Genomics and Genetic Engineering of Rice for Tolerance to Ozone and Anoxia

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Abstract

Rice is one of the dominant staple foods of Southeast Asia; its production has been affected by a number of abiotic and biotic factors. Oxides of nitrogen, carbon monoxide and volatile organic compounds produced from vehicles and industries interact with ultraviolet light and form tropospheric ozone. Excessive ozone in the troposphere and anoxia caused by submergence of plants are two important abiotic stresses causing extensive damage to the rice crop. The physiology and growth of rice is extremely susceptible to ozone stress, which can cause reduction in productivity of the crop. Therefore, it is a call of the time to address these abiotic stresses to safeguard the rice production system. Recent advancements in genomics facilitate the identification of various quantitative trait loci (QTLs) and single nucleotide polymorphisms related with tolerance to both these stressors. It is assumed that the trait is controlled by multiple medium-effect loci rather than by a single large-effect locus. However, the underlying mechanism of stress tolerance is yet to be investigated, or in other words, the target genes that could be engineered in high-yielding varieties are yet to be fished out. The present chapter summarises the available knowledge on molecular physiology of hyper ozone concentration and anoxia tolerance in rice plant.

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1 Introduction

Ozone is present in small amounts in the Earth's stratosphere and absorbs ultraviolet radiation. It is used in many industries as an oxidant and disinfecting agent. It has bactericidal effects against a broad array of organisms like gram-positive and -negative bacteria as well as spores and vegetative cells. Ozone is harmful when present in the troposphere and the majority of tropospheric ozone formation occurs when oxides of nitrogen (NOx), carbon monoxide (CO) and volatile organic compounds (VOCs) react in the atmosphere in presence of sunlight. Ozone enters the plant through stomatal opening and causes chlorosis, necrosis and leaf bronzing, which result in low yield from the crop (Felzer et al. 2007). Ozone pollution is one of the most important abiotic factors that affect the rice production system. The photosynthesis process is highly affected by ozone exposure along with decrease in the stomatal conductance and the rate of transpiration (Banerjee and Roychoudhury 2018).

Ozone is quickly degraded to reactive oxygen species (ROS) in the apoplast (Rao and Davis 2001; Baier et al. 2005). These ROS lead to direct necrosis or induce programmed cell death (Kangasjarvi et al. 2005; Frei et al. 2010a). Ozone tolerance can be developed by two ways, with the help of antioxidants or antioxidant enzymes. The response of rice plant to ozone varies from cultivar to cultivar, and the regulation of these traits takes place at gene, transcriptome, proteome and metabolome levels. There are various approaches for the development of ozone-tolerant rice varieties. Rice is also badly affected by environmental stresses such as anoxia and oxygen deficiency. Ethanolic fermentation is the principal pathway of anaerobic carbohydrate catabolism in rice during anoxic condition. Advance functional genomics studies, QTL mapping and genetic engineering approaches can lead to the development of ozone- and anoxia-tolerant rice varieties. The present chapter is an attempt to summarise the molecular mechanisms of ozone and anoxia tolerance and the genetic engineering approach to develop tolerant rice varieties.

2 Ozone Pollution and Mechanism of Hyper Ozone Tolerance

2.1 Ozone and Ozone Pollution

Ozone is the tri-atomic allotrope of oxygen, i.e. three atoms of oxygen (O) form a bent molecule with an O–O–O angle of 116.78°. It is mainly present in the stratosphere of the earth's atmosphere at a range of around 10–50 km from the ground at a concentration of about 8–10 ppm. This region of the earth's atmosphere is known as

the ozone layer, and it is the layer that absorbs most of the ultraviolet radiations (wavelength range of 200–315 nm) of the sunlight and protects all life forms from its harmful effects. Due to its highly unstable structure and high oxidisation property, it is used in many industries as an oxidising agent including pharmaceutical and lubricant industries. It reacts with iron and manganese in water and precipitates it, which can be filtered to make water drinkable. It is a highly reactive molecule and has bactericidal property; thus, it is used as a disinfectant.

Despite a wide range of beneficial effects and industrial applications, it has many harmful effects on all life forms including plants, animals and humans. In case of animals, ozone has been found to affect a wide range of metabolic functions, especially lung functions; in all the species including humans, rat, mouse, sheep, pigs, monkeys, and dogs, rapid and shallow breathing develops on exposure to ozone. Ozone exposure also causes inflammation and increases the susceptibility of animals to infections. Apart from this, it has been found to be having DNA mutational effects.

Ozone also causes devastating effects on plants, and it has been claimed that ozone causes more damage to plants than all other air pollutants combined (https://www.ars.usda.gov/southeast-area/raleigh-nc/plant-science-research/docs/climate-changeair-quality-laboratory/ozone-effects-on-plants/). Ozone enters the plants through the stromatal opening and causes several symptoms such as chlorosis, necrosis, flecks, bronzing and reddening, leading to loss of yield.

2.1.1 Sources of Ozone Pollution

Ozone is not directly synthesised or emitted from any natural or anthropogenic activity; rather, it is synthesised as a by-product of the photochemical reaction of many primary pollutants originating from various anthropogenic activities involving oxygen. These primary pollutants include oxides of nitrogen (NOx), methane, carbon monoxide and other VOCs. Fossil fuels are the primary sources of both nitrogen oxides and carbon monoxide. Apart from that, nitrogen oxides are generated from bio-mass burning, lightning, soils and other sources, whereas VOCs are produced from natural vegetation. These gases in the presence of sunlight react with oxygen to produce ozone. Thus, solar radiations play an important role in ozone production and its production is higher in stagnant high pressure systems in summer when the temperature is high (Fig. 1).

Ozone is also transported into a region by local winds and downward from the stratosphere. The different spatial distributions of NOx and VOC production, often result in the largest ozone concentrations downwind of urban centres, rather than in urban areas themselves (Felzer et al. 2007).

2.1.2 Effect of Ozone Pollution on Rice Plants

Rice is the most important staple food worldwide, especially in Asia, and one of the agricultural commodities with the highest production. In the year 2017, about 769 million metric tonne of rice was produced worldwide. China, followed by India, is the top lister among the rice-producing countries. China and India together produced around 382 million tonne of rice, which is approximately 50% of the total
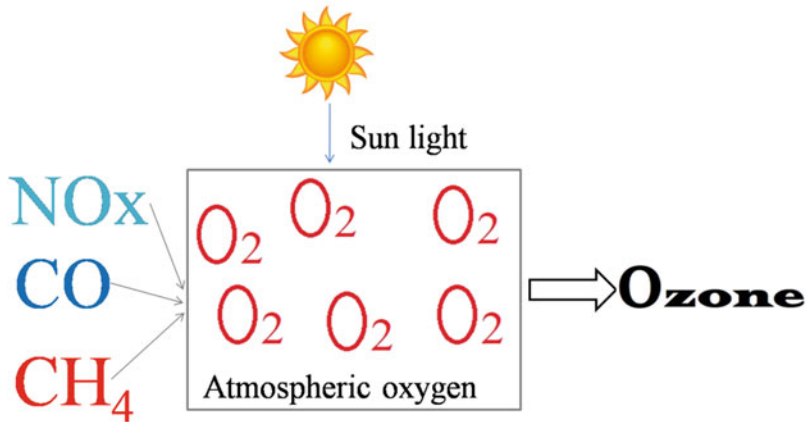
Fig. 1 The process of ozone synthesis in the atmosphere

rice production worldwide. The importance of rice in human nutrition can be understood from the fact that about 20% of the calories consumed by humans worldwide come from rice.

Rice production has grown steadily in the recent years from 538 metric tonne in 1994 to 769 million tonnes in 2017 due to better management practices including water, fertiliser and pest management. However, the rice production system is facing constant challenges, both biotic and abiotic stressors being the foremost challenges. Abiotic stressors include drought, submergence (excessive rain), salinity, and ozone pollution, whereas the abiotic stressors mainly include pests and pathogens.

Ozone pollution is one of the most important abiotic factors that affect the rice production system. It has been found that for an additional day with the ozone concentration of >120 ppb, there could be loss of about 1.12% in rice yield compared to a day with ozone concentration of <60 ppb (https://www.ucdavis.edu/news/surface-ozone-pollution-damages-rice-production-china/).

The physiological effects of ozone pollution on plants include reduced photosynthesis, increased turnover of antioxidant systems, damage to reproductive processes, increased dark respiration and lowered carbon transport to roots (Felzer et al. 2007; Ariyaphanphitak et al. 2005). The physiological aberrations ultimately lead to decline in productivity. Zong-wei have reported 49.1%, 26.1% and 8.2% decrease in grain yield per rice plant when exposed to 200 ppb, 100 ppb and 50 ppb of ozone. Ariyaphanphitak et al. reported a whopping 78% reduction in filled seed per ear in Pathumthani 1 rice cultivar when exposed to 150 ppb of ozone. Similarly, Shi et al. reported yield loss of 17.5% and 15% in SY63 and LYPJ rice varieties of China, respectively, when exposed to 50% more ozone than ambient.

2.1.2.1 Physiological and Molecular Effects on Rice Plants

The effect of high ozone concentration may vary from cultivar to cultivar. Leaf injury leading to severe necrosis and chlorosis is one of the primary visible effects of

high ozone exposure. The appearance of brown spots on rice leaves or otherwise called "leaf bronzing" is also observed in ozone-exposed plants. The photosynthesis process is highly affected by ozone exposure as synthesis of many of the proteins related with light harvesting and electron transport systems decline. The stomatal conductance and the rate of transpiration decline, which results in decline in photosynthesis, ultimately leading to chlorosis (Banerjee and Roychoudhury 2018). Along with the photosynthetic activity, there is a decline in grain yield. Even though these two are often simultaneous processes in ozone-exposed rice plants, the degree of visible leaf injury does not correlate well with loss in grain yield (Tsukahara et al. 2015; Sawada and Kohno 2009).

Ozone enters through the stomatal opening and it is quickly degraded to ROS such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in the apoplast (Rao and Davis 2001; Baier et al. 2005). These ROS can have dual effects; it may lead to direct necrotic tissue damage or may induce programmed cell death (PCD) (Kangasjarvi et al. 2005; Frei et al. 2010a).

On a biochemical level, ozone induces the synthesis of salicylic acid, jasmonic acid, abscisic acid and ethylene, which play important roles in plant defence mechanism including cell death (Rao and Davis 2001). Apart from these, the synthesis of other antioxidative enzymes like superoxide dismutase (SOD), catalases and ascorbate peroxidase have also been found to be increasing (Banerjee and Roychoudhury 2018). These biochemicals act on various interdependent or independent and sometimes antagonistic pathways related to the plant defence mechanism. For instance, salicylic acid has an inhibitory effect on enzymes such as catalase and ascorbate peroxidise. Catalase and ascorbate peroxidase are enzymes that are involved in the metabolism of H_2O_2; when there is increased synthesis of SA, it inhibits the synthesis of catalase and ascorbate peroxidise, leading to cell death of leaves (Rao and Davis 2001). Similarly, jasmonic acid and ethylene act synergistically and may antagonise or synergise the effects of salicylic acid depending upon the stimulus and rice variety. Ozone exposure has been found to induce the biosynthesis of ethylene, which is known to induce organ senescence.

Abscisic acid (ABA) is another plant hormone that has been found to have protective effects against ozone. Lin et al. (2001) have showed that treatment of rice plants with ABA significantly reduced H_2O_2 content in leaves of ozone-exposed plants. It also effectively reduced stomatal conductance and the degree of injury. They have suggested that ABA induced tolerance to ozone could be associated more with stomatal movement than on the modulation of antioxidant enzymes.

2.1.2.2 Effect of Ozone on Rice Yield

Ozone when present in the troposphere is called as bad ozone. This ozone is produced from methane, VOCs and nitric oxides (Ainsworth et al. 2012). Troposphere ozone significantly affects the growth of rice; the number of main stem leaves decrease and there is marked decrease in the plant height of rice. Significant reduction in the percentage of filled spikelet and individual grain mass in rice is due to bad ozone (Shi et al. 2009). Due to the increased concentration of ozone, carbon dioxide assimilation at the leaf level decreases, which significantly affects the

yield of crop (Fiscus et al. 2005). High level of ozone leads to stomata closing and affect the yield of rice (Imai and Kobori 2008). Grain yield is reduced significantly due to the exposure of rice to ozone (Pang et al. 2009). Exposure of plant to high concentration of ozone causes oxidative damage that leads to tissue damage, which can be subsequently detected from the damaged leaves (Baier et al. 2005; Fiscus et al. 2005; Rao and Davis 2001).

High concentration of ozone affects the quality of rice grain and straw. In case of grain, starch concentration decreases and protein content increases (Frei et al. 2012a, b; Wang et al. 2012; Zheng et al. 2013). Ozone enhances senescence, so nitrogen remobilisation takes place in the grain. High protein content is a good nutritional property but the quantity of rice grain decreases significantly so protein yield also decreases (Frei et al. 2012a, b; Zheng et al. 2013). It also affects the texture of rice as the grain becomes tougher and chewier (Hamaker 1994; Singh et al. 2011). High level of ozone induces grain chalkiness, which leads to change in the visual appearance of rice grain, ultimately affecting consumers' acceptance (Wang et al. 2014a, b). Frei et al. (2012a, b) found that lipid concentration increases in rice grain but the grain size becomes smaller.

Rice straw contains high amount of lignin and phenolics under ozone exposure than normal grown rice. Lignin and phenolics are antinutrients for ruminants. Straw quality is degraded on exposure to ozone, which is not suitable to feed herbivores (Devendra and Sevilla 2002; Frei et al. 2010b, 2011; Frei 2013; Wang and Frei 2011).

Ozone at a molecular level causes toxicity. It produces ROS like hydrogen peroxide, superoxide radicals and hydroxyl radicals. These radicals damage the lipids and proteins of the cell membrane. Membrane function and fluidity changes as a consequence of lipid damage caused due to ozone toxicity (Luwe et al. 1993). ROS also degrades proteins and DNA (Sharma et al. 2012). Ozone along with primary ROS produces secondary ROS, which degrades biomolecules (Choudhury et al. 2013). High ozone concentration induces chlorosis and necrotic lesions at a cellular level (Schlagnhaufer et al. 1995; Rao et al. 2000, 2002).

2.2   Mechanism of Ozone Tolerance

Ozone produces ROS in the apoplast region of plant. The ozone signalling mechanism in plants and its possible pathways are depicted in Fig. 2.

Mechanism of ozone tolerance is associated with the scavenging of ROS generated by oxidative stress. ROS detoxification involves antioxidants and antioxidant enzymes (Mittler 2002; Neill et al. 2002; Dietz 2003). Antioxidants are mainly ascorbic acid, glutathione and polyphenols. Antioxidant enzymes are SOD, catalase and peroxidase (Blokhina et al. 2003). The ascorbate-glutathione cycle works with the help of enzymes that use ascorbic acid and glutathione as substrate. Scavenging of ROS takes place and the reduced state of antioxidants is restored (Noctor and Foyer 1998). Ascorbic acid level and its reduced state are a critical factors for ozone detoxification (Conklin and Barth 2004). Polyamines and putrescine help in the
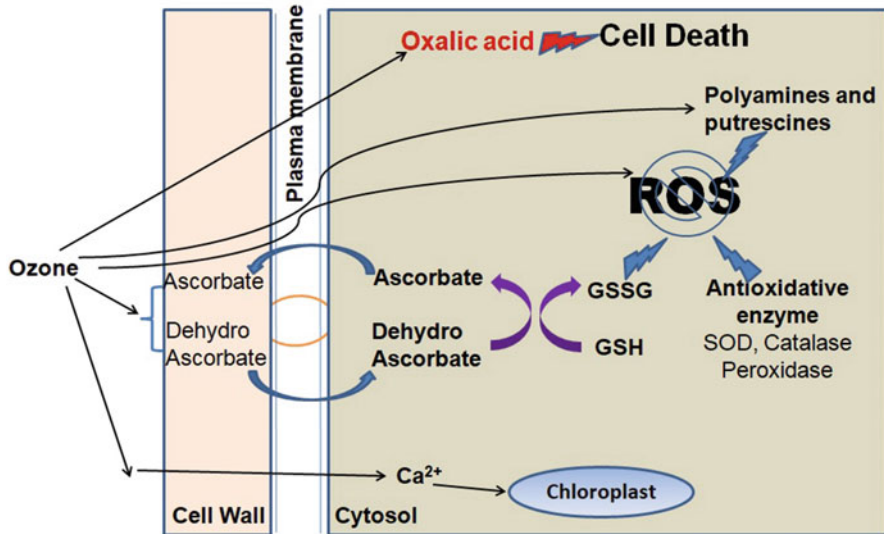
Fig. 2 Ozone signalling mechanism in plants

scavenging of intercellular ROS (Langebartels et al. 1991; Navakoudis et al. 2003; Nali et al. 2006; Bussotti et al. 2007). Similarly, isoprene has significant role in scavenging hydrogen peroxides and revert the effect of lipid peroxidation of membranes (Loreto et al. 2001; Loreto and Velikova 2001). SOD and monodehydroascorbate reductase play important roles in the protection of plants from ozone damage and provide tolerance to ozone (Eltayeb et al. 2007; Van Camp et al. 1994).

2.2.1   Molecular Responses of Rice Plant Against Ozone Toxicity

The response of rice plant to ozone varies from cultivar to cultivar; some of the varieties are tolerant, whereas others have been found to be susceptible. So, it is obvious to expect the regulation of these traits at the gene, transcriptome, proteome and metabolome level. There have been numerous studies on the mechanism of hyper ozone tolerance in plants; these studies are not limited to rice. In fact, only few studies have been carried out in rice. Some of such studies are discussed here.

Cho et al. (2008) have extensively studied the transcriptomic, proteomic and metabolomic alterations in rice plants to survey ozone response in leaves of rice seedling. Microarray-based transcriptomics study has, shown upregulation of 1266 genes and suppression of 267 genes involved in a wide range of pathways including photosynthesis, MAPK cascades, and jasmonic acid, ethylene and tryptophan biosynthesis. These results were also corroborated by proteomic and metabolomic investigations. One of the most highly represented genes were those of the WRKY domain proteins. OsWRKY1, 11, 24, 26, 28, 42, 68, 69, 71, 45, 55, 72, 76 and

77 were among the WRKY genes that were upregulated in one or other time points of ozone exposure. Many of these WRKY proteins have antagonising effects. For instance, OsWRKY72 and -77 activate the abscisic acid-inducible HVA22 promoter, whereas OsWRKY24 and 45 repress it. This study puts light on the molecular mechanism of the ozone-induced enhancement on the abscisic acid synthesis as discussed in the previous section. Further, the findings of the study suggested that ABA signalling is controlled by the competitive mechanism of positive regulators and negative regulators.

A number of previous studies have determined genetic factors associated with ozone tolerance in rice. Rice is among the very few crop plants for which putative QTLs for ozone tolerance have been identified (Frei et al. 2010a). It has been suggested that ozone tolerance is controlled by multiple medium-effect loci rather than by a single large-effect locus (Frei 2013; Ueda et al. 2015). Frei et al. (2008) have identified two QTLs for ozone tolerance through screening 23 rice varieties with a leaf bronzing score (leaf bronzing is one of the symptoms of ozone toxicity). Out of the 23 varieties, the indica variety 'Kasalath' showed no visible symptoms, whereas the japonica variety 'Nipponbare' was found to be moderately susceptible. Subsequent backcrosses identified two QTLs associated with leaf bronzing, i.e. OzT3 and OzT9. These two QTLs have been found to be having contrasting effects; while OzT3, located at 2 cM on chromosome 3, has been found to be enhancing LBS, the OzT9 has been found to be reducing it. Further, they characterised the QTLs through comparative gene expression analysis, which revealed that these genes are involved in PCD and antioxidative ROS generation synthesis (Frei et al. 2008).

Wang et al. (2014a, b) have shown that pyramiding the two QTLs OzT9 and OzT8 provides improved tolerance to ozone exposure. They created lines with tolerance alleles at both loci by crossing two chromosome segment substitution lines containing individual ozone tolerance QTLs *OzT8* and *OzT9*. It was found that the lines containing both the QTLs performed better than the Nipponbare parents SL46 (line with *OzT8*) and SL41 (line with *OzT9*) in terms of leaf bronzing, lipid peroxidation, chlorophyll level and stomatal conductance. These physiological advantages also led to better yield. It was observed that pyramiding of both the QTLs provided better yield as compared to a single QTL (Wang et al. 2014a, b).

An apoplastic protein, namely Ozone-Responsive Apoplastic Protein1 (OsORAP1), has been found to induce cell death in ozone-stressed rice plants. This gene is associated with the QTL *OzT9* and has a sequence similar to ascorbate oxidase. A knockout rice line of OsORAP1 has been found to have enhanced tolerance to ozone stress as evidenced from less formation of visible leaf symptoms (i.e. cell death), less lipid peroxidation and lower NADPH oxidase activity, which indicates reduced production of ROS (Ueda et al. 2015).

2.3   Genetic Engineering for Ozone-Tolerant Rice Variety

Given the emission of greenhouse gases from a wide range of anthropogenic activities, the atmospheric ozone concentration will surely increase in days to come. This would adversely affect all crop plants, especially rice plants, and thus, there is an urgent need to look for mitigation strategies. Even though multiple QTLs for ozone tolerance have been identified, there are speculations regarding their stable expression after the incorporation of these QTLs into high-yielding varieties through conventional breeding. Incorporation of novel genes through genetic engineering is one of the feasible strategies that could be employed to develop ozone-tolerant rice varieties.

Even though the molecular pathways involved in the process of ozone tolerance have not been extensively studied, there are some report on the transcriptomic and proteomic alterations in tolerant rice varieties in response to ozone. These studies have shown that the antioxidative enzyme systems and the pathways involved in ABA, JA, SA and ethylene metabolism play important role in ozone tolerance. Thus, genes involved in these metabolism processes are possible targets that could be engineered and overexpressed for providing ozone tolerance. For example, the wrky genes have been found to play an important role in ozone tolerance. The wrky gene identified in rice, OsWRKY89, known for its ROS scavenging activity, could possibly be overexpressed for ozone tolerance. Similarly, gene encoding enzymes associated with the AsA-GSH cycle like APX, dehydroascorbate reductase, monodehydroascorbate reductase and GR can be overexpressed to promote O_3 tolerance (Frei et al. 2012a, b). Downstream proteins like late embryogenesis abundant proteins can also be expressed to confer O_3 tolerance (Banerjee and Roychoudhury 2018).

3   Anoxia and Mechanism of Anoxia Tolerance

Plants usually face anoxia as an ecological challenge during their life cycle due to certain natural conditions like amalgamation of freezing and flooding stresses including spring floods and ice encasement. Aquatic plants have developed many adaptive metabolic structures to combat hypoxia or anoxia (Blokhina et al. 2003). Energy crisis is the major concern during anoxia so anoxia-tolerant plants develop mechanisms to adapt to energy crisis. Plant tissues enhance carbohydrate catabolism during the initial period and downregulate the rate of carbohydrate catabolism with time (Gibbs and Greenway 2003). Energy production is totally through glycolysis concurrent to ethanolic fermentation during the initial 3–4 h of anoxia in rice (Menegus et al. 1991; Ricard et al. 1994). There are reports that supply of high concentrations of exogenous glucose improves anoxia tolerance in rice coleoptile tips, as they do not lose K^+, P and Cl^- even up to 120 h of anoxia, indicating that these tips did not suffer injury. Energy-dependent solute transport also has priority during an energy crisis. During anoxia, the energy distribution in rice coleoptiles

favours increase in fresh weight instead of protein synthesis (Alpi and Beevers 1983).

Rice coleoptiles reacted to anoxia considerably at the level of changed metabolite pools. A set of enzymes, i.e. alcohol dehydrogenase (*ADH*), phosphoenolpyruvate carboxykinase (*PCK*), pyruvate decarboxylase (*PDC*) and pyruvate orthophosphate dikinase (*PPDK*), showed increased abundance in anoxic rice. There are reports of accumulation of certain amino acids such as Ser, Gly and Ala in anoxic rice coleoptiles. Many genes have been found to be downregulated under anoxia, especially those genes that code for enzymes requiring oxygen for their activity, indicating the existence of an energy-saving strategy in the regulation of gene expression. Similarly, various genes coding for signal transduction components (Baxter-Burrell et al. 2002), transcription factors (Liu et al. 2005), nitrogen metabolism (Mattana et al. 1994), nonsymbiotic haemoglobin (Dordas et al. 2004), ethylene biosynthesis (Vriezen et al. 1999), and cell wall loosening (Saab and Sachs 1996) were upregulated at low oxygen concentration. Anaerobic proteins like enzymes involved in sugar metabolism, glycolysis and fermentation pathways are overexpressed during anoxia (Huang et al. 2005).

3.1   Genetic Engineering for Anoxia-Tolerant Rice Varieties

Rice (*Oryza sativa* L.) is extremely sensitive to anoxia during germination and early growth of the embryo (Yang et al. 2019). Genetic engineering of novel genes into intolerant rice varieties could successfully resolve the limitations of anaerobic germination. Siangliw et al. (2003) reported that the *Sub1* locus strongly affects the survival of rice flooding tolerance varieties by synthesising ethylene response factors. Incorporation of the *Sub1* locus into the 'japonica' (intolerant; Toojinda et al. 2003) significantly increased its flooding tolerance (Fukao et al. 2006).

Very few papers have been published on the genetic engineering of anoxia-tolerant recombinant lines. Quimio et al. (2000) reported overexpression of PDC (*pdc1*) in the rice cultivar 'Taipei 309' that improved anoxia tolerance as a result of an increase in alcohol metabolism. Kretzschmar et al. (2015) explained that the expression of trehalose-6-phosphate (T6P) phosphatase gene (*OsTPP7*) increases starch mobilisation and the elongation of coleoptile, as a result of enhanced anoxia tolerance. However, the precise mechanism of anoxia tolerance is still not clear and merits further investigation. QTL mapping and genome-wide association studies were recently used to identify some QTLs associated with anoxia tolerance. Even though numerous tolerance loci have been identified, only one QTL (*qAG-9-2*) has been fine mapped and cloned as *OsTPP*7.

4   Conclusion

The chapter summarises the molecular mechanisms developed by rice to cope with ozone and anoxia. Recent developments in molecular biology and genetics have enhanced our understanding of mechanisms adapted by rice plant to survive under anoxia and ozone stress conditions. Further efforts are needed to develop transgenic rice varieties that can solve the global food demand in the era of climate change.

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