Chapter 8

Biotechnology of Drought-Tolerant Sugarcane

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72436

Abstract

Water stress exists in most sugarcane cultivation areas, which are not supported by irrigation system and have low rain precipitation. Better understanding of physiological and biochemical mechanism, underlying plants response to water stress, have been achieved to develop drought-tolerant plants by biotechnology approach. To survive and grow normally, plants use a range of strategies to cope the water stress such as changes in gene expression and accumulation of organic compound called compatible solutes. Observation of drought stress response in sugarcane found the presence of a drought-inducible protein called SoDip22 and that the expression was induced by drought stress and ABA hormone treatments. However, the function of this drought-inducible protein has not been elucidated and only suggested that the protein may play an important role in maintenance of water molecule during water deficit state. Biochemical studies on the drought-tolerance mechanism have shown that nontoxic small compound of compatible solute accumulated during water deficit condition. Genetic engineering of glycine betaine (GB), acting as a compatible solute, has been applied for enhancement of water stress tolerance. In sugarcane, bacterial *bet*A gene encodes for choline dehydrogenase (CDH) has successfully introduced and resulted in the transgenic drought-tolerance sugarcane. The CDH converts choline into betaine aldehyde, which is then converted to GB. The overexpression of *bet*A gene increased GB contents that act as an osmoprotectant and help sugarcane acclimate in water deficit condition. This chapter reports the development of biotechnology for drought-tolerant sugarcane.

Keywords: sugarcane, transgenic, drought-tolerance, *betA* gene, glycine betaine

1. Introduction

Climate changes have been considered as a serious issue in the past few decades and have an impact on the agriculture production and human health. The climate variability and change are projected to result in the frequency of extremely high-temperature events, floods, and

drought conditions. The global increase in temperature is predicted to enhance water losses due to high evapotranspiration rate and resulted in the increase of water stress. Many reports had been published that drought stress can impose decreasing of plants growth and losses in plant productivity. In addition, increasing human population that was predicted by US Census Bureau over 9 million in 2050, will need more demand for food, energy, and the residence. Expansion of city, as a consequence of increasing human occupancy, has significant impact on the displacement of farmland from well-irrigated system toward rain-fed marginal soil which might lose agriculture production.

Sugarcane is a major crop to produce sugar in tropical region and that sugar is extracted from sugarcane stem at sugarcane factory throughout the harvesting session. The stem is shredded, crushed, and pressed to produce juice that is separated from bagasse, the fibrous portion of sugarcane stem. The juice is then clarified and boiled to produce syrup, and through multiple rounds of crystallization to produce sucrose. The remaining black thick syrup called molasses is then separated as a by-product of sugarcane industry. Bagasse has several applications, including generation of power for the mill, papermaking, livestock feed and may be a useful source for production of various derivate of cellulose fiber, and fermentation of bagasse to produce ethanol. Due to still remaining high sugars, the molasses is used for alcohol and other fermentation products as well as a stock feed supplement. The molasses and another by-product called as filter cake are often used as a fertilizer on the cane fields. The various valuable products from sugarcane have put the plant as an economically important plant in tropical region. Increasing sugarcane production and processing would not only increase sugar supply and increase farmers income, but also enhance energy security by using bioethanol from sugarcane production and improve the environment.

Water is one of the most critical environments and plays a crucial role in the life of plants. The availability of water has a potential effect on plant's growth and productivity. The disruption of the plant water status due to drought stress condition reduces the plant survival, growth, and productivity in the environment. In the photosynthetically C4 plant species, sugarcane is considered a plant with high water-uptake efficiency. During day time, the C4 plants can slightly close their stomata to minimize evapotranspiration rate without any effect on photosynthetic carbon assimilation. Although sugarcane needs dry season before harvesting, the plant requires optimum water availability during the vegetative growth. Adequacy water supply during vegetative phase will enhance rapid growth, stem elongation, and internodes formation. On the other hand, limited water availability will stack sugarcane growth and seriously affect on sugar production [1]. Since sugarcane is a valuable crop in the tropical countries that is being used for sugar production and others products such as bioethanol, energy, feed, thus a strategy for development of new sugarcane cultivars tolerance to water stress will be an important issue.

The development of a new plant cultivar could be gained either by conventional breeding program or biotechnology approaches. Although sugarcane improvement by cross breeding program had been successfully implemented, creating a new variety through breeding program is laborious and take times around 12 years or even more. Sugarcane is a complex organism with high ploidy levels and chromosome number of 2n = 80 with a basic chromosome number (x) of 10 [2], and has limitation for the development of new cultivars. The flowering

occurrence under field condition is variable, influenced by variety and environmental conditions such as altitude and day length. Sugarcane is a cross-pollinating species although selfing occurs at low levels [3]. Sugarcane pollen is very small, rapidly desiccated, having a half-life only 12 minutes and no longer viable beyond 35 minutes and is immediately dried. Thus, biotechnological approach is believed to become crucial to overcome the limitations of classical sugarcane breeding. Development of transgenic sugarcane may foster the development for creation of new sugarcane cultivars with various important traits such as drought tolerance, high sucrose content, resistance to diseases, high yield of ethanol and biomass for fuels.

Recently, it has been reviewed that understanding of water stress mechanism in sugarcane from molecular, biochemical, and physiological perspectives will be the most promising strategies for developing the biotechnology [1]. From the physiological perspective, to survive and develop normally, plants adapt to water stress with various strategies including altered gene expression [4] and accumulation of specific compound called compatible solutes such as proline, sugar alcohol, and betaine [5, 6]. Water stress increases the level of ABA, and the hormone involved in the signal transduction of gene expression converting the adaptation to the water stress [7, 8]. The change in water stress-related gene expression associated with sucrose accumulation and the genes encoding enzymes involved in amino acid metabolism have been reported in sugarcane [9, 10]. In addition, Glycine betaine (GB) is a compatible solute that is believed to act as an osmoprotectant and converting plant to adapt to the water stress condition in several plants including sugarcane. Understanding molecular and physiological mechanism on the water stress is a major challenge in developing biotechnology of drought-tolerant sugarcane. The objective of this review is to report the development of biotechnology of drought-tolerant sugarcane using the gene that induces glycine betaine accumulation as well as to summarize an efficient method for genetic transformation method mediated by *Agrobacterium* for sugarcane.

2. Physiological and molecular drought stress responses in sugarcane

Water stress is one of the most critical environmental abiotic stresses that affect plant's growth and productivity. It was estimated by the International Water Management Institute that by the year 2025, one third of the world will be occupied with severe water scarcity. Moreover, the climate change will induce competition between the use for human consumption and irrigation, which in turn affects the displacement of agriculture to non-irrigated marginal area that reduced in agricultural productivity. When subjected to water deficit or drought stress, plants undergo alteration in physiological started with reduction in protein synthesis, stomatal conductance and photosynthetic rate. Depending on the plants species, drought stress condition will accumulate the compatible solutes to protect cell from serious damage in drought stress tolerant plants. Under rehydration after mild water deficit, almost every plant can return to normal growth, but if the stress was severe, some plants will not survive and dry.

Sugarcane is photosynthetically classified as C4 plant that adapted well in tropical climate. The C4 plants are often considered to be a better adapted to water limitation environments than most other crops, particularly as they are able to maintain leaf photosynthesis with slightly

stomatal close and increase in water-use efficiency. The C4 photosynthesis is characterized by the presence of phosphoenolpyruvate carboxylase (PEPC) as the primary carboxylation enzyme located in mesophyll cell, and by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) as the secondary carboxylation enzyme located in bundle sheath cells of anatomy C4 leaf. Fixation of CO_2 from atmosphere is catalyzed by PEPC in the mesophyll cells to form C4 acid (malate and/or aspartate) which is then transported into the bundle sheath cells. The metabolites transport process generates a much higher concentration of CO_2 in the bundle sheath cells than the external atmospheres. This elevated concentration of CO_2 at the carboxylation site of Rubisco results in suppression of photorespiration. It was believed that PEPC has high affinity to assimilate CO_2 from atmosphere [11, 12] and allow high rates of photosynthetic carbon assimilation to occur when stomata are slightly closed to prevent evapotranspiration. This PEPC has a significant role in C4 photosynthesis carbon assimilation and is regulated by environmental conditions such as light [13], water stress [14], and nitrogen availability [15]. Although limited reports, the enzymes involved in CO_2 concentration mechanism in C4 plant are considered to play significant role in water resistant [16, 17].

Sugarcane is an important crop to produce sugar and dry condition is commonly observed in sugarcane farm in tropical agriculture. Dry season or drying prior to harvest in irrigated sugarcane cultivation is an important strategy to enhance sucrose content in stem [18, 19]. Gradual water deficit during sugarcane maturation reduce stem elongation and leaf development, but more sucrose become available for storage in stem [18]. In the pathway of sucrose biosynthesis, sucrose-phosphate synthase (SPS) is believed to be a key enzyme for sucrose synthesis in plants [20]. A comparison study on the sucrose content in sugarcane cultivated in different agro-climate showed that dry-land cultivated sugarcane accumulated more sugar compared with wet-land and observation in *Saccharum* species showed that sucrose contents are fluctuated according to the SPS activities [21]. Further physiological study on drought stress revealed that stop watering increased sucrose-phosphate synthase (SPS) along with sucrose accumulation in sugarcane leaf (**Figure 1A**). Similar results were reported that water stress resulted in a stimulation of sucrose synthesis by activation of sucrose-phosphate synthase in spinach [22] and wheat [23]. Addition of ABA increased the SPS activities but did not increase other proteins levels (**Figure 1B**) since the hormone is involved in the signal transduction of gene expression conferring the adaptation [7, 8]. Identification of amino acid residue serine that is responsible for water-stress regulation by phosphorylation mechanism clearly showed that the amino acid is conserved in sugarcane SPS [24]. This experimental result suggests that drought stress induce sucrose accumulation in sugarcane as a mechanism helping the plant adapted to drought conditions. Moreover, overexpression of the gene for SPS has been reported to enhance SPS activity as well as sucrose accumulation in transgenic tomato [25]. The increasing of sucrose accumulation due to overexpression of the gene for SPS enhanced drought stress-tolerance will be an important study to be conducted in sugarcane.

Drought stress induces a wide range of physiological and biochemical responses in plants, including alteration in gene expression. The change in gene expression was triggered both by ABA-dependent and ABA-independent regulatory mechanism. Furthermore, identification by microarray analysis had classified two groups of drought-inducible genes in *Arabidopsis*. The first group is genes encoding for proteins with the function in abiotic stress tolerance and the second group is comprised of regulatory protein such as various transcription factors

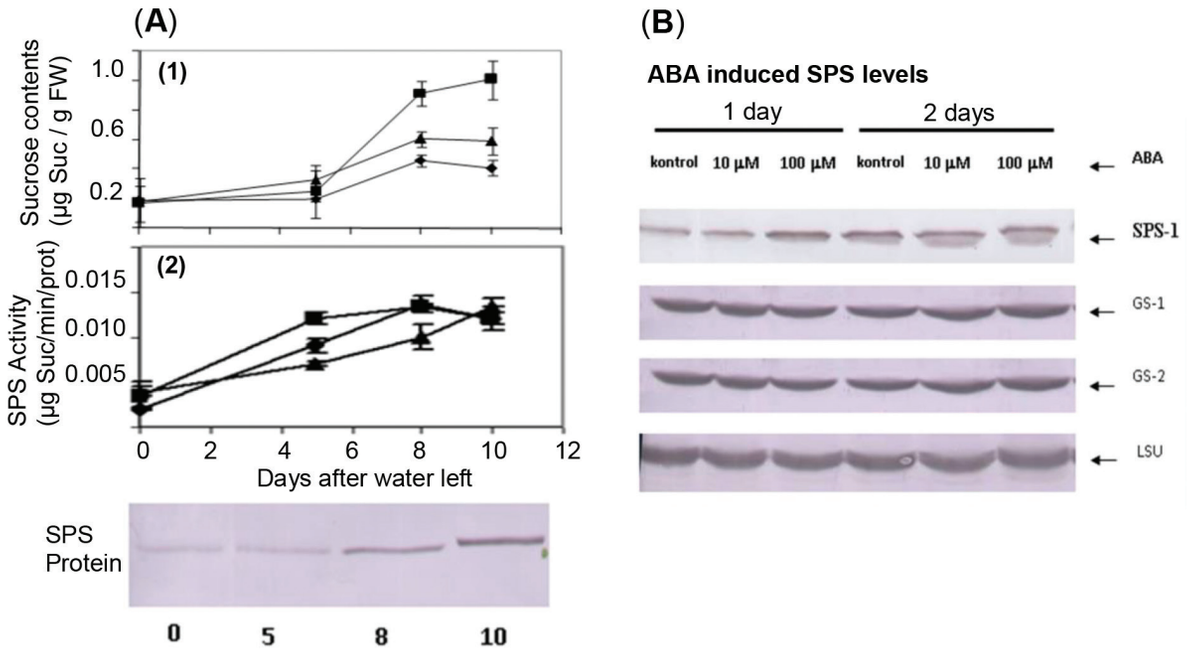


Figure 1. Enhancement of SPS levels in sugarcane leaves after drought stress (A) and ABA hormone (B) treatments. Two-months old sugarcane plants grown in green house were treated by either drought stress or ABA hormone. The drought stress was initiated by left sugarcane plants without watering and the SPS activity, SPS protein levels, and sucrose contents were measured at indicated times (A). (B) The fully developed youngest leafs were sprayed with ABA solution at indicated concentration for 1 and 2 days. Total proteins were extracted from the fully developed youngest leaves and the SPS activity was measured according to the method described in [21]. The levels of SPS, Rubisco-LSU, and GS (glutamine synthetase) proteins were detected by Western Blot analysis with specific polyclonal antibody against the proteins. The sucrose was extracted from the leaves using mixture of methanol-chloroform-water, and the sucrose content measured using HLPC. The figures were provided by Dr. Yudhi Rinanto.

and regulation of signal transduction [4]. Molecular study on the responses of sugarcane to drought stress found the presence of a drought inducible protein named SoDIP22 in the water stress tolerant phenotype of sugarcane [10]. A computer search of protein databases revealed that the sequence of the drought inducible protein exhibited significant similarity to that of members of the ABA stress and ripening-inducible (Asr) protein family, such as 73% identical to rice OsAsr1 protein. The expression of the drought-inducible from sugarcane SoDip22 protein was induced by drought stress and osmotic stress at −0.9 Mpa generated with PEG 6000 and 0.6 M mannitol. The expression of SoDip22 was controlled by the signal transduction pathway through ABA, since exogenous addition of ABA induced the SoDip22 expression, but not other growth regulators. Although the molecular size of SoDip22 was a small protein, only 22 kDa, and has similarity with Asr protein found in the nucleosome fraction which is predicted as a transcription factor, observation of transient expression of the SoDip22 protein did not support the nuclear localization. Interestingly, the protein was inclusively detected in bundle sheath cell of sugarcane leafs and the protein function is predicted to play an important role in the maintenance of water molecule during water deficit in the bundle sheath cell.

Water deficit causes various changes in biochemical reactions, including the production of a complex variety of secondary metabolites. Water stress induces the accumulation of reactive oxygen species (ROS) in plants which are highly reactive or toxic that causes damage to

cellular component such as proteins, lipids, carbohydrate, and DNA. The ROS also controls many processes such as cell cycle and programmed cell death [26]. Exposure of plants to drought condition increases production of ROS such as free radical (O_2*, superoxide radicals, OH* hydroxyl radical, HO_2* perhydroxy radical) and non-radical forms (H_2O_2, hydrogen peroxide and O_2, singlet oxygen). To ensure survival under drought stress condition, plants have developed efficient antioxidant machinery that is able to scavenger and detoxify ROS [27]. Plants possess enzymatic and non-enzymatic antioxidant defense system to protect plant cell from oxidative stress by scavenging ROS. The enzymatic activity such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and non-enzymatic antioxidants such as ascorbic acid, reduced glutathione, phenolic, alkaloids, and amino acids can work together to scavenge ROS. Water deficit induced the change activities of SOD, CAT, APX, and GR, according to variety and stress intensity in sugarcane. Drought-tolerant sugarcane exhibited higher CAT and APX activities in the early stage of drought, while the activity of GR was highest at the end of drought stress period compared with drought-sensitive sugarcane [28]. The increase of SOD, CAT and APX under drought stress was further confirmed in most tested sugarcane cultivars [29]. Thus, the activities of ROS scavenging enzymes may be used as a marker of water stress tolerant sugarcane.

Many plants respond to water deficit stress by accumulating non-enzymatic antioxidants defense system to protect from oxidative damage by ROS. Ascorbic acid is one of antioxidants that prevent or minimize the damage caused by ROS. The ascorbic acid has ability to donate electrons in numerous reactions and protect the cell membranes by scavenging the superoxide radical and hydroxyl radical [27]. Furthermore, glutathione is another important antioxidant that is capable of preventing damage caused by ROS. Glutathione exists either in reduced or in oxidized form and it is a crucial metabolite to perform multiple functions including plant responses modulation under abiotic and biotic stresses [30]. Despite ROS scavenging enzymatic and non-enzymatic antioxidants which had been reported to enhance drought tolerance in several transgenic plants [27], their application for developing drought-tolerant sugarcane is still meager.

The accumulation of non-toxic small molecule metabolites referred as compatible solutes or osmoprotectant such as sugar, proline and betaines help plants to survive under osmotic stress [5, 31]. These metabolites may have a role to protect cell membrane and maintain osmotic potential. Studies at physiological, biochemical, and molecular levels suggest that compatibles solutes perform important function in adjustment of plant against salinity and drought stress. Sugar and sugar alcohols have been accepted as osmoprotectant that provide membrane protection and scavenging ROS. The higher accumulation of sugar, such as trehalose, fructans, sucrose, acts as osmoprotectant under drought stress in plants [32]. Sugarcane can accumulate high content of sucrose in storage parenchyma of stem cell that may create osmotic gradient and act as osmoprotectant. Under water deficit, there was a change in stress-related gene expression and sucrose accumulation, but the mechanism responding to the water stress was different. Some genes expression such as genes encoding for asparagine synthase (AS), biosynthesis of proline (OAT), sugar transports were positively correlated, but the genes encoding for proline biosynthetic pathway (P5CS) and the bZIP transcription factor TF1 were negatively correlated with sucrose contents in sugarcane mature culm. The proline content was increased under water deficit condition, but was negatively correlated with sucrose concentration and suggested that proline has no osmoprotectant role in sugarcane [9].

Although the role of proline on osmotolerance remains controversial in plants, evaluation of transgenic sugarcane overexpressing heterologous *P5CS* gene indicated that proline content significantly increases after 9 days without watering. However, the increasing proline content has no effect on the osmotic adjustment, and the proline may protect sugarcane against the oxidative stress caused by water deficit. The results suggested that proline accumulation acts as a component of antioxidative defense system rather than as an osmotic adjustment [33].

The glycine betaine (GB) is one of the compatible solutes and an amphoteric quaternary amine that is considered as the most compatible solute that plays an important role in protecting plants under environmental stress [5, 34]. The GB is synthesized by plants at various capacity, such as spinach and barley accumulate high of GB, whereas *Arabidopsis* and tobacco do not synthesize GB. The role of GB is not only allowing cell to adjust the osmotic potential in their cytoplasm to maintain an appropriate water content, but also protecting protein from the water stress dissociation [34]. When plant cell is exposed to water stress or salt stress, GB stabilizes the structure of macromolecule, helping in maintaining the integrity and proper function of the membrane. Although the detail of the role of GB has not been firmly established, the objective of this book chapter is directed for developing of biotechnology of drought-tolerant sugarcane using production of GB in transgenic sugarcane.

Genetic transformation has a potential role to introduce a new trait in plant cell, including the introduction of new pathway for the biosynthesis of compatible solutes and resulting in transgenic plant with improved tolerance to environmental stress. There are many techniques for introducing a new gene into plant cell such as direct transformation using particle bombardments, micro injection or electroporation and indirect transformation using *Agrobacterium* as the vector. Depending on the plant species, *Agrobacterium*-mediated transformation is well established in dicotyledonous plants and less in monocotyledonous plants. The development of *Agrobacterium*-mediated transformation was successfully established for routine genetic transformation in monocotyledonous plants such as rice, maize, and sugarcane.

3. Development of an efficient method for *Agrobacterium*-mediated transformation for sugarcane

The genetic improvement of sugarcane cultivars has been achieved to increase sugar productivity with the cultivars created by conventional breeding. The sugarcane (*Saccharum officinarum*) cultivars contributed high sucrose production and the backcrosses of *S. officinarum* resulted in elite cultivars of *S. hybrid* with higher yield, improving ratooning ability and disease resistance. However, these improvise achievements would still enable the sugar industries to face current issues on climate changes as well as higher sugar demand due to human population growth. Sugarcane has a highly complex genome, low fertilization and tiny seed which make traditional breeding highly difficult and laborious to create new sugarcane cultivars. Recent progress on molecular techniques could be used in sugarcane varietal improvement as well as the combination between both traditional and molecular breeding. Beginning with development of cell and tissue culture of sugarcane that has emerged as a valuable tool for various research activity in sugarcane improvement and propagation,

sugarcane biotechnology has been used to introduce new traits that would be very difficult to conduct with conventional methods or almost not possible. The first establishment of genetic transformation method in sugarcane by Bower and Birch [35] and followed by engineering of agronomic traits using the method into various sugarcane genotypes are the important steps to support the development of sugarcane biotechnology. In addition, consideration also has been given to the development of molecular marker technologies for sugarcane breeding and variety identification [36].

Plant cell has a totipotency ability to regenerate and differentiate into whole plant that completed with leafs, stem and root. The totipotency ability has been used for plant multiplication or micropropagation by inducing meristematic plant tissue in the medium supplemented with plant growth regulator to produce somatic embryogenesis callus, which in turn regenerate into whole plants. In sugarcane, the somatic embryogenesis callus is produced by induction of meristematic leaf tissue on the MS (Murashige and Skoog) medium containing 2,4-dichlorophenoxyacetic acid (2,4 D), and the embryogenic callus is then regenerated into whole plant on the MS free hormone [37]. Thus, considerable effort has been expended to use this micropropagation technique for providing the demand of numerous sugarcane seed. However, the application of tissue culture through somatic embryogenesis induces somaclonal variation in sugarcane [38, 39] that causes variants of phenotype, although it will be reverted to original parental thereafter [38]. Moreover, the presence of somaclonal variation is frequently used to obtain new type of sugarcane cultivars such as resistance to Fiji disease and mildew [40] and resistance to eyespot disease [41]. Although there is wide diversity of the usage, the somatic embryogenesis has been widely used as a part in genetic transformation system for the improvement of sugarcane cultivars [35].

Micropropagation of sugarcane can be also performed by direct regeneration of both apical and axillary meristem buds. The regeneration from axillary buds reduces the somaclonal variation events and is routinely used for mass multiplication of sugarcane [42, 43]. However, axillary buds isolated from field grown sugarcane stalk were frequently contaminated with bacteria and should use unexpected strong sterilant such as mercury chloride ($HgCl_2$) before cultured on MS media. Thus, the proper concentration should be carefully selected since this $HgCl_2$ sterilant is extremely harmful. Alternatively, *in vitro* sugarcane shoot can be regenerated from meristematic shoot apical and multiplied on MS media. There are various methods to improve the multiplication sugarcane shoot *in vitro* using MS media. Using temporary immersion system, shoot formation for sugarcane micropropagation was improved [44]. The shoot induction and multiplication on MS containing higher benzylamino purine (BAP) hormone resulted in weak, tiny, and nonseparable shoots, whereas the lower concentration resulted in desirable quality, well grown, easily separable, and healthy plantlets [45]. The media composition is considered to play an important role for achieving maximum growth rates on sugarcane tissue culture. Amino acids mixtures such as glutamine, glycine, asparagine, cysteine, and casein hydrolysate are frequently used as source of organic nitrogen in culture media [46]. Recently, addition of the amino acids mixture to the MS media has been examined in shoot multiplication of sugarcane. Addition of 2 ppm of glycine did not induce shoot multiplication, and 100 ppm of glutamine gave induction of shoot regeneration, but shoot growth rate was low. However, addition of the combination of glycine and glutamine resulted in well growth and healthy sugarcane plantlets. These results indicate that inclusion

of amino acids of glutamine and glycine in the media for stimulating multiplication of healthy sugarcane shoot will be suitable for future use in micropropagation as well as genetic transformation method.

Genetic transformation technology serves as a useful and practical tool to introduce particular traits for crop improvement. Several genetic transformation methods have been attempted for delivery and expression of transgenes in plants. First genetic transformation methods for sugarcane were developed by direct introduction of desired genes using electroporation [47], polyethylene glycol (PEG) treatment [48], and particle bombardment [49]. These methods were considered less efficient compared to the indirect genetic transformation using *Agrobacterium*-mediated transformation. Furthermore, the direct transformation systems have certain limitations such as requires specific equipment, needs skill labor and results in high copy number integration in the plant genome [50]. The multiple gene copies frequently cause multiple gene arrangement, co-suppression and gene silencing [51]. Although, *Agrobacterium*-mediated transformation is restricted applicable in dicotyledonous plants due to the strictness of the host range of this bacteria, recent research indicated that *Agrobacterium*-mediated transformation is also possible in monocotyledonous plants such as in rice [52], maize [53] and banana [54]. In maize, the *Agrobacterium*-mediated transformation has been used for routine transformation using standard binary vector system and average stable transformation efficiency was 5.5% [55]. The evidences of the *Agrobacterium*-mediated transformation system were also reported in sugarcane using meristematic explants [56, 57]. The *Agrobacterium*-mediated transformation method offers several advantages such as technical simplicity, low copy number and minimal genome rearrangement. Although *Agrobacterium*-mediated method has been applied also to sugarcane, the lack of reproducible results has been an obstacle to establish effective transformation protocol for routine genetic manipulation in the plants. The cell is being traumatic due to *Agrobacterium* infection and poor survival rate thereafter. Oxidative burst, phenolization, and subsequent cell death are frequent phenomena after the *Agrobacterium* infection [58]. Development of the *Agrobacterium*-mediated transformation is necessary to have reproducible and efficient methods in sugarcane. This section demonstrates an efficient transformation system for sugarcane using explant *in vitro* shoot generated from apical shoot tips to minimize bacterial contamination as well as somaclonal variation.

Genetic transformation system has been developed for sugarcane with distinct agronomically important traits, transformation methods, explant and culture condition. However, the use of *Agrobacterium*-mediated transformation method that considered more efficient was limited applying in sugarcane [58, 59]. Moreover, embryogenic callus was mostly reported as the explant for the *Agrobacterium*-mediated transformation system, but the use of callus includes the somaclonal variation. Direct regeneration from explants without an intervening callus phase has several advantages for *Agrobacterium*-mediated transformation in sugarcane. The isolated axillary bud explants from 6-months old field grown sugarcane were infected with *Agrobacterium* harboring the T-DNA of binary vector and resulted in stable transgenic sugarcane. The results suggested that the method can be achieved to generate transgenic sugarcane in about 5 months with transformation efficiency as high as 50% [42]. However, this transformation system needs numerous axillary buds as explants that should be isolated from sugarcane stalk and it is very difficult to avoid bacterial contaminant in the tissue culture

media. By regeneration of *in vitro* shoot using meristematic shoot apical, subsequent multiplication in appropriate MS media will be suitable to overcome the problem of contaminant. The protocol for regeneration of *in vitro* shoot from shoot apical has been developed and with the method, the healthy shoot was rapidly multiplied in the MS media containing additional amino acid mixture of glutamine and glycine (**Figure 2A–F**). Green and healthy *in vitro* shoot from 2 to 4 weeks cultured was separated and basal segment that contains meristematic tissue was excised around 0.2–0.3 cm from the base (**Figure 2B** upper). These basal segments were injured with needles and used as the explant for *Agrobacterium*-mediated transformation. The injured or wounded tissue was suitable to induce *Agrobacterium* infection and allowed the *Agrobacterium* to penetrate into inner meristematic tissue of the basal segment. The presence of meristematic tissues provides young regenerable material that actively divided cell, competent for *Agrobacterium* infection, and improves the adhesion of *Agrobacterium* during co-cultivation [57]. Transient expression analysis showed that *Gus* gene expression was predominantly observed in the basal portion which was injured and contains meristematic tissue (**Figure 2B** lower). After cultured on selection medium containing the appropriate antibiotic for 2–3 weeks, the basal segment regenerates new axillar shoots, in which some of them become albino and bleached due to the presence of antibiotic in the media or regenerated green shoots. The explants with green shoots were transferred to the fresh selection medium and, after 5 times successive cycle, the putative transformant were acclimated. With this method, co-cultivation and antibiotic selection of putative transgenic shoot can be achieved in less than 4 months with transformation efficiencies around 6% when using 2 weeks-old shoot explant and the efficiencies sharply increased as high as 40% when using 4 weeks-old shoot explant. Genomic PCR and Southern Blot analysis indicated that most of the putative transformants contain insertion of the targeted DNA. All together these results suggest that basal segment of *in vitro* sugarcane shoot provides an effective explant for routinely *Agrobacterium*-mediated transformation protocol and produces transgenic sugarcane.

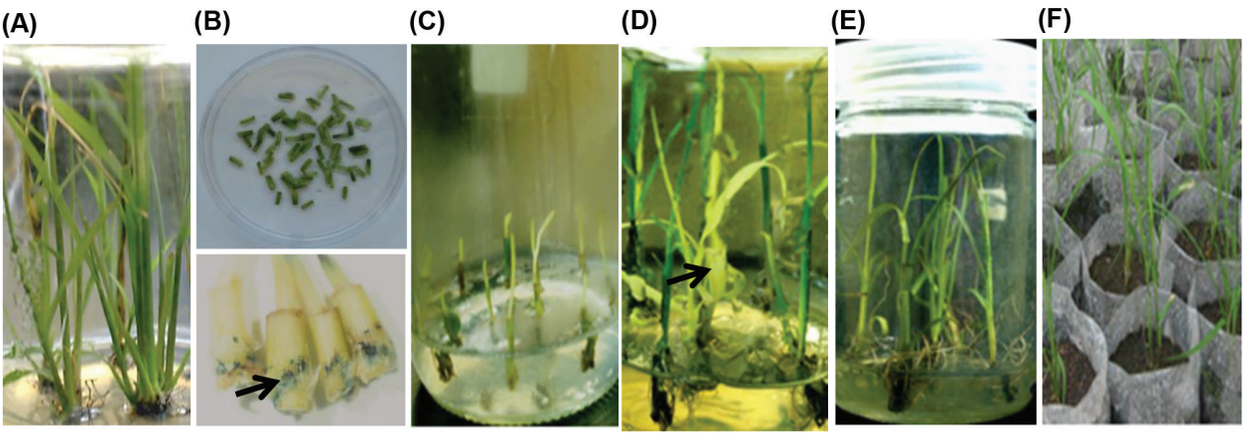


Figure 2. Workflow of the *Agrobacterium*-mediated transformation using explant base segments of *in vitro* sugarcane. Green and healthy sugarcane *in vitro* shoot were micro-propagated in MS media and used as source of explants (A). Excised base segments of *in vitro* sugarcane were used as explant for the transformation (B upper) and clear blue spots of *Gus* gene expression were observed in the basal segments (B lower). Elimination of non-transformant and multiplication of putative transformant shoot in the selection MS media containing appropriate antibiotic (C and D). After five cycles in the selection media (E), the putative transformant were acclimated in greenhouse for further analysis (F). Arrow represents clear blue spots in the basal segment and albino shoot of non-transformant.

The achievement of the current sugarcane transformation technology still needs further development. A number of undetermined conditions such as DNA promoter that drives the gene expression, selectable marker, *Agrobacterium* strain and some other factors are becoming important for improving genetic transformation efficiency. The promoter is a key DNA regulatory element that directs appropriate strength and pattern of gene expression in a constitutive or specific manner. Therefore, the promoter plays a crucial role in determining the transformation efficiency. There are some types of DNA promoters that drive strong, constitutive, or organ specificity expression. For example, the viral Cauliflower Mosaic Virus 35S (CaMV 35S) promoter has been widely used in the transformation of many dicot and monocot plants. However, it has been demonstrated that the expression activity of the 35S promoter was low in sugarcane [60]. The rice actin1 and Emu elements have shown to drive higher expression activity than CaMV 35S in different sugarcane tissues [61] and from the current research, it appeared that ubiquitin promoter has an emerging promoter for constitutive expression in sugarcane. The experiment concerning an effective promoter for sugarcane transformation has been also conducted using rice ubiquitin promoter (RUBQ2). The use of RUBQ2 promoter has increased transgene expression by about 1.6-fold over maize ubiquitin promoter in sugarcane [62]. Comparison study on GUS expression driven by CaMV35S and RUBQ2 promoter showed that RUBQ2 promoter produced high level GUS activity with clear blue spot in embryogenic callus and suspension cultures, while the CaMV35S promoter was not detected. Controversially, the GUS expression driven by sugarcane polyubiquitin promoter was dropped to very low or undetectable levels in the transgenic plants resulted from post-transcriptional gene silencing.

Among the factors considered as limiting the recovery of transgenic plant is the involvement of selection marker in genetic transformation system. The selection of genetically transformed cell can be conducted through positive selection and negative selection. The positive selection is referred as those that promote the growth of transformed tissue and negative selection is the use selective agents, killing or fully inhibiting the growth of untransformed cell [63]. The use of gene for selectable marker in combination with targeted gene is directed to identify and allow surviving the transformed cell, and inhibit the growth of non-transformed cell in the media containing appropriate selective agents. Therefore, the use of selectable marker provides easy protocol to support proliferation of transformed cell and remove the un-transformed cell. Among the widely used selectable markers, the genes responsible for resistance of antibiotic kanamycin (*nptII*), hygromycin (*hptII*) and herbicide resistance (*bar*) are frequently applied to develop transgenic plants. The genes of *nptII*, *hptII* and *bar* inactivate the enzymes that play in role of antibiotic resistances neomycin phosphotransferase, hygromycin phosphotransferse and phosphinothricin acetyltransferase, respectively. Determination of the explant sensitivity to the antibiotic and the antibiotic concentration can be potentially effects of the successful genetic transformation. Exceeding high level of antibiotic is not only to kill the nontransformed cell, but also to give retardation for the growth of the transformed cell [64]. Evaluation of resistance to antibiotic kanamycin and hygromycin showed that the antibiotics can be used as selectable marker to obtain stable transformants in the cell suspension culture of the Gramineae such as *Triticum monococcum*, *Panicum maximum*, and *Saccharum officinarum* [65]. Comparison study on grapevine transformation revealed that both antibiotic kanamycin and hygromycin inhibited growth of the untransformed explant at 16 and 1 ug/mL, respectively. Due to the hygromycin which can be applied at lower level than kanamycin, hygromycin appears an appropriate

selective agent [66]. Similar results reported that hygromycin is an effective selective marker for genetic transformation for monocot plants such as rice [67, 68], maize [69], and banana [70]. The herbicide Basta (*bar* gene) has been used as the selection marker for genetic transformation in rice [71], fescue—*Festuca arundinacea* [72], and oil palm [73]. However, limited reports have published the use of both *nptII*, *hptII*, and *bar* genes as effective selectable for sugarcane transformation. Using direct transformation with microprojectile bombardment, stable transformant was obtained after the selection of explant sugarcane callus on the media containing kanamycin and stepped increases in the antibiotic concentration allowed an active growing of callus, plantlets and completely inhibited untransformed callus [35]. When *Agrobacterium*-mediated transformation was performed, similar results were observed using explant callus and *nptII* gene as the selectable marker. Regeneration transformant was successfully conducted by culturing the explant callus on the media containing 150 mg/L paromomycin sulfate [74]. However, the first successful report on *Agrobacterium*-mediated transformation for sugarcane used selectable marker *hptII* gene [57]. There are many reasons to elaborate the discrepancy between the effectiveness of selectable markers *nptII* or *hptII* genes, but hygromycin is much more effective than kanamycin for the selection of transformed cell and at low concentration, the hygromycin provides strong discrimination between transformed and nontransformed cell. Thus, hygromycin at the concentration of 25 mg/L is sufficient for routinely used *Agrobacterium*-mediated transformation for sugarcane.

Agrobacterium strain and density frequently have an impact on the plant genetic transformation efficiency. There are many *Agrobacterium* strains used for genetic transformation in plant and, among of them, the LBA4404 *Agrobacterium* strain is widely used for genetic transformation. The LBA4404 has a higher transformation efficiency in several plants such as in tobacco [75], wheat [76], and herb of *Bacopa monnieri* [77]. However, GV3101 *Agrobacterium* strain has been reported with highest transformation rate than EHA105, AGL1, and MP90 *Agrobacterium* strains in tomato [78]. In addition, concentration of *Agrobacterium* and wounding explants are also considered as the factors influencing transgene expression in loblolly pine [79]. Concentration of *Agrobacterium* at OD_{600} = 0.5 improved the efficiency of transformation in cotton [80], whereas higher concentration will result in *Agrobacterium* overgrowth and difficulty to eliminate after co-cultivation. In sugarcane transformation system using explant *in vitro* shoot, GV3101 *Agrobacterium* concentration at 0.5 OD_{600} and wounding explants can be used for routinely transformation.

4. Genetic engineering of glycine betaine (GB) synthesis improves drought tolerance in sugarcane

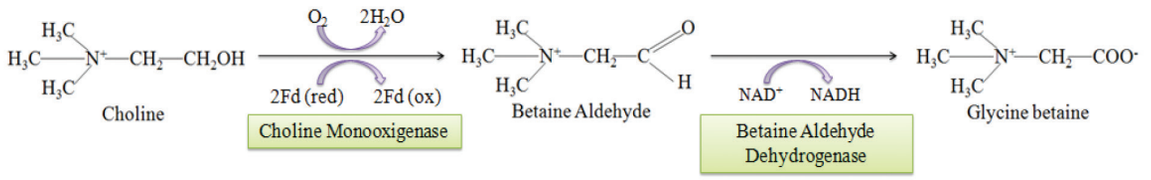
Glycine betaine (*N*,*N*,*N*-trimethyl glycine) is an amphoteric small organic compound, highly soluble and do not interfere with cellular metabolism even at high concentration. The molecular character of GB can interact with macromolecule such as enzyme, protein complexes, and cell membrane when cell is exposed to stress condition. Glycine betaine stabilizes the structure and activity of enzymes and proteins, and maintains integrity of membrane against damage caused by environmental stresses [81]. This GB accumulates in a variety of plant species in response to water stresses for osmotic adjustment. Depending on the plant species,

some plant species are accumulator of glycine betaine such as *Amaranthus*, sorghum, sugar beet and the other non-accumulators such as rice, sweet potato, and tobacco [5, 34]. Drought stress enhances accumulation of GB in the accumulator species for osmotic adjustment [82, 83]. Glycine betaine protects the plant cell by acting as an osmolyte, stabilizes protein and membrane cell, and maintains water balance during drought stress. It is widely accepted that the accumulation of GB plays an important role for the acclimatization of plant cell to drought stress. In many plants that do not accumulate GB, application of GB may help reduce adverse effects of the environment stress. The exogenous application of GB at 10 mM improved growth, leaf water content, and net photosynthesis, and increased growth and crop yield under environmental stress [84, 85]. However, consideration of economic and streamline useful application needs to be investigated. Determination of the GB concentration, timing, frequency of application, and a possibility of other disadvantage of exogenous GB application such as the risk of increasing pathogen attack should be well established [85]. Thus, genetic engineering for economically important crops such as rice, maize, and sugarcane that naturally are unable to accumulate GB will be an important target to improve.

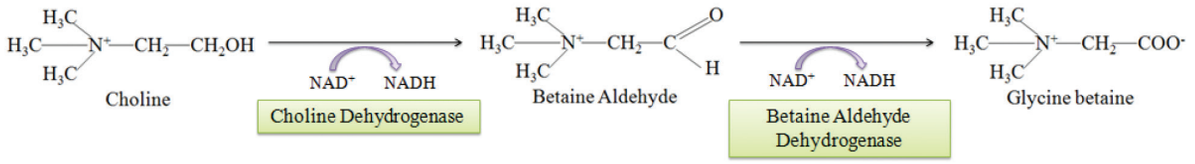
Glycine betaine is an osmoprotectant found in wide range of microorganisms, plants, and animals that are synthesized under various environmental stresses [5]. Glycine betaine is mainly synthesized from choline as the substrate through two-step reactions, dehydrogenation of choline, and oxygenation of betaine aldehyde (**Figure 3**). In higher plants, choline is converted by choline monoxygenase (CMO) to betaine aldehyde, and then converted into glycine betaine by betaine aldehyde dehydrogenase (BADH) to GB [34, 86]. In microorganism and mammalian cells, GB is also synthesized by two-step pathway, but choline is converted to betaine aldehyde by choline dehydrogenase (CDH) and not by CMO, and then to GB by same BADH activity [34, 87]. In contrast, a single step-reaction catalyzed by choline oxidase (COD) for synthesis of GB is found in some microorganism such as *Arthrobacter globiformis* and *Arthrobacter panescens* [88] . In addition, a distinct substrate for GB synthesis is found in two halophytic microorganism *Actinopolyspora halophila* and *Ectothiorhodospira halochloris*. The GB is synthesized from substrate glycine by glycine methylation pathway [89]. The increasing knowledge of physiological pathway for GB biosynthesis as well as genomic engineering technology allow to create transgenic plants that are properly tolerant to drought stress by engineering of glycine betaine biosynthesis.

The gene involved in the biochemical pathway can be used either to increase or diminish metabolite product by overexpressing or silencing the gene responsible for the metabolism. In the case of metabolite engineering of GB, the enzymes involved in the biochemical pathway have been focused as a potential target to engineer the content in the non-accumulator plants. For that reason, the genes encoding for the enzymes involved in pathway of GB biosynthesis have been cloned from various microorganisms and plants that accumulate GB. In microorganism, gene encoding CDH (*bet*A) and BADH (*bet*B) have been isolated from *Escherichia coli* [90, 91] and from salt-tolerant bacteria *Halomonas elongata* [92], whereas gene encoding COD or COX was cloned from soil-living bacteria *Arthrobacter panescens* and *Arthrobacter globiformis* [93, 94]. In higher plants, limited number of genes encoding CMO in combination with BADH has been cloned from spinach [95], sugar beet, and amaranth [96]. Genes responsible for GB synthesis from microorganism have become a major target in the genetic engineering of water stress-tolerant in plants that are unable to accumulate GB, such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), rice (*Oryza sativa*),

1. Plants



2. *Escherichia coli*



3. *Arthrobacter globiformis*

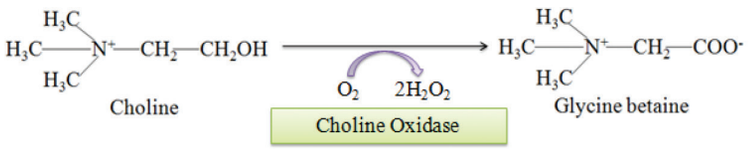


Figure 3. The biosynthesis pathway of glycine betaine (GB) in microorganism and plant cells. Choline is oxidized to GB by two enzymes, choline monoxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) in plant cells. Depending on the species of microorganism, they operate two distinct pathways. In *Escherichia coli*, choline is oxidized to GB by choline dehydrogenase (CDH) and BADH, but in *Arthrobacter globiformis* choline is converted to GB by single enzyme of choline oxidase (COD).

and maize (*Zea mays*) [97, 98]. The strategy to enhance glycine betaine contents in transgenic plant can be achieved by introduction of the relevant gene under transcriptional control of strong DNA promoter to ensure high-level expression. The genetic engineering of drought-tolerant sugarcane, an important crop for sugar production in tropical areas, with overexpression of the *bet*A gene encoding for CDH from *E. coli* under control of 35S CaMV promoter will be discussed.

Genetic engineering of GB synthesis in plants has focused only with individual gene involved in the pathway of GB biosynthesis. The gene encoding COD from *Arthrobacter globiformis* has been overexpressed in chloroplast of *Arabidopsis* and accumulated in low level of GB [99, 100]. Similar results were obtained with low accumulation level of GB, when the COD was overexpressed in the chloroplast of transgenic rice [101] and COX was expressed in the chloroplast of three diverse species, *Arabidopsis*, *Brassica napus*, and *Nicotiana tabacum* (tabacco) [102]. In addition, the expression of CMO which is catalyzed oxidation of choline in plant cell, accumulated very low GB content in the chloroplast of transgenic tobacco [103] and transgenic rice [104]. The low level of GB in the transgenic plant due to limitation of choline contents in the site targeted synthesis of GB, where the synthesis of choline occurred in cytosol. When COD is targeted to express in the cytosol, the synthesis of GB was 3–5 fold greater when the enzyme was targeted to chloroplast [34]. Substantial increase of GB level was obtained when the transgenic plants expressing COX in their chloroplast feed with choline [102] and in transgenic tobacco expressing chloroplast CMO were supplied with choline or phosphocholine [103]. The GB accumulation in transgenic plants is affected by choline availability, type of transgene, and promoter type [87]. Availability of choline as the substrate for GB synthesis either exogenous or endogenous supply

can enhance GB accumulation in transgenic plants. The GB accumulation that depend on the type of transgene and that of microorganism-derived gene for the pathway of GB synthesis is more potential, and constructing targeted gene under strong promoter is preferable. Although the GB was accumulated at low level, the transgenic plants expressing COD or COX showed enhanced environmental-stress tolerance and had better growth [34, 87].

Plant tolerance environmental stress should be achieved by genetic engineering of BADH since the enzyme acts oxidizing betaine aldehyde into GB. The betaine aldehyde is a toxic compound and should not be accumulated in the cell. Transgenic carrot (*Daucus carota*) expressing BADH in the chloroplast grew well in the presence of NaCl up to 400 mM, whereas control non-transgenic plant showed severe growth retardation [105]. BADH enzyme activity, GB contents, and growth rate were enhanced under salt stress 100 mM NaCl in transgenic carrot compared to the control non-transgenic. Expression of cDNA for BADH from GB accumulator of spinach and sugar beet targeted in the chloroplast of transgenic tobacco increased the level of BADH activity, but failed to accumulate GB in the absence exogenous supply of betaine aldehyde [106]. Similar result was reported in transgenic tobacco transformed with cDNA for BADH from barley [107]. These results suggest that the increase in expression of BADH alone is not sufficient for the increase of GB in transgenic plant and that BADH might possibly participate in other metabolite pathways [108].

Microorganism CDH is an useful enzyme for enhancing accumulation of GB into new species because the enzyme is able to catalyze in two reactions such as oxidation of choline to betaine aldehyde and also converting betaine aldehyde into GB [90, 109]. The purified recombinant CDH from *Halomonas elongata* showed similar subtract specificity with either choline or betaine aldehyde as the substrate [92]. Although gene encoding for CDH (*bet*A) has been poorly used for genetic engineering, increasing GB content and enhancing salt and drought tolerance have been proven in transgenic plants. Introducing *bet*A gene isolated from *E. coli* resulted in elevation of CDH activity and created salt tolerance phenotype of transgenic tobacco as well as an increase in the biomass dry weight [110]. The measurement of CDH activity in transgenic tobacco plants showed considerably higher CDH activity around 4.5–6 fold when compared with the wild type. In parallel with increasing CDH activity, the growth of transgenic tobacco was enhanced by salt stress at concentration 200–300 Mm NaCl. In addition, the level of endogenous GB was found to correlate with the degree of salt tolerance in maize lines and that the gene conferring GB plays a key role in osmotic adjustment [111]. Therefore, maize that transformed with the *bet*A gene from *E. coli* accumulated higher level of GB and more tolerant to drought stress than wild-type non-transgenic plants [98]. The measurement of CDH activities in transgenic maize showed increasing the activities 3-4 fold compared with non-transgenic maize and resulted in greater GB concentration compared with non-transgenic maize. The GB concentration was only 1.2 umol/g FW (fresh weight), but increased significantly up to 2.6–4.0 umol/g FW in the leaves of transgenic maize. Observation of total soluble sugar, free amino acids, and osmotic potential in the leaves of transgenic maize were not significantly increased, but photosystem II and net photosynthesis of transgenic maize were more stable than that in the control non-transgenic maize. These indicate that enhanced GB content has a beneficial effect on osmotic adjustment in condition of drought stress and protect from the damage due to dehydration. The most importantly, the grain yield of transgenic

maize, overexpressing *bet*A gene from *E. coli*, was significantly higher than that of the control non-transgenic after drought treatments [98]. Similar result is also reported that transgenic cotton (*Gossypium hirsutum* L.) expressing *bet*A gene from *E. coli* enhanced GB accumulation and drought tolerance [112]. The GB concentration in the leaves of transgenic cotton was higher compared to wild-type plants in non-stress condition and the GB concentration was much elevated after drought stress condition reached at 282.3–308.4 umol/g dry weight or around 2.3–2.6 fold compared with wild-type plants. The measurement of osmotic pressure showed that the osmotic adjustment was higher in transgenic than in the wild type plant, and the higher osmotic adjustment was significantly correlated with the GB concentration in drought stress condition. The results indicate that GB plays a more important role in osmotic adjustment and maintains the membrane stability than that in slightly increased of soluble sugar and amino acid contents in transgenic cotton. Moreover, after 4 days drought stress treatments, the wild-type cotton showed starting to wilt and decreased leaf relative water contents, whereas wilting symptom did not observe and the leaf relative water content remained higher until 10 days of the drought treatments in transgenic cotton. As observed in the transgenic maize, the transgenic cotton seed yield is significantly greater than in wild-type non-transgenic cotton under drought stress [112]. All together, these studies imply that engineering of GB content using *bet*A gene for CDH from *E. coli* enhances GB content that acts not only as an osmoprotectant, but also stabilizes the structure and activities of protein-enzymes, and maintains the integrity of membrane against damage caused by the drought stress, which in turn increase growth and productivity of the transgenic plants.

Several experiments on the effect of addition exogenous GB have been reported to improve sprouting sugarcane bud under heat and chilling stresses [113]. However, there is almost no report concerning accumulation of GB content in sugarcane. Therefore, enhancing GB synthesis with genetic engineering is considered to be a potential method for improving drought stress tolerance in sugarcane. The drought-tolerance transgenic sugarcane has been developed by introduction of *betA* gene encoding for CDH from *Rhizobium meliloti* (Australian Patent Office, Patent No. 737600 – Inventor(s); Naoki Katsurada, Tsushi Hayakawa, Haruhumi Miwa). The *bet*A gene was constructed in binary vector under the control of strong promoter CaMV35S by Ajinomoto Co., Inc., Japan and used for sugarcane transformation. The *Agrobacterium*-mediated transformation was conducted using explant from BL sugarcane cultivars by the state-run sugarcane producer PT Perkebunan Nusantara XI Indonesia in collaboration with Ajinomoto company and University of Jember. After screening of transformed plantlet in selection media containing hygromycin antibiotic, the resulted plantlet was acclimated in green house and used for analysis. The characterization of the transgenic sugarcane was carried out by analysis of the plants grown in greenhouse experiments.

Genomic analysis by PCR (polymerase chain reaction) and Southern Blot confirmed the presence of stable insertion of *bet*A in the genome of the transgenic sugarcane. As expected, the transgenic sugarcane lines appeared to contain a low-copy insertion of *bet*A gene, whereas non-transgenic plant almost had no DNA hybridization. The stable integration of *bet*A gene was confirmed by PCR analysis in third generation of transgenic sugarcane after vegetative propagation. Although the expressions of gene *bet*A at transcriptional and translational levels were not examined, GB contents in the transgenic sugarcane were detected using HPLC

with Inertsil ODS-3 column. The GB content highly elevated in the leaves of transgenic sugarcane ranged 182–880 ppm, but almost not detected in the control non-transgenic sugarcane after drought condition. The increasing of GB contents sugarcane enhanced drought-tolerance of transgenic sugarcane. Observation of plant morphology during exposure to drought stress by stop watering showed that non-transgenic sugarcane started to wilt at 8 days and permanently wilt at 28 days after drought stress. However, the transgenic sugarcane still vigorously growth at 8 days and start to wilt at 12 days, and then permanently wilt after more than 30 days of drought stress treatments (**Figure 4A, B**). Moreover, expressing gene *betA* also induced salt-tolerance of the transgenic sugarcane. When cultured in media containing 200 mM NaCl for 3 weeks, the drought-tolerance sugarcane showed stay-green, but the wilt-type leaves were yellowish and partly dried. Interestingly, observation of the root profile of the transgenic sugarcane showed a wider and longer root system compared to the wild-type sugarcane, but there was no change in the appearance of the shoot morphology (**Figure 4C**). The improved root system has a good water absorption system to extract limited water availability from deep soil and this is a criterion of drought-tolerance sugarcane [114, 115]. These results imply that the enhanced GB contents in transgenic sugarcane provides an osmoprotectant, stabilizes the structure of macromolecule, maintains the integrity and proper function of the cell membranes, and helps the sugarcane acclimate to drought- and salt-stress condition.

To investigate the growth and productivity of transgenic sugarcane under water limited condition, the sugarcane was grown in non-irrigated dry land of experiment station. Cultivation of the transgenic sugarcane was carried out under confined and limited field trial system according to the regulation for assessment of genetically modified organisms (GMO). Comparison of the drought-tolerance transgenic sugarcane with the wild-type showed almost no difference in the germination of lateral buds and the initial growth rate.

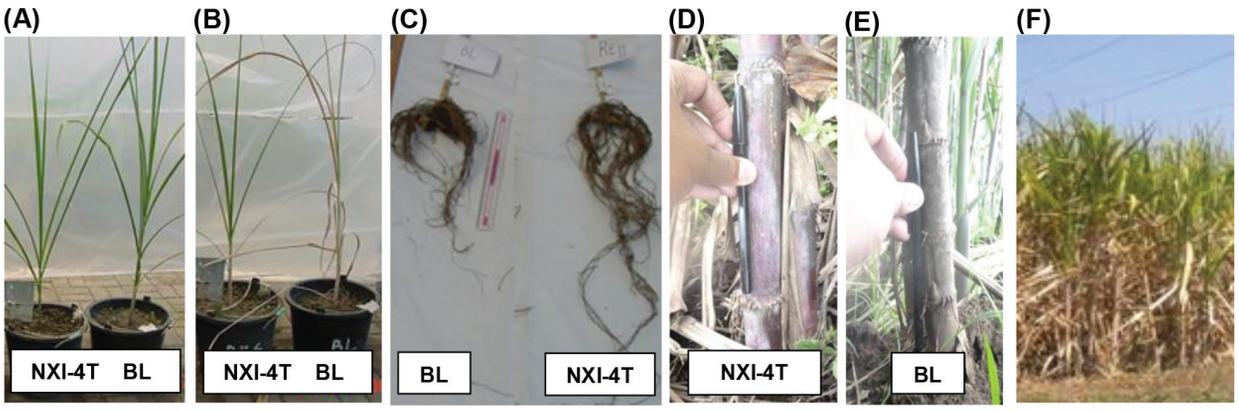


Figure 4. Growth performance of drought-tolerant sugarcane overexpressed *betA* gene in greenhouse and in non-irrigated experiment field station using confined trials system. Two-months old NXI-4T (transgenic drought-tolerant) and BL (non-transgenic) sugarcane cultivars were grown in greenhouse and treated with drought stress by stop watering. The transgenic drought-tolerant NXI-4T sugarcane stay green (left) and non-transgenic BL sugarcane cultivars (right) started wilting (A) after 8 days drought stress, then BL sugarcane being permanently wilting and dried after 28 days without watering (B). Wider and longer root profile of NXI-4T (right) is compared to BL (left) sugarcane (C). Normally growth internode of NXI-4T (D) and retarded internode of control BL sugarcane cultivars (E). Growth performance of nine-months old drought-tolerant sugarcane (F). The figures were provided by Dr. Nurmalasari of PT. Perkebunan Nusantara XI, Surabaya, Indonesia.

However, with the start of dry season, non-transgenic sugarcane showed retardation and elongation of stem. The internode of non-transgenic sugarcane becomes shorten during the dry season, but not the internode of transgenic sugarcane (**Figure 4D, E**). The internode of transgenic sugarcane was normally elongated at the similar size as expected and was not affected by dry season. The measurement of sugarcane yield of cane stalk significantly increased compared with non-transgenic plants, although the sucrose content was not different. Similar results were observed that drought-tolerance sugarcane lines have higher productivity stalk height and stalk weight than the susceptible line [116, 117]. In conclusion, all together the results showed that the transgenic sugarcane expression *betA* gene is a drought-tolerant sugarcane and this sugarcane should be the first drought-tolerant sugarcane developed by biotechnology approach.

The Cartagena Protocol on Biosafety (2000), which protects biological diversity from the potentially risk due to the use of transgenic plants, has been ratified by Indonesian government. Thus, for the commercialization of drought-tolerance sugarcane, biosafety assessment has been completed such as environmental safety, food, and feed safety. The environment safety assessment claimed that the drought-tolerance sugarcane has no effect on biodiversity, the occurrence of gene flow, and potentially to be an invasive crop. Bioinformatics BLASTP analysis suggested CDH protein encoded by *bet A* did not have similarity with allergen data base (NCBI Entrez) and potentially to be allergen. Further analysis using animal feeding experiment and simulation digestion system found that the drought-tolerance sugarcane did not potentially toxic or allergen. Based on the biosafety assessment, the drought-tolerance of sugarcane has been approved by the National Genetically Modified Product Biosafety Commission for commercial cultivation in the state-run sugarcane producer PT. Perkebunan Nusantara XI [118]. The company claimed that the drought-tolerance sugarcane produces 10–30% higher sugar productivity under dry land than in conventional parental lines.

Acknowledgements

The author thanks Dr. Nurmalasari Darsono and Dr. Yudhi Rinanto for providing the experimental results, and also Suvia Widyaningrum and Intan Ria Neliana for their assistances. The authors also thank the PUSNAS 2017 - Research Grant and University of Jember for the support of funding.

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