plants within the range of 0–16.6%. These data indicated that the protein content with a range of 0.8–1.3% of the total soluble protein was able to protect plants from YSB attacks. However, the efficacy of the translational fusion gene in transgenic rice lines should be conducted in the field under natural conditions (Ho et al. 2006).

Another strategy to prevent or to delay the emergence of resistance among the target insects to *Bt* toxin is the use of wound-inducible promoters (Breitler et al. 2004; Breitler et al. 2001). One of the wound-inducible genes is the maize proteinase inhibitor (*mpi*) gene encoding for MPI protein. The accumulation of MPI was observed in response to mechanical wounding or insect feeding (Tamayo et al. 2000). A monocot codon-optimized *cry1B* synthetic gene driven by a wound-inducible promoter of *mpi* gene had been transformed into widely grown Mediterranean Japonica rice cultivar, Ariete. The homozygous transgenic line harboring *cry1B* gene under the control of *mpi* promoter, A9.1 line was protected from SSB attacks compared to the original Ariete cultivar under greenhouse and field conditions (Breitler et al. 2001, 2004). Moreover, A9.1 line has the same level of resistance as A64.1 line against YSB and the accumulation of Cry1B in insect-wounded leaves of A.9 line was comparable to the A.64.1 line i.e. 0.2%–0.4% of total soluble protein. A64.1 is a transgenic rice line expressing the *cry1B* gene under the control of the constitutive maize ubiquitin promoter and show a highly resistant against SSB (Breitler et al. 2000, 2004).

To study the role of a wound-inducible promoter of *mpi* gene in relation to Cry1B accumulation in transgenic rice

plants, Western analyses on both unwounded and wounded of leaf blade, leaf sheath, stem and root tissues had been conducted. Cry1B was not detected in unwounded tissues but can be found adjacent the insect-wounded tissues of transgenic rice line (A9.1) harboring *cry1B* gene under the control of *mpi* promoter (pMpi-*cry1B*). On the contrary, Cry1B was detected even in unwounded tissues of transgenic rice line, A64.1 expressing the same gene driven by the constitutive maize ubiquitin promoter (pUbi-*cry1B*). The results indicated that the activity of *mpi* promoter was triggered by wounding (Breitler et al. 2004).

Moreover, the accumulation of Cry1B was not detected in seed parts (embryo, mature endosperm, polished grain and cooked polished grain) of A9.1 line whereas the toxin was found in comparable tissues except in cooked rice of A64.1 line (Breitler et al. 2004). This finding supports the previous statement that the activity of the *mpi* promoter was triggered under wound induced conditions.

The absence of Cry1B toxin in cooked polished grain of both transgenic rice lines, A9.1 and A64.1 is due to the boiling process. Heating can destroy the heat-sensitive Cry1B (Breitler et al. 2004). Although Cry toxins have been reported to be safe for human consumption (Bashir et al. 2004), additional evidence related to the absence of Cry1B in cooked rice provides an added value in increasing consumer confidence in the acceptance of *Bt* rice (Breitler et al. 2004).

The development of Javanica rice cultivar, Rojolele expressing the *cry1B* gene had been conducted in our laboratory. The goal of our research is to build the resistance of susceptible Rojolele cultivar against YSB. Rojolele cultivar was chosen since it has a good quality in taste and texture and aromatic as well, which makes this cultivar popular in Indonesia. The construct of *cry1B* gene driven by a wound-inducible promoter of *mpi* gene, was provided by Dr. Emmanuel Guiderdoni (CIRAD, France).

Agrobacterium-mediated transformation had been chosen to introduce *cry1B* gene from *Bt* into a local Javanica rice cultivar, Rojolele. Four homozygous transgenic rice lines harboring *cry1B* gene under the control of *mpi* promoter have been obtained. The efficacy of *cry1B* gene in four homozygous transgenic rice lines was examined using *in vitro* and *in planta* bioassays under laboratory and greenhouse conditions. Both assays were conducted at the vegetative stage. *In vitro* bioassay using the cut-leaf method showed that the percentage larval mortality of YSB in four homozygous transgenic rice lines ranged from 93.62 to 100% within 3 days after infestation. Meanwhile, the number of mortality of YSB larvae in non-transgenic Rojolele cultivar only reached 11.11% and the remaining larvae still alive (Estiati et al. 2020). Due to the very few surviving larvae in transgenic tissues, it is suggested that the toxin level of Cry1B in four homozygous transgenic lines was sufficient to kill YSB larvae (Fig. 3).

In planta bioassay using the whole plant showed that the percentage of deadhearts in four homozygous transgenic rice lines ranged from 0 to 9.83%. Conversely, in non-transgenic Rojolele and IR64 cultivars as susceptible checks reached 93.5% and 98.18%, respectively. The contrasting appearance between transgenic rice lines and untransformed control cultivars can also be seen (Fig. 4). Based on the scale from 0 to 9 (IRRI 2013), the four homozygous transgenic rice lines have score of 0 and 1 and they are categorized as resistant lines, whereas the non-transgenic Rojolele and

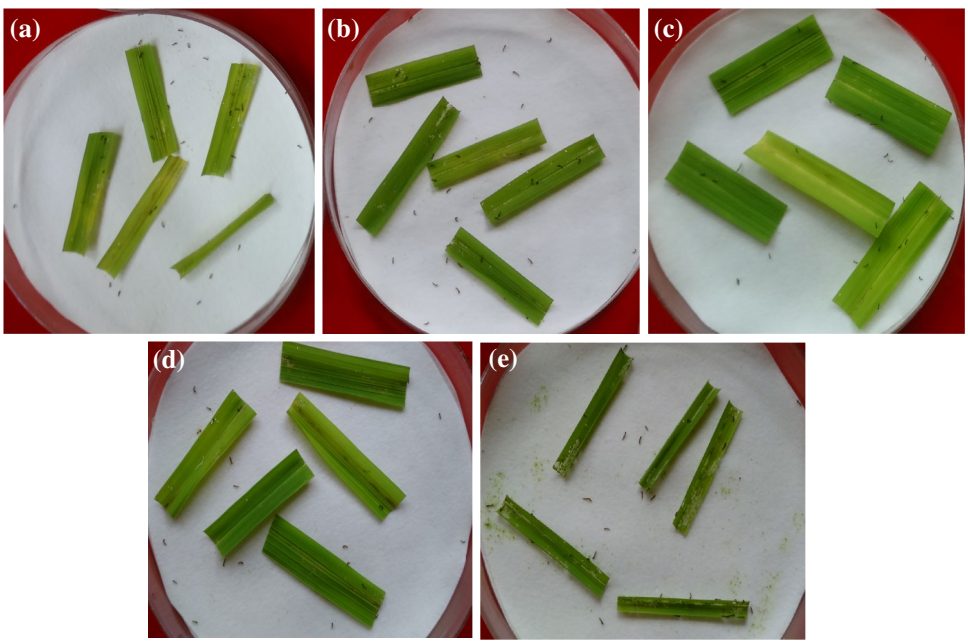

Fig. 3 *In vitro* bioassay on homozygous transgenic rice lines harboring *cry1B* gene and non-transformed Rojolele as a control of susceptible cultivar. Each leaf section was infested with 10 larvae of YSB. **a–d** Homozygous transgenic rice lines; **e** non-transgenic Rojolele cultivar. The observations were conducted at 3 days after infestation. The leaves of four homozygous transgenic rice lines exhibited little detectable damage. Meanwhile, severely damaged leaves were seen in non-transgenic Rojolele plant as a result of YSB feeding (Source: Estiati et al. 2020)

IR64 cultivars have a score of 9 and they are categorized as highly susceptible cultivars against YSB (Estiati et al. 2020).

From insect bioassays under *in vitro* and *in planta* conditions exhibited that the expression of *cry1B* gene in transgenic rice provides satisfactory plant protection against YSB and SSB (Estiati et al. 2020; Breitler et al. 2004).

Conclusion

YSB is difficult to control by using pesticides since the insect larvae stay only for a short period outside the plant before enter the plant and live in the stem (Pathak and Khan 1994). Planting the YSB-resistant cultivars in the field is the right approach. Since no rice germplasm with sufficient level of resistance to YSB has been identified, plant improvement by utilizing the resistance genes from rice plants is less effective (Breitler et al. 2000; Deka and Barthakur 2010). Genetic engineering is an alternative. Genetic engineering overcomes the problem of sexual incompatibilities thus it is possible to transfer or to introduce the desirable genes between any two unrelated species of organisms (Ye et al. 2009; Kumar et al. 2010). Introducing *cry* genes from *Bt* into various rice cultivars has been widely carried out. The *cry* genes have been introduced into rice cultivars adapted to local growth conditions and consumer requirements. The efficacy of the genes in transgenic rice plants under laboratory, greenhouse and field conditions exhibited a high level of protection from damages by YSB (Breitler et al. 2000). It has been reported that the higher concentration of Cry protein in transgenic plants correlates with the higher mortality of insect larvae (Manikandan et al. 2016; Kumar et al. 2010; Breiler et al. 2000). Thus, the level of Cry protein correlates with the level of insect resistance.

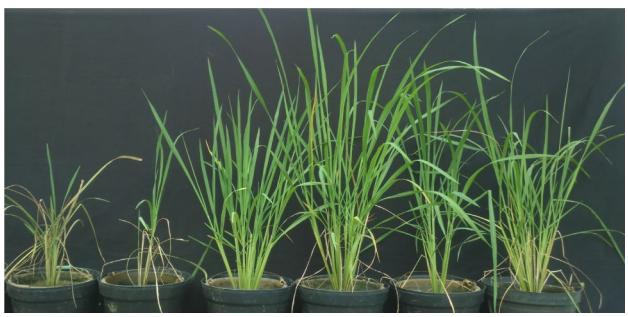

Fig. 4 The appearance of transgenic rice lines and susceptible checks following infestation with the larvae of YSB at 14 days after inoculation. **a** Non-transgenc IR64 cultivar; **b** non-transgenic Rojolele cultivar; **c–f** homozygous transgenic rice lines. The deadhearts symptoms were visible both in non-transgenic Rojolele and IR64 cultivars but were absent in four homozygous transgenic rice lines (Source: Estiati et al. 2020)

A number of strategies have been conducted to increase the expression of *cry* genes in transgenic rice and to prevent or to delay the emergence of insect resistance in the stem borer populations to *Bt* toxins. These including the use of strong promoters such as the constitutive ubiquitin (*ubi*) promoter from maize, a green tissue-specific promoter (*rbc*S) to target protein to the chloroplast, optimized codon usage to match codon preference in plants, discovery of novel insecticidal genes with novel modes of action, gene stacking and the use of wound-inducible promoters (Breitler et al. 2000; 2004; Cheng et al. 1998; Chakraborty et al. 2016; Manikandan et al. 2016).

The advantages of using a green tissue-specific *rbcS1* promoter from *Chrysanthemum morifolium* and a wound-inducible *mpi* promoter from maize in addition to increase the expression of *cry* genes, also prevent the accumulation of Cry toxins in the rice seed parts as well (Chakraborty et al. 2016; Breitler et al. 2004). The absence of Cry accumulation in the rice seed parts gives a positive value in increasing consumer confidence in the acceptance of *Bt* rice.

Gene stacking or combining two or more *cry* genes with different modes of action in one plant is a strategy to prevent or to delay the insect pest from evolving to become resistant to *Bt* toxins. Insect has the ability to overcome the efffectiveness of a *Bt* toxin with mutation in one of insect's gene. Thus, in order to survive, multiple mutations are required by the insect to break the effectiveness of multiple *Bt* toxins and this will not be realized in a short time (Bashir et al. 2005; Chen et al. 2005; Tang et al. 2006; Ho et al. 2006; Bravo and Soberón 2008).

The level of insect resistance against YSB is depends on the accumulation of Cry protein in plant (Tang et al. 2006). The concentration of Cry in transgenic rice plants varied within the range of 0.1–0.5% of total soluble protein, even in some transgenic rice plants reached 1% of total soluble protein. These levels of Cry are sufficient to protect plants from YSB attacks. However, high toxin accumulation at a level of 3–5% of total soluble protein have been reported but the plants showed phenotypically abnormal leading to stunting, leaf chlorophyll bleaching, sterility and decrease of yield (Bano-Maqbool et al. 1998; Breitler et al. 2000; Chen et al. 2005; Chakraborty et al. 2016).

Although the accumulation of Cry toxins with different gene constructs are different among independent transgenic rice lines, the toxins are still providing full-plant protection against YSB compared to the non-transformed control cultivars. Transgenic rice plants expressing Cry toxins resulted in 90–100% insect mortality and showed almost zero percentage of deadhearts and whiteheads. Meanwhile, in non-transgenic rice plants that do not contain Cry toxins, the insect larvae grow normally and the dead larvae is only less than 10%. The non-transformed plants also produce a high percentage of deadhearts and whiteheads. Moreover,

Bt toxins are highly specific to target insect thus they are harmless to non-target organisms especially for mammals (Tu et al. 2000; Kumar et al. 2008). Thus, the development of environmentally friendly YSB-resistant transgenic rice plants by introducing and expressing the *cry* genes from *Bt* is a good strategy to overcome yield losses due to YSB attacks in paddy field.

Compliance with ethical standards

Conflict of interest The author declares that there is no conflict of interest.

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