Effects of Water Deficit on Pollen Development in Rice

by

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STATEMENT OF ORIGINALITY

The research presented in this thesis is the result of my own investigation, except where acknowledgement is given, and has not previously been submitted for the award of a degree at any institution.

Giao Ngoc Nguyen
ABSTRACT

Rice (*Oryza sativa* L.) is very susceptible to water deficit at any time during its life cycle as a semi-aquatic cereal crop. However, the consequential damage is particularly severe if water deficit occurs during reproductive phases. The conspicuous injury often observed in rice plants exposed to water stress during meiosis of the pollen mother cell is the reduction of grain set, which is attributed to the decline of male fertility. In spite of much research on drought-induced male sterility in rice, the underlying mechanisms of the problem are poorly understood.

This project was therefore conducted to investigate the molecular mechanisms of water deficit-induced pollen sterility in rice. In this study three consecutive days of water deficit treatment at -0.5 MPa osmotic potential during anther development effectively reduced the leaf water potential ($\psi_{\text{leaf}}$) and the number of viable pollen which later led to a decrease in grain set. Moreover, this thesis demonstrates that the immediate deleterious effects of water deficit to plant fertility could be estimated using a young microspore viability index, which showed a strong correlation with mature viable pollen and grain set. The present work has also illustrated that oxidative stress appears to be a plausible cause for the decline of male fertility and grain set. Water deficit has induced the excessive production of reactive oxygen species (ROS) above the redox balance, which in turn caused detrimental effects to cellular DNA and might result in programmed cell death (PCD) in the anthers. Moreover, ROS accumulation effectively influenced ATP synthesis leading to a decrease in the level of ATP in the anthers. Excessive ROS accumulation after drought could be the consequence of insufficient activity of the antioxidant system, which has been illustrated by qRT-PCR expression analysis of major antioxidant genes. Down-regulation of those genes would increase the incidence of oxidative damage. In contrast, stable or up-regulated expression of these genes resulted in less oxidative damage.
Detailed investigations of sugar metabolism in anthers has provided supplemental data to develop a model of sugar unloading and transport within anther using \textit{in situ} hybridisation to mRNA techniques. Analysis of sugar transportation within the cellular compartments of anther has unveiled the role of sugar metabolism on pollen sterility in rice. qRT-PCR assays of genes associated with the sugar metabolic pathway has demonstrated that the supply of both sucrose and hexoses from the anther walls to the locules was not restricted after water deficit stress. The results indicate that water deficit might not cause sugar starvation for developing microspores as previously thought, nor inhibit the initial steps of sugar utilisation such as glycolysis. This thesis has suggested new ideas regarding the role of rising sugar levels to cope with oxidative stress in anthers. Sugar accumulation might have provided protection against oxidant damage by strengthening the antioxidant system. However, the interplay between sugar and oxidative stress is not straightforward and needs to be further characterised. In-depth investigations on the interaction between sugar signalling and oxidative stress responses may help indentify the role of sugars in protecting anthers under water deficit.

Although many studies on drought and chilling stresses in rice anthers have been performed, the causal mechanism of male sterility still remains to be elucidated. Findings presented in this thesis may contribute to understanding molecular mechanisms of male sterility in rice as a response to drought stress. A more detailed investigation of mitochondrial respiration in rice anthers is required to further examine this problem. Finally, this thesis suggests that signalling molecules such as 14-3-3 proteins and abscisic acid (ABA) might act upstream of ROS production and antioxidant defence in plants. Further work on these molecules might therefore further illustrate how they influence plant fertility under water shortage conditions.
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<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>AD</td>
<td>Auricle distance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylene pyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MST</td>
<td>Monosaccharide transporter</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PPT</td>
<td>Parts per thousand</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>q RT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride sodium citrate buffer</td>
</tr>
<tr>
<td>SUT</td>
<td>Sucrose transporter</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>t-RNA</td>
<td>Transfer ribonucleic acid</td>
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<td>TUNEL</td>
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CHAPTER 1: REVIEW OF LITERATURE AND AIMS

1.1. Introduction

Rice (*Oryza sativa* L.) is one of the most widely adapted crops and is cultivated on approximately 154 million hectares worldwide, occupying about 11% of the world’s arable land. The plant is considered the world’s single most important cereal crop providing food for more than half the world’s population (Khush, 2005), and constituting over 30% of the calories consumed in Asia (Narciso & Hossain, 2005). Due to its importance, total global rice production has been increasing steadily from 126 million tons in 1961 to 619 million tons in 2005 through adoption of technical advances such as modern cultivars, increased investment in irrigation, greater use of fertiliser, and expansion in areas cultivated (Narciso & Hossain, 2005). The most important constraint on world rice production is the limited area of irrigated land available, in a context of increasing global population. According to Mygatt (2006), the world’s irrigated area per capita has dropped from 48 ha to below 44 ha per 1000 people between the late 1970s and 2003 respectively. Still, rice production needs to be increased by 40% by 2030 in order to meet the growing demand (Khush, 2005).

Although rice can be adapted to different growing ecosystems such as irrigated land, rainfed lowland, upland and flood prone land (Khush, 1984), as a semi-aquatic plant the most productive growth of rice requires abundant water supplies compared to other cereal crops. Bhuiyan (1992) calculated that the amount of water required to produce 1 kg of rice is significantly larger than for other cereal crops. Productivity of rice in terms of water input is only 0.3-0.7 g of rice per kg of water while this parameter is 0.8-1.6 g for wheat and 1.6-3.9 g for corn, respectively (Tuong, 1999). Despite the strong requirement of non-flooded rice cultural practices for the industry, research on the production of rice under non-flooded conditions conducted by several investigators
showed a large decrease in grain yield as a result of reduction of grain bearing panicles, increased floret sterility and lower individual grain mass (Blackwell et al., 1985; Muirhead et al., 1989; Vories et al., 2002). Among factors limiting rice yield, drought is considered one of the main constraints in rainfed and poorly irrigated areas (O'Toole & Chang, 1979). There are at least 23 million ha of drought-prone rice growing land occupying 20% of the total rice production area in Asia (Pandey et al., 2007). It is estimated that drought can cause a rice annual yield loss of up to 18 million metric tons which can have a value of $US3,600 million (Widawsky & O'Toole, 1990; Evenson et al., 1996).

Of the strategies to improve rice production in drought prone areas, breeding for tolerant or resistant varieties is the most promising. However, progress in breeding for drought tolerant cultivars has been reported to be slow for a number of reasons. Problems include difficulty in precisely defining the target environment, complex interactions of drought tolerance with the environment, and a lack of appropriate, systematic screening methods of rice germplasm (Cooper et al., 1999; Wade et al., 1999). Moreover, genetic characterisations of drought tolerance are difficult as resistance is a complicated trait often consisting of different physiological and biological processes at different cellular levels and stages of plant development (Tripathy et al., 2000). This combined with a lack of knowledge of the genetic basis and mechanism of drought resistance in real field conditions, especially at reproductive stages, restricts progress in breeding for drought tolerant varieties (Yue et al., 2005; 2006). In addition, the drought damage depends not only on the level of water deficit but also on the timing of the stress. Loss of grain is particularly more serious if drought occurs during reproductive development which usually determines grain yield (Saini & Westgate, 2000; Pantuwan et al., 2002). Thus, strategic breeding criteria for drought
resistance at reproductive stages are crucially important (Yue et al., 2006). Although some factors conferring greater drought tolerance in regards to pollen viability have been identified, the actual underlying mechanism of water stress damage remains poorly understood to some extent. This chapter will therefore outline the current knowledge of abiotic stress-induced pollen abortion in this context, with emphasis on water stress induced sterility in rice.

1.2. Studies on male gametophyte development in rice

The rice inflorescence, or panicle, usually consists of the rachis, rachilla, primary, secondary or tertiary branches, pedicel, and florets (Takeoka et al., 1992). The rice floret has six distinct stamens as the male organ of the flower (Figure 1.1).

![Morphology of a rice floret](http://www.gramene.org/species/oryza/rice_illustrations.html#sativapan)

**Figure 1.1:** Morphology of a rice floret.

(Adapted from [http://www.gramene.org/species/oryza/rice_illustrations.html#sativapan](http://www.gramene.org/species/oryza/rice_illustrations.html#sativapan))
Male gametophytes form within the reproductive floral organ, the stamen. Each stamen has two morphologically distinct parts: a stalk-like filament and the anther that is situated on the top of the filament. The filament is tube-like and consists largely of vascular tissue that bears the stamen to the flower and serves as a conduit for the movement of water and nutrients. Male sporogenous cells enveloped in the anther differentiate and undergo meiosis to produce microspores, which develop further to pollen grains, while other cell types contribute to pollen maturation, protection, or release (Scott et al., 2004). There have been several excellent reviews of male gametophyte development of angiosperms in the past (Mascarenhas, 1989; Goldberg et al., 1993; McCormick, 1993). Figure 1.2 illustrates a schematic of typical microsporegenesis of most angiosperms (McCormick, 2004).

Numerous studies on male gametophyte development in rice have also been published (Raghavan, 1988; Wada et al., 1990; 1992; Li, 2005; Mamun et al., 2005b, a). An ultrastructural study of normal anthers at different stages of development is shown in Figure 1.3. Like other species, pollen development in rice occurs within the locules of the anther. At the pre-meiotic stage, the sporogenous cells are large, containing cytoplasm with some scattered organelles. Sporogenous cells are interconnected by plasmodesmal connections (Mamun et al., 2005a). At the start of meiosis, the sporogenous cells are surrounded by a special callose wall (β-1, 3 glucan) and then differentiate into pollen mother cells (Figure 1.3B) (Li, 2005). No plasmodesmatal connection is observed between the meiocytes or meiocytes and the tapetum layer (Mamun et al., 2005b). The pollen mother cells separate from each other and undergo two distinct episodes of meiotic division to produce haploid meiocytes, the dyads, after the first meiosis and the tetrads after the second meiosis. The newly formed tetrads are enveloped by a callose wall (Figure 1.3C) (Li, 2005; Mamun et al., 2005b).
Figure 1.2: Scheme of microsporegenesis in angiosperm (McCormick, 2004)

After the breakdown of the callose wall, single haploid microspores are released into the locules (Figure 1.3D). The process of microsporegenesis is continued soon after that with the production of multiple small vacuoles in the microspore cytoplasm. At the end of the microspore stage, the small vacuoles are fused into a single large one which causes the nucleus to migrate to the peripheral region opposite the germpore, about nine
days before anthesis (Figure 1.3E) (Li, 2005). These uninucleate microspores undergo the first mitotic division resulting in binucleate cells known as pollen. Pollen contains two types of cells: the vegetative and generative cells (Figure 1.3F) (Li, 2005). Before pollen is shed from the plants, the generative cell undergoes a second mitosis to form two sperm cells. Starch granules start to increase rapidly in the cytoplasm of the vegetative cell about five days before anthesis (Wada et al., 1992; Li, 2005). As pollen grains enlarge, the central vacuole decreases in size and starch deposition increases further. At anthesis, the starch granules mainly localize near the germ pore (Wada et al., 1992; Li, 2005). This starch is an important source of energy for pollen germination and pollen tube growth during pollination.

Concurrent with the development of pollen, anther walls also evolve. Normally, a rice anther consists of four microsporangia, which are seen as four circular lobes in transverse sections and are linked by connective tissue. Each anther lobe differentiates into four distinct layers: epidermis, endothecium, middle layer and the tapetum with sporogenous cells at the centre of the lobe (Figure 1.3) (Li, 2005; Mamun et al., 2005a, b). Different layers of the anther perform different functions during pollen development. The epidermis is the outermost layer of the anther and is composed of highly vacuolated and quadrate cells with peripheral cytoplasm. The cuticle coating the outer wall of the anther epidermis is a protective layer to prevent water loss during anther development (Goldberg et al., 1993; Mamun et al., 2005b). The endothecium underlying the epidermis has been considered as a supportive layer for the development of sporogenous cells and may play a role in pollen dehiscence (Bonner & Dickinson, 1989; Pacini, 1994; Matsui et al., 1999). In addition, it has also been suggested as a storage organ which controls the level of sugars being transported to the developing meiocytes (Clement & Audran, 1995).
**Figure 1.3:** TEM images of anther development in rice at different stages. Photographs kindly provided by Li (2005).

A- Transverse section of a typical anther (microspore stage).

B- Pollen mother cell stage.

C- Tetrad stage.

D- Early microspore stage.

E- Vacuolated microspore stage.

F- Binucleate stage.

Il large lobes; sl small lobes; ct connective tissue; st stomia; ep epidermis; en endothecium; ml middle layer; t tapetum; lo locule containing sporogenous cells; bs bundle sheath; vb vascular bundle. x xylem elements; ste sieve tube element. cw callose wall; pmc pollen mother cell; te tetrad; mi microspore.
The middle layer is the inner layer to the endothecium. During anther development, the middle layer may serve as an ordinary structure to support the anther walls (Goldberg et al., 1993) or may be involved in sugar unloading (Li, 2005). The tapetum is the layer situated between the somatic cells and sporogenous cells (Mamun et al., 2005a). This tissue envelops the pollen sac at the onset of anther development, self degenerates during the later phase, and does not exist as a systematised organ in mature anthers (Esau, 1977). The distinct role of the tapetum during anther development has been well documented in the literature. It is generally considered as a nourishing layer which provides nutrients and structural components for developing microspores (Pacini et al., 1985; Pacini, 1990; Piffanelli & Murphy, 1998). All nutrients required for the pollen mother cell and developing microspore must pass through this specialised secretory layer. Also, the tapetum secretes 1,3-β-glucanase to break down the callose wall, facilitating the release of young microspores into the locule and synthesizes precursors such as sporopollenin and pollen coat (Pacini, 1994; Piffanelli et al., 1998). Frequently, organelles like mitochondria have been seen with increasing numbers in the tapetum during anther development, implying a role of supplying nutrition to developing microspores (Mamun et al., 2005b).

1.3. Water stress causes male sterility: sensitive stages and nature of the damage

Water deficit occurring during reproduction results in a decline of grain yield in rice (O'Toole & Moya, 1981). The nature of the damage is dependent on the degree, duration and the particular stages that stress occurred (O'Toole & Moya, 1981). The physical and molecular effects of drought stress on the reproductive development of cereal crops in general and rice in particular have been reviewed intensively in the past (Saini, 1997; Saini & Lalonde, 1998; Saini & Westgate, 2000; Barnabas et al., 2008 and references cited therein). Two stages of high sensitivity to water deficit have been well documented in the literature. The first vulnerable stage to water deficit centres on anther
meiosis, while the second one is during anthesis or flowering of rice plants (Saini & Westgate, 2000).

The obvious damage often observed in rice plants exposed to water stress during meiosis of the pollen mother cell is the decline of the grain set and number of engorged pollen, which are similar to symptoms for rice under chilling stress. The decrease in the number of engorged pollen has been regarded as the main cause for sterility of rice plants if chilling occurs during the microspore stage (Nishiyama, 1983; Heenan, 1984; Matsuo et al., 1995). The reduction in the number of engorged pollen could be attributed to several factors such as failure of young microspores to differentiate (Satake, 1991), the decline in the number of dehisced anthers (Sawada, 1978), and the repression of anther development (Nishiyama, 1984). Consequently, the reduction in the number of pollen grains will result in a decrease in the number of available pollen grains falling on stigma, ultimately leading to unsuccessful or poor pollination (Nishiyama, 1984). Gunawardena et al. (2003) have concluded that 830 engorged pollen grains per anther are generally required to maintain levels of sterility less than 10% if chilling occurred during the microspore stage.

Likewise drought stress has been reported to induce adverse effects on male gametophyte development resulting in a reduction in the number of viable pollen in rice (Namuco & O'Toole, 1986; Sheoran & Saini, 1996). The similarities between the responses of rice, wheat and other crops during reproductive stages to water deficit have been well documented (Sheoran & Saini, 1996). Experiments on wheat have confirmed that the critical drought sensitive stage is during the period from meiosis to tetrad breakup in anthers (Bingham, 1966; Saini & Aspinall, 1981; Dembinska et al., 1992). Interestingly, while anthers suffer a serious fertility loss, female fertility is not affected at the same stress level and only starts to deteriorate under much more severe stress (Saini & Aspinall, 1981). Rice plants subjected to water deficit during anthesis decrease
grain yield as a result of failure in full panicle exsertion, delayed flowering, spikelet desiccation, inhibition of spikelet opening and fertilisation (O'Toole & Namuco, 1983; Cruz & O'Toole, 1984; Ekanayake et al., 1989).

Although pollen abortion of cereals caused by environmental stresses such as drought has been the subject of numerous studies and attempts have been made to describe the cellular and metabolic processes associated with this event (Saini & Westgate, 2000; Barnabas et al., 2008), the primary trigger for this fertility reduction remains to be identified. In the following sections mechanisms that have been suggested to be the causes of pollen failure in rice will be discussed.

1.4. **Studies on mechanisms of male sterility in rice**

1.4.1. **Disturbance of sugar metabolism in anthers**

1.4.1.1. **Lack of starch in mature pollen and accumulation of sugar**

During reproductive development, starch is reserved as a temporary source of sugar in anthers to provide energy for microspore development (Bhandari, 1984). Starch is considered a major source of the energy required for pollen development, pollination, and pollen tube growth (Pacini & Franchi, 1988; Clement et al., 1994). Generally, sterile pollen grains of cereals, such as wheat, have been shown to contain no starch granules (Saini & Aspinall, 1981; Saini et al., 1984). Due to this important role, the starch content in mature pollen has been used as a measure to assess pollen viability in rice (Gunawardena et al., 2003). Two main phases have been reported to be involved with changes to starch levels in anthers of *Lilium* (Clement et al., 1994; Castro & Clement, 2007). The first phase is from anther meiosis to the vacuolated microspore stage. Starch is mainly located in the anther walls and decreases gradually during this phase. This degradation of starch is assumed to provide energy for developing microspores (Clement et al., 1994). The second phase is from the late vacuolated stage to pollen maturation. This phase is characterised by intensive starch deposition in pollen
grains (Clement et al., 1994; Castro & Clement, 2007). Spatially, starch of wheat anthers has been shown to be deposited in anther walls during meiosis, disappearing and deposited again in the mature pollen (Saini et al., 1984). Likewise, deposition of starch in rice anthers was mainly found in the anther walls from the pollen mother cell to the later vacuolated stage (Li, 2005). Moreover, starch levels have been shown to first decline from meiosis to the microspore stage and then steadily increase from pollen mitosis until maturation in rice and wheat anthers (Dorion et al., 1996; Sheoran & Saini, 1996). These observations suggest that starch is perhaps stored mainly in the anther walls at early stages as a reserve material for pollen development, while its deposition in pollen grains at maturation is to provide energy for pollen germination and pollen tube growth in rice as well as other cereals.

A common feature of stress-induced sterile mature pollen is a lack of starch granules, in contrast to the number of starch granules observed in fertile pollen. Starch levels have been shown to be reduced in pollen from rice and wheat plants exposed to drought stress (Saini & Aspinall, 1981; Dorion et al., 1996; Sheoran & Saini, 1996; Lalonde et al., 1997a) and chilling stress (Gunawardena et al., 2003; Imin et al., 2004; Oliver et al., 2005). Dorion et al. (1996) measured the starch content in wheat anthers after water stress and also determined that there was approximately 30% less starch than the controls at anthesis. Similarly, Sheoran & Saini (1996) demonstrated that mature pollen from rice plants subjected to water stress at meiotic stages had low levels of starch, and the stressed anthers contained only about half the amount of starch as normal anthers at pollen maturity. Likewise, a 70% reduction in anther starch content at anthesis has been reported in rice plants chilled for four days at meiosis (Ito, 1978). As discussed above, the second starch deposition in the anther is mainly in mature pollen. These observations suggest that both chilling and drought stress have resulted in mature pollen with lower levels of starch content, indicating less viability.
Concomitant with the lack of starch deposition in mature pollen, stressed anthers from identical plants show an abnormal accumulation of sugars. Earlier studies clearly demonstrated that water deficit stress at the meiotic stage resulted in an accumulation of non-reducing sugars in wheat and rice anthers (Dorion et al., 1996; Sheoran & Saini, 1996). Dorion et al. (1996) reported that sucrose levels in wheat anthers from plants subjected to water deficit for four days at the meiotic stage, increased by 32% compared to the controls during the treatment period. Likewise, levels of non-reducing sugars in water stressed rice anthers were twice as great as the controls (Sheoran & Saini, 1996). This abnormal accumulation of sugar probably preludes disturbance of sugar metabolism in anthers under stress.

1.4.1.2. Activity of relevant metabolic enzymes

Paradoxically, while stressed mature pollen lacks starch to supply energy for further developmental processes, the sugar precursors involved in starch synthesis appear to increase under stress (Dorion et al., 1996; Sheoran & Saini, 1996). This phenomenon suggests that downstream pathways of sugar utilisation may have been disrupted. Division of carbon into starch reservoirs in plant tissues depends on photosynthate supply as well as its requirement (Preiss, 1982). The reduction of starch in water stressed pollen as discussed above could, therefore, be attributed either to insufficient assimilate during this intensive reserve period or to a direct deterioration or down regulation of enzymes in the pathway leading to starch synthesis (Dorion et al., 1996; Sheoran & Saini, 1996). Indeed, decreased activity of starch synthetic enzymes such as ADP-glucose pyrophosphorylase and soluble starch synthase might result in poor starch deposition in mature pollen of drought stressed rice (Sheoran & Saini, 1996). However, this was not the case for drought stressed wheat (Dorion et al., 1996).

Of the other enzymes involved in the downstream sugar utilisation pathways, invertase enzymes are the most interesting. Cleavage of sucrose in plants can be
catalysed by either invertase or sucrose synthase (Sturm & Tang, 1999). Sucrose synthase (EC 2.4.1.13) reversibly hydrolyses sucrose into glucose and fructose (Copeland, 1990). Coupled with sucrose synthases, invertases (β-D-fructofuranosidase) (EC. 3.2.1.26) play a crucial role in maintaining the sucrose concentration in sink organs (Sturm & Tang, 1999). Invertase catalyses irreversible cleavage of sucrose into glucose and fructose, an initial step in sucrose metabolism pathways (Sturm, 1999). Nevertheless, the domination of invertases over sucrose synthase during anther development has been illustrated in several reports (Dorion et al., 1996; Sheoran & Saini, 1996). In higher plants, invertases are classified into several types on the basis of their biochemical characteristics such as pH optima, solubility and cellular location (Tymowska-Lalanne & Kreis, 1998). Three main classes of invertase are generally accepted to exist in plants: the soluble acid invertase located in the vacuole sometimes called vacuolar invertase, the insoluble acid invertase localised in the cell wall, which has been known by different names such as cell wall or extracellular or apoplastic acid invertase, and the alkaline soluble invertase or neutral invertase present in the cytoplasm (Tymowska-Lalanne & Kreis, 1998). Environmental stresses have been shown to affect activity of invertases in anthers, which was associated with rising levels of sugars and pollen abortion in cereal plants (Dorion et al., 1996; Sheoran & Saini, 1996; Koonjul et al., 2005; Oliver et al., 2005). Decreased activity of acid invertases is predicted to block the supply of hexoses to developing pollen, which later results in male sterility (Koonjul et al., 2005; Oliver et al., 2005). The transcriptional regulation of acid invertase genes during pollen development has been first demonstrated using genetic engineering technology. Goetz et al. (2001) found that antisense inhibition of a cell wall invertase gene Nin88, which is specifically expressed in the anther tapetum and developing pollen, resulted in male sterility in tobacco. Likewise, the repression of expression of vacuolar and cell wall invertase genes in pollen has been claimed to be associated with
pollen abortion in drought stressed wheat (Koonjul et al., 2005). Similarly, down regulation of an anther specific cell wall invertase gene, OsINV4, in the tapetum has been shown to result in disruption of hexose supply to developing pollen and caused their death (Oliver et al., 2005). However, the causal relationship between sugars and their associated metabolic enzymes needs to be carefully assessed in a specific context as sugars can modulate gene expression and hence their translated protein (Koch, 1996; Roitsch, 1999; Smeekens, 2000).

1.4.1.3. Transportation of sugar to sink tissues

Whilst glucose is the dominant transport sugar in animals, sucrose is the major substrate of phloem sap in plants due to its non-reducing characteristics that help it avoid reactions with proteins and other compounds (Lambers et al., 1998a). Sucrose synthesised in the mesophyll cells of the source leaves is initially loaded into the sieve element (SE) - companion cell (CC) complex (phloem loading) either by plasmodesmatal connections (symplastically) or by active sucrose carriers (apoplastically) and then it is translocated to other tissues of the plants via long distance transport (phloem transport) (Lalonde et al., 2003). In sink tissues, transported sucrose can be unloaded either symplastically or apoplastically depending on the sink type. Several sink cells employ symplastic connections to the phloem to allow direct importation of sucrose and other assimilates (Oparka & Gates, 1981; Patrick & Offler, 1995; Imlau et al., 1999), whilst in others both symplastic and apoplastic pathways are active concomitantly (Clement & Audran, 1995; Wu et al., 2004). Ladone (2003) proposed a model of sugar unloading in the sink tissue, in which sucrose could be discharged from the SE - CC complex either symplastically or apoplastically (Figure 1.4). In the case of rice anthers, the pollen mother cell has been shown to be symplastically isolated after meiosis and the formation of the callose wall (Li, 2005; Mamun et al., 2005b). Therefore the exportation of sucrose into the locules and
developing microspores might require active transporters. The sucrose can be exported directly to the inner cell layers by active carriers or can be first hydrolysed by cell wall invertases into glucose and fructose which are then taken up via monosaccharide transporters (Lalonde et al., 1999; Sturm, 1999). Sugar carriers and cell wall invertases may work coordinately to regulate the supply of sugar for developing microspores. Recent publications have indicated that down regulation of a cell wall invertase gene, OsINV4, and a monosaccharide transporter, OsMST8, might lead to insufficient supplies of hexoses for anther development (Alfred, 2006; Mamun et al., 2006) resulting in suppression of young microspore development after chilling. In addition, abnormal starch accumulation in anther walls has been reported for chilling rice and drought stressed wheat (Saini et al., 1984; Lalonde et al., 1997a; Li, 2005; Mamun et al., 2006). However, Clement & Audran (1995) demonstrated that although excessive sugar would result in accumulation of starch in the anther walls of Lilium, the anthers continued to develop normally and produced viable pollen. This observation suggests that abnormal deposition of starch in the anther walls after stress is not necessarily relevant to pollen abortion. Moreover, this abnormal accumulation of starch in anther walls was assumed to result from the redirection of sugar flux in anthers by active sugar carriers (Oliver et al., 2005). These observations and assumptions need to be further validated under drought stress conditions.
Figure 1.4: Model of phloem unloading of assimilates in the sink cells during development.

Phloem unloading could proceed via either (A) the symplastic route or (B) apoplastic pathway. In some sinks, sucrose influx (red) has been demonstrated to occur by facilitated diffusion or by proton symport (orange transporter). For other sinks, sucrose could be first cleaved into hexoses (light blue molecules) by cell wall invertases. These hexoses are then subsequently taken up by sink cells via hexose transporters (blue/purple). An apoplastic barrier of cell wall layers (yellow diamond) hinders the back flow of nutrients. In both symplastic and apoplastic pathways, sugar may be accumulated into vacuoles for temporary or more long-term storage by specific transporters. Alternatively storage may occur in the form of starch in amyloplasts. All of these transporters are driven by the proton motive force generated by an H⁺-ATPase (green). cc, companion cell; mc, mesophyll cell; vp, vascular parenchyma; se, sieve element. Adapted from Lalonde et al. (2003).
1.4.2.  Oxidative stress and programmed cell death

1.4.2.1  Death terminologies

The Nomenclature Committee on Cell Death has defined the phenomenon of dead cells based on three molecular or morphological categories: 1) plasma membrane of the cell has lost integrity; 2) the cell including its nucleus has completely fragmented into apoptotic bodies; 3) cell fragments have been engulfed by a neighbouring cell (Kroemer et al., 2005). Based on these categories, cell death has been further classified into several types such as apoptosis, autophagy, necrosis, mitotic catastrophe, anoikis, excitotoxicity, wallerian degeneration, and cornification (Kroemer et al., 2005).

Of those, three main types of cell death are of particular interest. Apoptosis is a type of cell death that is characterised by specific cytological and morphological hallmarks including chromatin and nuclear condensation; endonuclease-mediated DNA fragmentation into nucleosomal units known as DNA laddering; nuclear blebbing and the formation of apoptotic bodies (Kerr et al., 1972; Kerr & Harmon, 1991; Cohen, 1993; Lawen, 2003). Apoptosis can be understood as a cellular self-destruction process under normal conditions so that the organism benefits from getting rid of unwanted or harmful cells (Zong & Thompson, 2006). Another type of cell death is autophagy (“self-eating”), a catabolic process involving self-degradation of cytoplasmic components within lysosomal vacuoles (Klionsky & Emr, 2000). Autophagy occurs without chromatin condensation and is characterised by a massive accumulation of double membrane autophagic vacuoles in the cytoplasm (Levine & Klionsky, 2004; Kondo et al., 2005). Necrosis is a passive form of cell death that results from acute cellular injury in which the cell rapidly becomes unable to maintain homeostasis, and undergoes early plasma membrane rupture and dilatation of cytoplasmic organelles (Kerr & Harmon, 1991; Denecker et al., 2001; Golstein & Kroemer, 2007). This type of accidental cell death is commonly considered to be a pathological form of cell death.
death only occurs when induced by external stress stimuli. There are few features to distinguish between apoptosis and necrosis. Apoptosis is an energy independent process while necrosis is often characterised by bioenergetic catastrophe (Zong & Thompson, 2006). The key events of necrosis are bioenergetic failure, loss of membrane integrity, often associated with an increasing production of reactive oxygen species (ROS) and a failure to generate ATP (Zong & Thompson, 2006).

Another death phenomenon regarded as programmed cell death (PCD) has sometimes been used as a synonym to apoptosis. PCD is used to imply that the death of the cells has been genetically programmed (Heemels, 2000; Varnier et al., 2005) and is therefore opposite to necrosis. Nevertheless, this synonymous use perhaps should be avoided as sometimes it is difficult to distinguish whether the dead cells underwent a programmed or necrotic death (Kroemer et al., 2005). Necrosis has been found not absolutely accidental and can be programmed (Galluzzi et al., 2007). However, van Doorn & Woltering (2005) illustrated that apoptosis might not exist in plants and that many other forms of cell death belonging to the autophagic type may be apparent during plant development. Mea et al. (2007) argued that it is sometimes difficult to differentiate either programmed or accidental cell death in many cases because the above mentioned definitions are only based on morphological observation in vitro without clear confirmation of biochemical mechanisms. For instance, adding H$_2$O$_2$ into cell culture has been reported to induce PCD in Arabidopsis (Tiwari et al., 2002); actually it should be considered necrosis. Moreover, Li et al. (2004) also claimed that pollen of cytoplasmic male sterile (CMS) rice underwent PCD during development, which might have been recognised as apoptosis due to the plant showing hallmarks of apoptosis such as TUNEL positive staining and DNA laddering. In addition, this process was genetically programmed, as there were no stress stimuli in this case. Zong
& Thompson (2006) concluded that stimulants can induce apoptosis at lower concentrations and necrosis at higher doses and hence necrosis can be a controlled form of cell death independent of apoptosis.

In this context, Van Breusegem & Dat (2006) proposed two terminologies to indicate some forms of cell death in plants. The first type, necrosis, is a passive form of cell death, indiscriminate and often followed by irreversible injury. The second type, PCD or active cell death, is a form of cell death involving a single or series of molecular and cellular orderly processes, which can be induced. Therefore, despite some controversy over terminologies, the term PCD hereafter will be used in a general sense to indicate the death of cells in this document, whereas a more detailed term will be used in some specific circumstances as appropriate.

1.4.2.2 Role of PCD during reproductive development

In vivo, PCD plays a critical role in the normal development of angiosperms, as well as in response to a variety of diseases, by controlling cell number or strategically eliminating infected or damaged cells in defence (Greenberg, 1996, 1997; Vaux & Korsmeyer, 1999). During reproduction, several tissues of plants such as the developing xylem, the anther stomium and the tapetum have been found to experience a PCD as part of their normal development (Greenberg, 1996; Beers, 1997; Pennell & Lamb, 1997; Wu & Cheung, 2000; Varnier et al., 2005).

Failure or improper function of PCD in anthers has been reported to result in abnormal pollen development and subsequent sterility (Sanders et al., 2000; Wu & Cheung, 2000). The degeneration of the tapetum in anthers is a typical example of PCD during reproductive development. In normal development, the self-degraded process of the tapetum is characterised by cytoplasm shrinkage and subsequent separation from the cell wall. As the tapetum undergoes PCD, tapetal cells produce components for the coat
of the mature pollen grains. This degeneration of the tapetal cells in anthers has been well known as a tightly controlled PCD, which must occur in an orderly manner (Bedinger, 1992; Papini et al., 1999). Failure to do so frequently results in pollen sterility (Ku et al., 2003; Li et al., 2004). Moreover, abiotic stresses such as heat (Chen et al., 1999) and oxidative stress (Slater et al., 1995; Dumont et al., 1999) are known to induce PCD in plants. It is therefore interesting to know if pollen sterility under drought stress associates with PCD in anthers.

1.4.2.3 Reactive oxygen species (ROS) and antioxidants

ROS is a terminology used to indicate some reduced forms of oxygen, which result from aerobic respiration and enzymatic activities, such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH') and nitric oxide (NO') (Smirnoff, 1993; Halliwell, 2006). The mitochondrial electron transport chain is the major source of ROS in plants (Moller, 2001). Meanwhile, by-products of enzymatic reactions such as those catalysed by glycolate oxidase in the peroxisomes during photorespiration and the production of O$_2^+$ on the outer surface of the plasma membrane by NADPH oxidase are also important sources of ROS (Moller et al., 2007). The formation of some ROS can be summarised by the following four consecutive reactions (reactions 1-4) (Smirnoff, 1993):

1. $\text{O}_2 + e^- \rightarrow \text{O}_2^-$
2. $2\text{O}_2^- + e^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2$
3. $\text{H}_2\text{O}_2 + e^- + \text{H}^+ \rightarrow \text{OH}^- + \text{H}_2\text{O}$
4. $\text{OH}^- + e^- + \text{H}^+ \rightarrow \text{H}_2\text{O}$
When the level of ROS is low, the antioxidant system of the plant can neutralise them and maintain the redox balance. However, under some adverse conditions this redox homeostasis is easily violated, potentially resulting in a significant ROS accumulation (Van Breusegem & Dat, 2006). Excessive production of this ROS in cells can lead to oxidative stress and potentially cause damage to various constituents such as proteins, lipids, DNA and other components of the cell (Beckman & Ames, 1997; Berlett & Stadtman, 1997; Halliwell & Whiteman, 2004).

Naturally, the plants cope with excessive accumulation of ROS by minimizing the level of ROS production by using a system of scavenging enzymes to neutralise the species (Halliwell, 2006). Table 1.1 lists the major enzymes of the scavenging system.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>EC number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
<td>1.15.1.1</td>
</tr>
<tr>
<td>Ascorbate peroxidase</td>
<td>APX</td>
<td>1.11.1.11</td>
</tr>
<tr>
<td>Monodehydroascorbate reductase</td>
<td>MDHAR</td>
<td>1.6.5.4</td>
</tr>
<tr>
<td>Dehydroascorbate reductase</td>
<td>DHAR</td>
<td>1.8.5.1</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>GR</td>
<td>1.6.4.2</td>
</tr>
<tr>
<td>Catalase</td>
<td>CAT</td>
<td>1.11.1.6</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>GPX</td>
<td>1.11.1.9</td>
</tr>
<tr>
<td>Guaiacol-type peroxidases</td>
<td>—</td>
<td>1.11.1.7</td>
</tr>
<tr>
<td>Glutathione S-transferases</td>
<td>GST</td>
<td>2.5.1.18</td>
</tr>
</tbody>
</table>

Table 1.1: List of major antioxidant enzymes (Noctor & Foyer, 1998)

The principal enzymatic reactions of ROS scavenging are presented below (reactions 5 – 12) (Apel & Hirt, 2004):
Within the cell, superoxide dismutases (SODs) are amongst the most important enzymes that catalyse the first scavenging reactions against ROS. Three distinctive types of SOD have been well characterised in plants namely: Cu/ZnSOD, MnSOD, and FeSOD (Alscher et al., 2002). MnSOD has shown to result in better tolerance to drought in engineered transgenic rice compared to the wild type (Wang et al., 2005).

The SODs catalyse the disproportionation of superoxide to hydrogen peroxide (reaction 5). Subsequently hydrogen peroxide produced from the above reaction can be broken down to water by some enzymes in different pathways. Hydrogen peroxide can be neutralised by catalase (CAT) to form water and molecular oxygen (reaction 6) (Willekens et al., 1995). The ascorbate –glutathione cycle is initiated by ascorbate peroxidase (APX) to convert hydrogen peroxide to water (reaction 7) following a series of reactions to regenerate the ascorbate substrate (reaction 8-10). Similarly to APX, glutathione peroxidase (GPX) can detoxify hydrogen peroxide to water using

\[
\begin{align*}
\text{O}_2^- + 2\text{H}^+ & \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 & \xrightarrow{\text{CAT}} \text{H}_2\text{O} + \frac{1}{2}\text{O}_2 \\
\text{H}_2\text{O}_2 + \text{Ascorbate} & \xrightarrow{\text{APX}} \text{H}_2\text{O} + \text{Monodehydroascorbate (MDA)} \\
\text{MDA} + \text{NAD(P)H} & \xrightarrow{\text{MDAR}} \text{Ascorbate} + \text{NAD(P)}^+ \\
\text{Dehydroascorbate} + \text{GSH}^+ & \xrightarrow{\text{DHAR}} \text{Ascorbate} + \text{GSSG}^{**} \\
\text{GSSG} + \text{NAD(P)H} & \xrightarrow{\text{GR}} \text{GSH} + \text{NAD(P)}^+ \\
\text{H}_2\text{O}_2 + \text{GSH} & \xrightarrow{\text{GPX}} \text{H}_2\text{O} + \frac{1}{2}\text{O}_2 \\
\text{GSSG} + \text{NAD(P)H} & \xrightarrow{\text{GR}} \text{GSH} + \text{NAD(P)}^+ 
\end{align*}
\]

*GSH glutathione; **GSSG oxidised glutathione.

* * * 

Within the cell, superoxide dismutases (SODs) are amongst the most important enzymes that catalyse the first scavenging reactions against ROS. Three distinctive types of SOD have been well characterised in plants namely: Cu/ZnSOD, MnSOD, and FeSOD (Alscher et al., 2002). MnSOD has shown to result in better tolerance to drought in engineered transgenic rice compared to the wild type (Wang et al., 2005). The SODs catalyse the disproportionation of superoxide to hydrogen peroxide (reaction 5). Subsequently hydrogen peroxide produced from the above reaction can be broken down to water by some enzymes in different pathways. Hydrogen peroxide can be neutralised by catalase (CAT) to form water and molecular oxygen (reaction 6) (Willekens et al., 1995). The ascorbate –glutathione cycle is initiated by ascorbate peroxidase (APX) to convert hydrogen peroxide to water (reaction 7) following a series of reactions to regenerate the ascorbate substrate (reaction 8-10). Similarly to APX, glutathione peroxidase (GPX) can detoxify hydrogen peroxide to water using
glutathione (GSH) directly as a reducing reagent (reaction 11). The GPX cycle is completed with a reaction to regenerate GSH from oxidised glutathione (GSSG) catalysed by glutathione reductase (GR) (reaction 12).

1.4.2.4 Relationship between oxidative stress and PCD

The association of ROS and PCD has been the object of several reviews (Lam, 2004; Van Breusegem & Dat, 2006; Zong & Thompson, 2006; Ryter et al., 2007; Scherz-Shouval & Elazar, 2007). The accumulation of ROS to induce PCD was first illustrated based on the correlation between increased ROS levels and cell death by Levine et al. (1994). Evidence of exogenous hydrogen peroxide application triggering PCD in cell suspension cultures of soybean (Glycine max L.) showed that increasing levels of hydrogen peroxide caused a corresponding level of cell death (Levine et al., 1994).

Foyer & Noctor (2005b) proposed that ROS could trigger PCD in compartments with low antioxidant buffering capacity or where ROS production is high. The crucial role of antioxidants in maintaining the redox balance has been demonstrated in transgenic tobacco plants. Plants with a low level of antioxidants to detoxify ROS have been shown to activate PCD in response to minor pathogen attacks which did not trigger the activation of PCD in control plants (Mittler et al., 1999). Likewise, high levels of hydrogen peroxide accumulated under photorespiratory conditions have been found to cause an active cell death program which also exhibits several PCD hallmarks in transgenic tobacco leaves with deficient catalase activity (Dat et al., 2003). Li et al. (2004) concluded that excessive accumulation of ROS causes a chronic oxidative stress which may trigger the tissue-specific PCD in anthers of CMS rice.

In summary, there is accumulating evidence of PCD in plants triggered by ROS. ROS-dependent PCD usually takes place when the level of ROS exceeds the scavenging
capacity of plants leading to oxidative stress. This oxidative stress results in necrotic cell death in extreme cases while lower levels of ROS will switch the direct execution of the cell to PCD (Van Breusegem & Dat, 2006).

1.4.2.5 Oxidative stress and pollen sterility

Although a considerable amount of information is available on water deficit induced oxidative stress and reduction of antioxidants (Bowler et al., 1992; Baisak et al., 1994; Moran et al., 1994; Sairam & Srivastava, 2001; Pan et al., 2006), there is little work regarding the sequential effects of the oxidative stress on microsporegenesis in plants. To date only a few reports demonstrate the involvement of oxidative stress on pollen sterility in CMS rice and cotton (Li et al., 2004; Jiang et al., 2007; Wan et al., 2007). In these plants, increased levels of ROS coupled with reduced antioxidant capability have resulted in oxidative damage to developing pollen. Moreover, oxidative stress has hampered mitochondrial activity leading to a depletion of the ATP pool in the anthers. Therefore a high percentage of sterile pollen in CMS plants has been considered as the result of oxidative damage and energy shortage in comparison to the controls (Wan et al., 2007). Results from studies by Selote & Khanna-Chopra (2004) indicate that an inefficient antioxidant system in panicles may be the cause of drought induced spikelet sterility in rice. However, direct sequential impacts of drought stress on pollen formation have not been explored.

1.5. Aims of this project

Abiotic stresses in general, or water deficit-induced male sterility in rice in particular, is not a new phenomenon. Although a vast amount of literature has discussed this symptom from different aspects, no conclusive evidence for the causes of pollen sterility under drought conditions has been achieved so far. There are still large gaps in our understanding of some of the fundamental processes involved in pollen formation
and its response to water deficit. The overall objectives of this project are, therefore, to gain insight of the possible mechanisms or signalling processes that lead to pollen sterility in rice under water shortage conditions. The concrete aims of the project are to:

1. Determine the critical level where a short transitory period of water deficit effectively influences male fertility using a reliable and easily reproducible drought-induction method. Experiments will be conducted to test if mild water deficit levels do reduce pollen viability and grain set. Male fertility will be tested immediately after exposure to drought stress to detect any changes in young microspore viability.

2. Examine possible molecular mechanisms of pollen sterility with emphasis on carbohydrate metabolism in rice anthers under drought. These experiments will be carried out in order to test the hypothesis that disturbances in carbohydrate metabolism in rice anthers actually repress pollen development and subsequently affect formation of grain set.

3. Investigate the association of pollen sterility with PCD and oxidative stress in rice anthers under drought. On the basis of accumulating evidence of involvement of PCD and oxidative stress in plants under environmental stresses, experiments will be conducted to test hypotheses that oxidative conditions occur during drought and that drought induces a PCD in rice anthers. In addition, it will be determined if these biological events are physiologically relevant to pollen sterility.
CHAPTER 2: MATERIALS AND GENERAL METHODS

2.1. Plant materials

*Oryza sativa* L. *japonica* cv R31, also known as Lijiangheigu, was used as the plant material for all experiments. R31 is a short growing duration variety with approximately 110-115 days from sowing to maturity. It is well adapted to growth in a hydroponic system and has a strong tillering capacity (Li, 2005). Fertility, determined by seed set and pollen viability, is stable (Li, 2005). Rice plants were grown in a hydroponic system developed by Dr Xiaochun Zhao, Plant Breeding Institute, The University of Sydney, Australia and a soil system in glasshouse at 24°C day/night temperature. Seed of R31 was kindly provided by Dr Xiaochun Zhao.

2.2.1. Soil system for initial trials

Rice seeds were soaked in water for 48 hours at room temperature and then transferred to a tray spread with wet blotting paper. The trays were then covered with lids to avoid water evaporation and incubated at 32°C for another 48 hours. Germinated seeds were sown in nursery trainers containing soil collected from Camden, New South Wales, Australia. The trainer is made from plastic and includes two foldable flaps that form four separate cavities, when fully assembled. Figure 2.1A illustrates rice plants grown in nursery trainers. The trainers were supported by a steel frame and were placed into 30 litre plastic boxes filled with tap water. The use of trainers improves handling of dialysis tubing as described in section 2.3.3. To protect the dialysis tubing from being punctured by developing rice roots, a hand-made sock was applied. The socks were made by sewing two pieces of ordinary cotton fabric (25 cm length and 8 cm width) to form a sock which can then be filled with soil for growth of plants. The sock filled with the soil and plants was then placed in a single cavity of the nursery plastic trainer. Each
trainer could hold four plants. The water level in the tray was kept approximately 3-5 cm above the trainer's surface. Nutrients including urea (10 g); KH₂PO₄ (10 g); KCl (5 g); Ca(NO₃)₂ (2 g) and chelated micronutrient mixture (1.5 g) (BMX, Librel, Ciba Speciality Chemicals, UK) were applied to every trainer before sowing (Figure 2.1).

### 2.2.2. Hydroponic system

The hydroponic system used in this study was assembled from several plastic channels. Each channel contained holes cut to the size of the plastic pots. The channels were interconnected by plastic irrigation pipes with a barrel storing the hydroponic medium and an immersed pump (Pond Master, PM-1000) that continuously circulated nutrients from the storage barrel to the channels. A floating cistern valve connected to the main water supply was used to maintain the level of water in the barrel (Figure 2.2).

About 10 geminated seeds were sown every two weeks in a support medium consisting of coarse sand, perlite and pine bark (1:1:10) in 80 mm Jiffy pots (Yates, Australia) to avoid leakage of this mixture into the hydroponic system. To prevent degradation, each Jiffy pot was placed inside a 100 mm plastic pot. All pots were secured to the plastic channels through the holes. The culture medium contained (NH₄)₂SO₄ (0.15 g), KNO₃ (0.04 g), KH₂PO₄ (0.3 g), NH₄NO₃ (0.3 g), Mg(NO₃)₂ (0.15 g), Ca(NO₃)₂ (0.16 g) and chelated micronutrient mixture (0.15 g) (BMX, Librel, Ciba Speciality Chemicals, UK) per litre of tap water.

The nutrient concentration and pH were controlled using a pH/electrical conductivity meter (Combo HI 98130, Hanna Instruments Inc.). The pH of the solution was checked every two or three days and kept between 5.0 - 6.0. The pH value was adjusted using either 1 M KOH or 1 M HNO₃. The level of total dissolved solids (TDSs) was kept between 0.7-1.0 parts per thousand (ppt) and adjusted with either the stock nutrient solution or fresh water. About two months after sowing, plants were pruned and only 2-3 main culms were allowed to continue growing.
Figure 2.1: Rice grown using a soil system. A- Plants with roots covered by sock and dialysis membrane supported by a plastic trainer. The dialysis membrane and sock was only used when the plants were exposed to water stress using PEG. B- Plants held upright and grown in plastic boxes.

Figure 2.2: The hydroponic system.
2.3. Water stress system

2.3.1. Water stress inducer

Polyethylene glycol (PEG) molecular weight 20,000, pharmaceutical grade (Merck, Germany) was used as a water stress inducer. The osmotic potential of the PEG solution was measured using a vapor pressure osmometer (WESCO 5500). The osmometer was first stabilised using commercial standard solutions of NaCl 100, 290 and 1,000 mOsmoles (WESCO Inc). Measurements were made after calibration with NaCl solutions (Lang, 1967). The osmotic potential of the culture medium was controlled by either adding PEG or fresh water to maintain the water potential of the solution at the desired level. The PEG solution can be reused several times without reduction in its osmotic effects (Plaut & Federman, 1985).

2.3.2. Plants grown in hydroponic system

Water stress was imposed by moving plants from the hydroponic system and placing them directly into a 2 L pot containing a PEG solution of the designated osmotic potential (Figure 2.3A). It is critical to avoid physical damage to plant roots at this step. Plants were kept upright by a home-made plastic supporting holder. To minimize PEG precipitation and to provide oxygen for plant growth, the PEG solution was slightly aerated by an air-stone connected to an air-pump (Figure 2.3B). Water lost through evaporation was replenished several times during the days of stress treatment. A piece of plastic cling wrap was also used to cover plants around the edge of the pot to minimize the rate of evaporation.
2.3.3. Plants grown in soil system

Water stress for the plants growing in soil was conducted similarly to the hydroponic stress as described above with some additional steps. About three months after sowing, plants growing in soil were carefully taken out of the supporting trainers and placed into a MEMBRA-CEL dialysis tubing MC-97 (Viskase Inc, USA). The tubing membrane size was 104 mm wide (dry flat) with a molecular weight cut off of 12,000-16,000 and a pore size about 25 Å. The aim of using the tubing membrane was to help plant roots avoid direct contact with PEG. Therefore, care was taken to make sure that the whole plant root system was covered with the membrane. Plants with roots wrapped in dialysis tubing were moved back into trainers and continued to grow until the desired stages. Water stress was induced by placing plants into 25 L plastic storage boxes containing the PEG solution of designated potential.

Water stress was released by simply removing the plants from the PEG solutions and returning them to the normal growing conditions. It was essential to ensure that all
PEG residues were completely washed off the root system. Stressed plants were carefully washed by hand under running water to remove PEG from the roots. These plants were then soaked in a new storage box containing nutrient solution for four hours; the solution was changed every two hours. After soaking they were returned to the hydroponic system to continue normal growth.

2.4. Anther sampling

Rice anthers at three developmental stages were routinely harvested; the tetrad stage, the microspore stage and the vacuolated microspore stage. All stages were distinguished based on definitions by Wada et al. (1990; 1992). Anthers from parallel control plants were sampled at identical stages of development. The stages of development were confirmed by microscopic inspection of representative anthers. The spikelets of the top three primary branches were dissected with a scalpel and fine forceps using a dissection microscope to remove the enclosed anthers. In order to take more synchronous anther samples, only the main tillers of the rice plants were used for the experiments. Anthers within the same length range were collected and immediately frozen in liquid nitrogen and stored at -80°C until use. Anther samples belonging to the same anther developmental stages were pooled to obtain sufficient materials for each developmental stage (Figure 2.4).
Figure 2.4: Light microscopic observation of three stages of anther development: ① Pollen mother cell stage; ② Tetrad stage; ③ Microspore stage; ④ & ⑤ Vacuolated microspore. pmc pollen mother cell. te tetrad. mi microspore. vmi vacuolated microspore. Scale bars: 20 µm (①②③④) and 10 µm (⑤).
2.5. RNA extractions

Total RNA, from frozen anthers of desired stages (tetrad, microspore and vacuolated microspore) of both treated and control plants, was extracted using a RNeasy Plant Mini kit (Qiagen, Australia) following the manufacturer’s protocol. Briefly, anthers were ground to a fine powder in liquid nitrogen. Approximately 10-15 mg of this anther powder was transferred to a microcentrifuge tube containing 450 µL of RLC buffer with 4.5 µL β-mercaptoethanol and vortexed vigorously. The homogenised mixture was then transferred directly to a Qiagen shredder column placed in a 2 mL collection tube and centrifuged for 2 minutes at 13,000 rpm in a microcentrifuge. The clear flow-through lysate was then transferred to a new collection tube and mixed with 225 µL of 96% ethanol. This mixture was transferred to a RNeasy column in a 2 mL centrifuge tube and spun for 15 seconds at 11,000 rpm. The RNeasy column was subsequently washed with 350 µL of RW1 buffer and centrifuged for 15 seconds at 11,000 rpm. Potential contaminant genomic DNA was digested on-column with RNase-free DNase set (Qiagen, Australia). A DNase I mix containing 10 µL DNase I stock solution and 70 µL buffer RDD was pipetted directly onto the RNeasy spin column membrane and incubated for 15 minutes at room temperature. After incubation, the spin column was washed with 350 µL of RW1 buffer and centrifuged for 15 seconds at 11,000 rpm. The spin column was washed twice with 500 µL of 96% ethanol-added RPE buffer by centrifugation. The total RNA was eluted from the spin column with 50 µL RNase free water. Total RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (Biosciences, Australia). Total RNA was diluted with RNase free PCR grade water to desired concentrations for RT-PCR as required. The RNA was stored at -80°C.
2.6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

2.6.1. Primer design

Gene specific primers were either sought from the literature or designed based on sequence information from the gene bank (http://www.ncbi.nlm.nih.gov). Primers were designed to cross exon-exon junctions of the genomic DNA to avoid possible amplification of contaminant DNA during the RT-PCR process (Bustin, 2000).

2.6.2. cDNA amplification and sequencing analysis

Initial conventional RT-PCR tests were applied to all genes under investigation. Transcripts of the specific genes were reverse transcribed into corresponding cDNA using Superscript™ III One-Step RT-PCR Platinum® Taq (Invitrogen, Australia). Briefly, a 20 µL reaction mixture consisted of 10 µL of 2X reaction mix, 0.4 µL SuperScript™ III RT/platinum Taq enzyme, 0.4 µL of 10 µM gene specific primers, RNase free PCR grade water, and 1 µL total RNA. RT-PCR was performed on a Mastercycler® Gradient (Eppendorf, Australia). The cycling profile of the standard RT-PCR was initiated with a reverse transcription step at 50°C for 40 minutes, initial denaturation at 94°C for 2 minutes followed by 30-35 cycles of 94°C for 20 seconds denaturing, 55-65°C for 30 seconds annealing, 62-72°C for 25 seconds elongation depending on gene target. Amplified products were electrophoresed in a 1% agarose gel for 1.5 hours at 90V. A commercial standard 1 Kb plus DNA marker (Invitrogen, Australia) was run in parallel. The gel was stained in a water bath containing ethidium bromide (0.5 µg/mL) for 20 minutes. The DNA bands on the gel were then visualised under a UV light. An image of the gel was captured using Gel Doc (Bio-Rad, Australia) digital imaging system. Complete removal of possible contaminating genomic DNA in the RNA extract was also confirmed by replicating the RT-PCR in another tube containing gene specific primers but Platinum® Taq DNA Polymerase (Invitrogen,
Australia) was substituted for SuperScript™ III RT/platinum Taq enzyme. cDNA was purified using a QIAquick PCR Purification Kit (QIAGEN, Australia) according to the manufacturer's instructions. In brief, 5 volumes of supplied buffer PB were pre-mixed with 1 volume of the PCR sample and applied to the binding column with subsequent centrifugation at 13,000 rpm for 1 minute. After removal of the flow through, the spin column was washed with 0.75 mL of buffer PE by centrifugation for 1 minute at 13,000 rpm. An additional 1 minute centrifugation at 13,000 rpm was applied to completely remove any ethanol from the PE buffer. cDNA was eluted in 50 µL EB buffer (10 mM Tris-Cl, pH 8.5).

The purified cDNA sample was sent to Sydney University Prince Alfred Molecular Analysis Centre (SUPAMAC) for sequencing. Procedures of sample preparation for sequencing analysis using Applied Biosystems DNA Analyser (ABI 3730) were followed (http://www.supamac.com/content/index.php). Sequencing data was analysed with BioEdit and Chromas software. This sequence data was aligned against the gene bank using Basic Local Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) to confirm the identity of cloned cDNAs with target sequences.

2.6.3. House keeping gene for standard control and qRT-PCR reaction

Rice OsTuba1 gene (Genebank ID AF182523) was chosen as an internal standard control for the qRT-PCR system in this study. OsTuba1 gene specific primers were as follows: forward primer 5’-GGCTTGTGTCTCAGGTTATCTCATC and reverse primer 5’-CATGGAGGATGGCTCGAAGG (Oliver et al., 2005). OsTuba1 gene has been reported as being unregulated by any known environmental stimulus (Yamaguchi et al., 2004; Oliver et al., 2005) and was recently used as a standard in real-time qRT-PCR for rice anthers (Kong et al., 2007).
The real-time qRT-PCR reaction was carried out using Superscript™ III Platinum® SYBR® Green One-Step qRT-PCR (Invitrogen, Australia). Components and reaction conditions are similar to standard RT-PCR with some minor modifications. In short, a 20 µL reaction mixture containing 1x SYBR® Green reaction mix, 0.4 µL SuperScript™ III RT/platinum Taq enzyme, 200 nM gene specific primers, PCR grade water, and 40 ng total RNA was pipetted into a real time PCR tube (Integrated Sciences, Australia). Detection and quantification of the RNA transcripts of targeted genes in water stressed and control samples in real time was performed on the Rotor-Gene 2000 (Corbett, Australia). A 2-fold dilution series of total RNA with RNase free PCR grade water (160, 80, 40, 20 and 10 ng) was used to develop a standard curve for each target gene.

Duplicate qRT-PCR reactions for OsTuba1 gene generally consisted of an initial reverse transcription step at 50°C for 20 minutes, initial denaturation at 94°C for 5 minutes following 35-40 cycles of 94°C for 20 seconds denaturing, 65°C for 30 seconds annealing, 68°C for 25 seconds elongation. Specificity of amplified products and primer dimer formation were monitored by an additional step at 79°C for 15 seconds during cycles, and a melt curve at the end of the RT-PCR reaction. Fluorescence signals were also acquired at the last step during each cycle (Figure 2-5). qRT-PCR was independently run on two different RNA extractions with double replicates for each sample.

2.6.4. Statistical analysis of raw data

Raw signals from real-time amplification reactions were analysed using Rotor-Gene 2000 software version 5.0. Cycle crossing point (CP), sometimes called cycle threshold (Ct), is the point at which the raw fluorescent signals of a real time PCR reaction becomes statistically significantly higher than the background level during the
exponential phase of the amplification (Bustin, 2000). The CP value is reported for every sample and has been illustrated to be proportional to the number of copies of mRNA in the sample (Heid et al., 1996). Therefore the CP value can be used to indicate the absolute number of mRNA copies in the sample by developing a standard curve (Bustin, 2000). The CP value of individual reactions in this study was calculated based on a standard curve which was generated by plotting the logarithm of input amount of total RNA versus cycle crossing point values (Figure 2.6). The real-time PCR efficiency (E) was calculated by Rotor-Gene 2000 software which is based on the slope of the standard curve. Relative expression ratios of treated and controlled samples at different stages were calculated and compared by individual reaction CPs and reaction efficiency according to the following formula (Pfaffl, 2001):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(\text{Control-Sample})}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(\text{Control-Sample})}}$$

where: $E_{\text{target}}$ is the real-time PCR efficiency of the target gene transcript; $E_{\text{ref}}$ is the real-time PCR efficiency of a reference gene transcript; $\Delta \text{CP}_{\text{target}}$ is the CP deviation of control – sample of the target gene transcript; $\Delta \text{CP}_{\text{ref}}$ is CP deviation of control – sample of reference gene transcript.

Statistical analysis of real time RT-PCR results, the randomisation test, was performed by using a Relative Expression Software Tool (REST©) (Pfaffl et al., 2002). The expression of the targeted gene in the control samples at the tetrad stage was normalised at 1 and used to compare the relative expression of the control and treated samples from other stages.
**Figure 2.5:** Normalised fluorescence of OsTuba1 at different anther developmental stages. Channels indicate fluorescence signals of different individual samples. The red horizontal bar indicates threshold cycle of PCR reactions.

**Figure 2.6:** Standard curve of OsTuba1 gene generated by Rotor-Gene 2000 software using two fold dilution of total RNA. $R^2$ is the correlation coefficient of the curve (0.99125); $M$ is the slope (-2.975); $B$ is the intercept (19.889); $E$ is the PCR reaction efficiency (1.17).
2.7. Sample preparation for immunological localization

Florets of desired stages of anther development were selected for this experiment. After removal of the tips, the florets were immediately fixed in 1X phosphate buffered saline (PBS) solution (pH 7.2) containing 4% (v/v) (para) formaldehyde (ProSciTech, Australia) and 2.5 % (w/v) sucrose, in a vacuum desiccator at room temperature overnight (~14 hours). The samples were dehydrated through an ethanol series of 30, 50, 70, 95 and 100% on the following day. The samples were then pre-infiltrated in xylene for 2 hours and manually embedded in Paraplast (Sigma-Aldrich, Australia) at 60°C. The blocks were microtomed into 8 μm sections for further procedures.

The structure of the embedded section was confirmed by light microscopy with toluidine blue O staining as follows: 8 μm sections were dewaxed twice in fresh xylene for 10 minutes each time, and rehydrated in a series of ethanol washes (100%, 95%, and 70%), once for 5 minutes each series, and washed in distilled water twice for 2 minutes each. Dewaxed sections were then incubated in an aqueous solution containing 0.05% (w/v) toluidine blue O in 0.1 M phosphate buffer at pH 6.8 for 5 minutes. The sections were then washed twice with distilled water for one minute each, air-dried and observed immediately under the light microscope. Figure 2.7 represents the detailed structure of a paraffin embedded transverse section of an anther.
Figure 2.7: Paraffin embedded cross section of a normal anther stained with toluidine blue O (microspore stage).

A. Transverse section of an anther

B. Details of a single lobe

c_t connective tissue; e_p epidermis; e_n endothecium; m_l middle layer; t apetum; l_o locule; v_b vascular bundle; m_i microspore. Scale bars: 20 µm.
CHAPTER 3: EFFECTS OF WATER DEFICIT ON MALE FERTILITY IN RICE

3.1. Introduction

Water deficit has been known to impair the growth and development of rice plants (Oryza sativa. L) with the nature of the damage strongly dependent on the level and duration of the stress at the time of exposure. Water stress occurring at the vegetative stage mainly reduces tiller number and appears less detrimental to grain yield (De Datta et al., 1975; Lilley & Fukai, 1994). However, the consequential damage of water deficit is particularly serious if the stress occurs during the reproductive phase (Steponkus et al., 1980; O'Toole & Moya, 1981). Effects of drought stress during anther development have been well characterised for wheat (Saini & Aspinall, 1981, 1982; Saini et al., 1984; Dorion et al., 1996; Lalonde et al., 1997a; 1997b; Koonjul et al., 2005) and to a much lesser extent for rice. The major effect of water deficit at meiosis is the decline in the number of engorged pollen that leads to poor, or failure of, pollination at flowering (Namuco & O'Toole, 1986; Sheoran & Saini, 1996). Therefore, pollen viability has been employed as a indicator of fertility for cereal plants exposed to abiotic stresses (Saini & Aspinall, 1981; Heenan, 1984; Satake, 1991; Gunawardena et al., 2003; Oliver et al., 2005).

Grain yield reductions ranging from 20% to 70% of the control have been observed in rice under water deficit treatment during the reproductive stage (Lilley & Fukai, 1994). Sheoran and Saini (1996) reported a serious decline of grain set from 92% to 22% by withholding water for 4 days during the meiotic stage of pollen mother cells. This decrease of grain set corresponds to the reduction of leaf water potential from -1.2 MPa to -2.3 MPa (Figure 3.1). However, those experiments have not quantitatively assessed the immediate effects of drought on male fertility at the time of exposure. The
direct relationship between grain set and pollen viability which attributes to the success of pollination has not been validated.

**Figure 3.1:** Changes of leaf water potential in rice plants withheld water for four days during reproductive development (Reproduced from Sheoran & Saini, 1996).

O'Toole & Cruz (1980) reported that, in practice, the leaf water potential of the above mentioned experiment can only be observed in fully rolled leaves of some cultivars and thus is considered too low. The sharp changes of leaf water potential observed in this experiment indicate that plant water status was uncontrolled and therefore it might be difficult to reproduce in later trials. On the other hand, the development of spikelets within the same panicle of cereals like wheat and rice is not simultaneous; the top spikelets are generally senescent and ripen earlier than the lower ones. Consequently, the top spikelets appear to suffer less detrimental effects than the lower ones do. Due to the difficulties of regulating drought experimentally (Graham-Bryce, 1967; Krizek, 1985), a reliable and easily reproducible method of inducing water stress in this context is required.

This experiment was therefore designed to determine the critical level of drought stress that starts affecting fertility of rice plants during anther meiosis using a reliable
and experimentally reproducible stressing method. The association between leaf water potential ($\Psi_{\text{leaf}}$), pollen viability and grain set will be presented and discussed. Moreover, results about the immediate effects of drought on young microspore fertility and its causal relationship with grain set will also be discussed.

3.2. Materials and methods

3.2.1. Plant materials and water stress treatment

The rice cultivar used for this experiment was as described in Chapter 2 (Section 2.1). Tentative experiments were first conducted with plants grown in soil (Section 2.2.1 & 2.3.3). Due to problems with the semi-permeable membrane, which will be discussed below, plants for all other experiments were grown hydroponically and stressed as described in Section 2.2.2 & 2.3.2, Chapter 2, unless otherwise stated. Three levels of water stress, -0.25 MPa, -0.5 MPa and -0.75 MPa were applied.

3.2.2. Auricle distance scale and timing of stress

In order to stress plants at a designated, specific stage, an auricle distance measurement method was applied (Matsushima, 1957; Satake & Hayase, 1970; Heenan, 1984). The auricle distance (AD) is the distance between the auricles of the flag leaf and the penultimate leaf (Figure 3.2). By measuring the auricle distance, the developmental stages of rice anthers can be estimated. If the auricle of the flag leaf is inside the penultimate leaf, the AD value is negative. By contrast, if the auricle of the flag leaf is outside the penultimate leaf, the AD value is positive. Figure 3.3 presents a relative empirical scale of AD for R31 cultivar, and water stress treatment scheme.
**Figure 3.2:** Auricle distance of rice plants

(Adapted from [http://www.ikisan.com/links/ap_ricemorp.shtml](http://www.ikisan.com/links/ap_ricemorp.shtml))

**Figure 3.3:** Panel (A) Auricle distance scale, and corresponding anther developmental stage. Stages in the rectangles indicate the time for anther sampling. Panel (B) Relative stress schemes for seed set measurement of cultivar R31. Broken arrows indicate the period at which the plant was experiencing maximum stress. PMC = pollen mother cell.
3.2.3. Plant water status

The leaf water potential from control and treated plants was measured using the pressure bomb technique as described by Scholander et al. (1965). Leaf measurements were conducted at predawn (5.00 - 6.00 am) commencing at day zero (exposure to water stress) and throughout the duration of the experiment (six days). Second to the youngest fully expanded leaves were cut about 2.0 cm below the leaf collar. These leaves were kept in a sealed polyethylene bag to protect from vapour pressure loss. The measuring leaf was placed in a pressure chamber in such a way that the cut portion of the surface was just protruding into the atmosphere through the seal on the top of the chamber. The pressure to the leaf blade was adjusted slowly until the meniscus just returned to the cut surface. This pressure was taken as the water potential of the leaves.

3.2.4. Gas exchange analysis

Net photosynthesis, transpiration rate, and stomatal conductance of the second fully expanded leaf from the top were measured using a portable infrared gas analyzer (LI-6400, Li-COR Inc.). The quantum flux density at the leaf surface, flow rate in the chamber, and leaf temperature were maintained at 1,000 µmol m\(^{-2}\) s\(^{-1}\), 500 µmol s\(^{-1}\) and 25°C, respectively. Leaf gas exchange measurements were conducted at midday on day zero (exposure to water stress) and continued throughout the duration of the experiment (six days).

3.2.5. Grain set measurement

Water deficit was imposed at several times during the development of anthers from the PMC to the vacuolated microspore stage, estimated by using the AD scale (Figure 3.3). Because florets from the same panicle do not develop synchronously, a three consecutive day water deficit treatment at either -0.25, -0.5 or -0.75 MPa was used to stress a maximal number of spikelets at the desired sensitive stage. Appropriate
untreated plants were conducted at the same time. After the stress treatment, the treated plant roots were washed carefully to remove all residues of PEG and returned to the glasshouse hydroponics system and allowed to continue development until mature seed was formed. Grain set, expressed as the number of grains produced as a percentage of fully developed florets, both in control and water stress treatments, was recorded when grains were mature.

3.2.6. Mature pollen viability

Starch pollen was stained with KI/I$_2$ (0.5 g of I$_2$ and 2 g of KI in 100 mL of H$_2$O) according to the method of Gunawardena et al. (2003) with slight modifications. Five florets were sampled from the uppermost primary branches per panicle and four panicles from different plants were used for pollen viability. Pollen contained in the anthers of each floret was released onto a slide in a drop of KI/I$_2$. Slides were then incubated at room temperature for one minute and covered with cover slips. Observation and photography was conducted using a light microscope with a digital camera attached to a computer. Ten view areas were photographed for pollen viability counting of each sample. Viable and unviable pollen were immediately manually counted by using Adobe Photoshop software.

3.2.7. Young microspore viability

Young microspores were dually stained with a mixture of fluorescein diacetate (FDA) and propidium iodide (PI) (Li, 2005; Oliver et al., 2005). Anthers from a few representative plants were selected for this experiment. Anthers were dissected from the panicle and pressed onto a glass slide by a scalpel to release young microspores. One drop of anther culture medium containing 2 µg/mL FDA and 10 µg/mL PI was applied directly to the newly released microspores, covered and incubated for 5 minutes in the dark. The recipe for the anther culture medium was adapted from Li (2005) and
consisted of KCl (1,500 mg/L), KH$_2$PO$_4$ (500 mg/L), MgSO$_4$·7H$_2$O (180 mg/L), EDTA (32 mg/L), MnSO$_4$·H$_2$O (17 mg/L), KI 0.8 (mg/L), ZnSO$_4$·7H$_2$O (8.6 mg/L), Ca(NO$_3$)$_2$ (300 mg/L) and sucrose (80 g/L) (pH 6.5). Observation and photography was analyzed using a fluorescence microscope (Olympus BX51, Japan) and ten view areas were captured for further analysis. Viable and unviable young microspores were immediately manually counted as described for mature pollen staining.

3.2.8. Statistical analysis

The results are reported as means ± standard error (SE). The significance of results was determined by analysis of variance (ANOVA) using Genstat statistical software (version 8.0).

3.3. Results

3.3.1. Influence of PEG on root morphology

Figure 3.4 compares the morphology of rice roots before (A) and after (B) three days exposure to osmotic stress induced by PEG at -0.5MPa. The images illustrate that the water deficit treatment did not inhibit development of the roots; rather it appeared to stimulate the elongation of the roots after stress. The roots appeared healthy and no symptoms of root necrosis or toxicity were observed (Figure 3.4B). Roots of the non-treated control showed less elongated after three days than the treated one (Figure 3.4C & D).
Figure 3.4: Effect of PEG on root morphology of rice plants after three days exposure to water deficit stress at -0.5MPa. A. Roots before PEG exposure. B. Roots after PEG exposure. C. Roots of control plants. D. Roots of control plants after three days.

3.3.2. Influence of water deficit on leaf water status

Rice plants subjected to three different levels of water deficit stress; -0.25 MPa, -0.5 MPa and -0.75 MPa showed a significant and continuous decline of predawn leaf water potential ($\Psi_{leaf}$) compared with the controls at the same period (P<0.01; Figure 3.5). Greater reductions of $\Psi_{leaf}$ were observed in plants exposed to higher levels of osmotic stress e.g. -0.5 MPa and -0.75 MPa (Figure 3.5), while, osmotic stress at -0.25 MPa only reduced $\Psi_{leaf}$ slightly and caused mild leaf rolling. Two days after the application of stress, the $\Psi_{leaf}$ stayed constant for each treatment throughout the duration of the stress (Figure 3.5). Once the water deficit stress was removed at day three, the plant leaves rehydrated to the normal $\Psi_{leaf}$ after one day (Figure 3.5). Complete
recovery of the $\Psi_{\text{leaf}}$ within one day after the removal of stress indicates that the stress caused by PEG in this instance is reversible.

![Graph showing leaf water potential over days with different treatments](image)

**Figure 3.5:** Pre-dawn leaf water potential ($\Psi_{\text{leaf}}$) of rice plants exposed to different water deficit stress treatments. Water stress treatments were initiated at day zero and removed at day three. Error bars represent standard error (SE). Data points labelled with the same letter are not significantly different ($P<0.05$) as determined by ANOVA (n=10). WD = water deficit. ns not significant.

### 3.3.3. Changes in leaf gas exchange parameters under water deficit stress

Different osmotic levels in the growing media induced by PEG significantly inhibited gas exchange of leaves in all treatments. Similar to the $\Psi_{\text{leaf}}$, water deficit caused an instant decline in leaf net photosynthesis ($A$) (Figure 3.6), transpiration rate ($E$) (Figure 3.7) and stomatal conductance ($g_s$) (Figure 3.8) after one day of exposure. When water deficit stress was relieved $A$, $E$ and $g_s$ returned completely to the initial level within two days (Figures 3.6, 3.7 & 3.8). Interestingly, the osmotic effect of the growing media appeared to impact on the activities of leaf gas exchange more rapidly than it did the $\Psi_{\text{leaf}}$ (Figure 3.4). While it took only one day to reach a constant level of leaf gas exchange, the water deficit required two days to completely reduce the $\Psi_{\text{leaf}}$ to a constant level. The level of $A$, $E$ and $g_s$ was constant during the stress period, and the
lower osmotic potentials of the growing media caused a greater reduction in A, E and g,
(Figures 3.6, 3.7 & 3.8).

Figure 3.6: Leaf net photosynthesis (A) of rice plants exposed to different water stress
treatment. Water stress treatments were imposed at day zero and removed at day three.
Data are the means of ten replicates with standard errors (SE) shown by vertical bars.
WD = water deficit.

Figure 3.7: Leaf transpiration rate (E) of rice plants exposed to different water stress
treatments. Water stress treatments were imposed at day zero and removed at day three.
Data are the means of ten replicates with standard errors (SE) shown by vertical bars.
WD = water deficit.
Figure 3.8: Leaf stomatal conductance ($g_s$) of rice plants exposed to different water stress treatment. Water stress treatments were imposed at day zero and removed at day three. Data are the means of ten replicates with standard errors (SE) shown by vertical bars. WD = water deficit.

3.3.4. Water deficit affects grain set

To assess the effect of water stress on the grain set of rice plants, osmotic stress was imposed for three consecutive days at -0.25, -0.5 and -0.75 MPa during anther development. The results showed that low stress levels (-0.25 MPa) did not significantly decrease the percentage of grain set compared with the control at any stage (Figure 3.9). Meanwhile, higher levels of osmotic stress; -0.5 MPa and -0.75 MPa, caused a serious decline in grain set (P<0.001). On average, a reduction of 24% and 34% of grain set was recorded for plants subjected to osmotic stress at -0.5 and -0.75 MPa, respectively (Figure 3.9).
Figure 3.9: Grain set (%) of rice plants exposed to different water deficit treatments during anther development. Data are the mean of five replicates with standard errors (SE) shown by vertical bars. Bars showing the same letter at any stage are not significantly different (P<0.001) as determined by ANOVA (n=5). LSD$_{0.05}$ = 10.79. PMC = pollen mother cell, VM = vacuolated microspore.

The greatest decrease in grain set was observed in the -0.75 MPa treatment imposed during the PMC and tetrad stages (P<0.001; Figure 3.9). In general, the percentage of grain set was less affected by water deficit treatment imposed during microspore and vacuolated microspore stages.

3.3.5. Water deficit affects pollen viability and young microspore viability

Microscopic observations of vital staining for mature pollens and young microspores of control and stressed plants by KI/I$_2$ and FDA/PI respectively are shown in Figure 3.10.
Figure 3.10: Microscopic observation of mature pollen and young microspores stained with KI/I$_2$ and FDA/PI, respectively. Control (A) and stressed (B) mature pollen stained with KI/I$_2$. Unviable pollen is not stained with KI/I$_2$ resulting in a yellow colour (white arrows) in contrast to viable pollen which is stained dark blue. Control (C) and stressed (D) young microspores. PI stained unviable microspores resulting in red fluorescence (white arrows) in contrast to viable microspores stained by FDA (yellow to green).

Table 3.1 represents the percentage of viable mature pollen, and young microspores of plants under different levels of water deficit stress during the PMC stage. The data show a significant reduction of viable pollen at -0.5 and -0.75 MPa of osmotic stress, while the percentage of viable pollen at osmotic stress of -0.25 MPa remained unchanged when compared to the control viable pollen (P<0.001). Osmotic stress caused approximately 16% and 24.5% reduction of viable pollen at -0.5 and -0.75 MPa, respectively (Table 3.1). Vital staining of young microspores with dual dyes...
(FDA/PI) showed a similar decreasing trend of young microspore viability during water deficit stress (Table 3.1). Mild water stress of -0.25 MPa did not cause a significant reduction of viable young microspores in comparison with the control (P<0.001), however, higher levels of osmotic stress at -0.5 and -0.75 MPa caused a 20% and 35% decline in viability of young microspores, respectively (P<0.001).

<table>
<thead>
<tr>
<th>Viability (%)</th>
<th>Young microspore</th>
<th>Pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86.9 ± 6.0a</td>
<td>83.07 ± 6.85a</td>
</tr>
<tr>
<td>-0.25 MPa</td>
<td>82.7 ± 6.4a</td>
<td>79.09 ± 4.29a</td>
</tr>
<tr>
<td>-0.50 MPa</td>
<td>66.8 ± 6.6b</td>
<td>67.06 ± 14.46b</td>
</tr>
<tr>
<td>-0.75 MPa</td>
<td>52.1 ± 5.2c</td>
<td>53.63 ± 15.66c</td>
</tr>
<tr>
<td>LSD&lt;sub&gt;0.05&lt;/sub&gt;</td>
<td>7.32</td>
<td>11.1</td>
</tr>
</tbody>
</table>

**Table 3.1:** Percentage of young microspore and mature pollen viability of plants under different levels of water deficit stress during the PMC stage, as determined by FDA/PI and KI/I<sub>2</sub> vital staining, respectively. Data are the mean of five replicates with standard errors (SE). Data in a column showing the same letter are not significantly different (P<0.001) as determined by ANOVA.

### 3.3.6. Relationship between grain set, pollen viability and microspore viability under water deficit stress at the PMC stage

To investigate the relationship between grain set, pollen viability and microspore viability, a linear correlation was examined. Figure 3.11 presents the linear regression analysis between grain set and pollen viability and Figure 3.12 presents the linear regression analysis between grain set and young microspore viability. The data indicates that there is a strong correlation between grain set and pollen viability (P<0.001, r<sup>2</sup> =
0.8168; Figure 3.11) at these stages. Likewise, grain set also exhibited a strong correlation with young microspore viability (P<0.001, $r^2 = 0.8223$; Figure 3.12). These data indicate the important role of pollen viability and young microspore viability in successful pollination and grain set.

**Figure 3.11**: Relationship between grain set and pollen viability of rice plants exposed to water deficit stress during PMC stage.

**Figure 3.12**: Relationship between grain set and young microspore viability of rice plants exposed to water deficit stress during PMC stage.
3.3.7. Association of $\Psi_{\text{leaf}}$ with grain set, pollen viability and microspore viability under water deficit stress at PMC stage

The relationship between $\Psi_{\text{leaf}}$ with grain set, pollen viability and microspore viability was also determined. Under water deficit stress during the PMC stage, a strong correlation between percentage of grain set and $\Psi_{\text{leaf}}$ was observed ($P<0.001$, $r^2 = 0.8165$; Figure 3.13). Similarly, $\Psi_{\text{leaf}}$ also exhibited a strong relationship with the number of viable mature pollen ($P<0.001$, $r^2 = 0.8016$; Figure 3.14) and viable young microspores ($P<0.001$, $r^2 = 0.8728$; Figure 3.15). These results indicate the crucial influence of the $\Psi_{\text{leaf}}$ on percentage of viable pollen as well as grain set.

![Figure 3.13: Relationship between grain set and leaf water potential ($\Psi_{\text{leaf}}$) of rice plants exposed to water deficit stress during PMC stage.](image)

$y = 60.289x + 98.856$

$R^2 = 0.8165$
Figure 3.14: Relationship between percentage viability of pollen and leaf water potential ($\Psi_{\text{leaf}}$) of rice plants exposed to water deficit stress during PMC stage.

Figure 3.15: Relationship between percentage viability of young microspore and leaf water potential ($\Psi_{\text{leaf}}$) of rice plants exposed to water deficit stress during the PMC stage.
3.4. Discussion

3.4.1. PEG as an ideal osmotic inducer to control levels of water deficit

The concept of using an osmoticum such as PEG for controlling continuous and constant soil water potential has been reported in the past (Painter, 1966; Zur, 1966). Before the advent of PEG, withholding water from the soil was a widely accepted technique to experimentally induce water stress to plants (Krizek, 1985). While the water potential of a PEG solution can be controllable, it is almost impossible to know the water potential at the root surface of plants grown in soil using water withholding methods as water is continuously taken up by the root system (Krizek, 1985). High solubility in water, low toxicity to mammals (Lawlor, 1970) and ability to control the water potential are characteristics that make PEG popular as an osmoticum to decrease the water potential of the growing medium for studying responses of higher plants to water deficit.

Often, the application of PEG as an osmotic stress inducer involves the use of a semi-permeable membrane to overcome problems of ionic effects. There have been concerns about the ionic toxicity of PEG absorbed by plants (Janes, 1966; Lawlor, 1970; Kaufmann & Eckard, 1971; Janes, 1974). The deposition of high molecular weight PEG can be found in leaves causing leaf tip necrosis (Lawlor, 1970) and large amounts of heavy metals, especially aluminum and magnesium, in PEG compounds may be toxic to plants (Lagerwerff et al., 1961). PEG has also been found to inhibit the passage of phosphorus and water into plants (Emmert, 1974). However, PEG only causes detrimental effects once it enters the tissue via damaged roots (Lawlor, 1970). Carpita et al. (1979) reported that PEG molecules with molecular weights $\geq 6,000$ are unable to penetrate the cell wall pores and therefore, as a result of movement of water out of the cell wall, only causes plasmolysis in severe cases (Oparka, 1994). To ensure...
there are no ionic effects, only PEG with a high molecular weight (20,000) and high purification (pharmaceutical grade) was used in these experiments.

At the onset of this study, many attempts were made to apply the osmotic stress produced by PEG to rice plants growing in soil using a semi-permeable membrane (Chapter 2, Section 2.2.1). Using a membrane might potentially help overcome the problem of direct contact between PEG and roots that might cause toxicity to the root system. However, after several trials, it was decided that the membrane appeared to be causing a restriction of movement of water across the membrane (personal observations). In addition, it was technically very difficult to keep the membrane intact during the experimental period. The membrane degraded quickly due to natural microbial action. In addition, strong root development punctured the membrane within one to two weeks. Replacing the membrane repeatedly and using other protective materials (hand-made socks) did not aid in maintaining membrane integrity.

Another approach was to expose plants grown in the soil directly to the PEG solution, but this was also ineffective. Because the water retention capacity of the soil was high, it took too long for the water deficit stress to take effect. A trial was conducted by withholding water to some plants and observing how long it would take for them to be stressed. The plants did not show any stress symptoms (leaf rolling) after 4 - 5 days. This would make it very difficult to coincide the stress with the sensitive developmental stages of the plants e.g. pollen mother cell stage. In addition, PEG can potentially permeate through the soil, which might result in a permanent water deficit stress, as PEG could not be removed completely after exposure.

In this case growing plants hydroponically was decided to be more feasible and the most reliable method for stress induction. The idea was that potting mix (mainly tree bark and coarse sand) is very poor in water holding capacity (and perhaps once applied
stress would take effect very quickly). Moreover, the root system of plants grown hydroponically can easily come into contact with the hydroponic medium containing PEG. This characteristic makes it easy to stress as all roots are exposed directly to the chemical. Successful employment of PEG to induce water stress in cotton, maize (Lawlor, 1970; Stikic & Davies, 2000), pepper (Janes, 1974), potato (Bussis et al., 1998; Zgallai et al., 2005), wheat, oats and barley (Kaul, 1966) and rice (Choi et al., 2000; Cabuslay et al., 2002; Lian et al., 2004; Zhou et al., 2007) support the use of PEG as an osmotic stress inducer without a membrane.

The results from the experiments conducted in this study show that rice plants subjected to osmotic stress induced by PEG, at any level, significantly decreased $\Psi_{\text{leaf}}$ in comparison to the control (Figure 3.5). Moreover, water stress also decreased the level of $A$ (Figure 3.6), $E$ (Figure 3.7) and $g_s$ (Figure 3.8). In general, the advantage of PEG to induce water deficit compared to a withholding water method (Figure 3.1) is to generate more precise and constant osmotic stress to plants. The results from $\Psi_{\text{leaf}}$ (Figure 3.5) and gas exchange measurements (Figures 3.6, 3.7 & 3.8) indicate that the PEG solution produced an almost constant osmotic level throughout the stress period. Moreover, no symptoms of toxicity were found in rice plants exposed to the PEG solution for three successive days in this experiment. Complete recovery of $\Psi_{\text{leaf}}$, $A$, $E$ and $g_s$ (Figures 3.5, 3.6, 3.7 & 3.8) indicates that the stress caused by PEG was not permanent and could be reversed when PEG was removed.

There are several hypotheses on how PEG causes osmotic effects. Some authors suggest that PEG may block transpiration pathways of plants and thus cause stress (Janes, 1961; Lawlor, 1970). Janes (1966) conducted experiments on bean and pepper and concluded that imposing an osmotic stress on the roots reduces the rate of water entry and thus the water supply to leaves.
However, the chemical structure of PEG may be the most reasonable explanation for its osmotic effect. Structurally, PEG is described as a linear polymer of ethylene oxide with a hydroxyl terminal. In water it is soluble at moderate temperatures in any concentration over a wide range of degrees of polymerization (Hui et al., 1999). The chains of repeating ethylene oxide monomers form helical coils which maximize hydrogen bonding while minimizing the number of exposed hydrophobic groups (Kjellander & Florin, 1981). Therefore, it is believed that the water solubility of PEG is the result of the coil structure of its backbone (Beily & Koleske, 1987; Schiller et al., 1988), where the hydrogen of water interacts with the oxygen of the ethylene oxide group by hydrogen bonding (Graham & Chen, 1993). Although the oxygen atom of the helical chain has been shown to associate with one or several molecules of water to form various hydrates (Liu, 1968; Maxfield & Shepherd, 1975), it has been demonstrated that each ethylene oxide group in the helix chain requires three water molecules to form a stable hydrated complex (Liu & Parsons, 1969; Graham et al., 1982). This interaction between PEG and water has resulted in a substantially lower diffusion coefficient of water near the polymer chain than that in bulk water. Blow et al. (1978) demonstrated that there is no free water at 45% PEG. This high affinity for water is responsible for the unusual osmotic reduction of PEG solutions.

The decline of available oxygen in PEG solutions (with molecular weight $\geq 4,000$ and osmotic potential $\leq -0.7$ MPa) has also been considered a possible cause of root growth inhibition (Mexal et al., 1975). This possibility, however, may not be the case in rice plants because of the ability of rice shoots to supply sufficient oxygen to roots while growing in anaerobic environments (John et al., 1974). Moreover, healthy roots observed after osmotic stress by PEG in this study (Figure 3.4) eliminate this
hypothesis. Perhaps more branched and elongated roots after stress treatment might indicate the response of the rice roots in the search for water.

3.4.2. Water deficit stress reduced male fertility

Three consecutive days of exposure to mild water deficit at -0.25 MPa did not significantly reduce grain set, whilst a serious decline of grain set was obvious at -0.5 MPa and -0.75 MPa of osmotic stress (Figure 3.9). Water deficit occurring at any time during pollen development from PMC to VM stages resulted in significant grain loss (Figure 3.9). This characteristic has been well documented in the literature (Saini & Westgate, 2000). Although analysis of variance was only meaningfully different at the microspore stage at -0.5 MPa osmotic stress, plants subjected to water deficit stress at PMC to tetrad stage seemed to suffer the most damage (Figure 3.9). These data are supported by earlier findings of sensitive stages of rice plants to water stress which claimed that the most sensitive stage centres around meiosis (Saini & Lalonde, 1998; Saini & Westgate, 2000; Yang et al., 2007). The reduction of grain set could be ascribed to the decrease in number of viable pollen in the present study (Table 3.1). Theoretically, only a single pollen grain is needed for fertilization in every individual floret. In practice, however, a large number of engorged pollen is required for the successful pollination of the floret. Therefore, the number of viable pollen has been used as an indicator of fertility in rice (Gunawardena et al., 2003; Oliver et al., 2005) and other cereals (Saini & Aspinall, 1981; Schoper et al., 1986). The strong positive linear relationship between grain set and pollen viability confirms this hypothesis (Figure 3.11).

The result of pollen vital staining with KI/I\textsubscript{2} showed a considerable amount of unstained pollen that signifies a lack of starch (Figure 3.10B & Table 3.1). Starch is considered a major energy source for pollen development and germination (Pacini &
Franchi, 1988; Clement et al., 1994), hence the absence of this energy source could lead to pollen sterility. The level of starch has been found to be reduced in anthers from plants exposed to water stress (Dorion et al., 1996; Sheoran & Saini, 1996; Lalonde et al., 1997a) and chilling stress (Gunawardena et al., 2003; Oliver et al., 2005). These findings indicate that the process of starch biosynthesis and deposition in pollen grains is somehow inhibited by stresses. However, this starch staining method by KI/I₂ can only be used to validate mature pollen at the late developmental stages that may not truly tell the immediate effects of water deficit on the developing microspores. Moreover, starch filling in rice pollen occurs a few days prior to anthesis (Raghavan, 1988) and it is probable that water stress could have already caused the failure of young microspores before they reach the starch filling stage.

Heslop-Harrison & Heslop-Harrison (1970) developed a vital staining method using FDA to test the integrity of the cellular plasma membrane which was claimed to be closely correlated with pollen viability. The principle of the method is that the fluorescent dye in the form of an esterase is readily hydrolyzed by enzymes secreted from intact cellular plasma membranes. The fluorescein will be intracellularly entrapped and therefore can be detected by its fluorescence. Meanwhile, damaged cellular membranes cannot hold the intracellular fluorescein and it will diffuse back to the culture medium. Huang et al. (1986) developed a method using a mixture of double dyes FDA and PI to test the viability of aleurone protoplasts from barley. By contrast to FDA, PI cannot generally enter the intact cells, but it can easily penetrate the cells with damaged plasma membranes resulting in fluorochromasia of both the nucleus and cytoplasm in dead cells (Huang et al., 1986). Therefore double staining using a mixture of FDA/PI dyes can differentiate the intact cells (potentially viable) and damaged cells (potentially unviable). Dual staining with FDA and PI has been applied successfully in
the past by some investigators (Zhang et al., 1992; Li, 2005; Oliver et al., 2005) to test young microspore viability. Therefore, a vital staining method combining FDA and PI has been the method of choice in this study in addition to KI/I₂. Interestingly, the results from dual staining for young microspore viability demonstrated a serious reduction of fertility immediately after the water deficit was removed (Figure 3.10D & Table 3.1). A strong positive relationship between grain set and number of viable young microspores (Figure 3.12) supports this idea. This reduction of viable young microspores might therefore be considered to result from instant effects of water deficit on developing microspores.

3.4.3. Association of $\Psi_{\text{leaf}}$ with reduction of grain set and pollen viability

Amongst the criteria representing plant-water relationships, $\Psi_{\text{leaf}}$ is most widely used and often recognized as an index for whole plant water status (Turner, 1982). Under drought conditions, rice lines which could maintain a high $\Psi_{\text{leaf}}$ had a lower percentage of spikelet sterility (Jongdee et al., 2002) and maintenance of a high $\Psi_{\text{leaf}}$ during the flowering stage has been reported to be associated with high spikelet fertility (O’Toole & Namuco, 1983; Ekanayake et al., 1989). An increase in spikelet sterility has been shown to be related to a serious decline in the panicle turgor potential and relative water content at anthesis (Selote & Khanna-Chopra, 2004). A decline in the flag leaf $\Psi_{\text{leaf}}$ from -0.5 MPa to -0.9 MPa in comparison to the corresponding controls has been reported to result in an approximate reduction of 30% grain set (Selote & Khanna-Chopra, 2004). Therefore, $\Psi_{\text{leaf}}$ has been proposed as an indicator for selecting drought tolerant rice varieties (Jongdee et al., 2002). Consistent with previous reports, the results in this study show that there was a strong positive linear relationship between $\Psi_{\text{leaf}}$ at day three of water deficit stress during PMC with grain set ($P<0.001$, $r^2 = 0.8165$; Figure 3.13), mature pollen viability ($P<0.001$, $r^2 = 0.8016$; Figure 3.14) and
young microspore viability (P<0.001, r² = 0.8728; Figure 3.15). On average, the decline of the predawn $\Psi_{leaf}$ during the six days since the initiation of water deficit stress from -0.26 MPa (control) to -0.35 Mpa (-0.25 MPa treatment) did not cause a significant reduction of plant fertility, meanwhile the predawn $\Psi_{leaf}$ of -0.49 MPa (-0.5 MPa treatment) and -0.58 MPa (-0.75 MPa treatment) significantly decreased plant fertility (Figures 3.5 & 3.9). The decrease of the grain set and plant fertility in the present study could directly be attributed to the decline in $\Psi_{leaf}$ under water deficit stress induced by PEG.

3.5. Conclusion

This chapter has illustrated the feasibility of using PEG as an osmotic regulator for controlling, precisely and constantly, water deficit stress in rice plants grown hydroponically. Some concerns regarding the possible toxicity of PEG in inducing osmotic stress could be negated by using high molecular weight and high grade PEG. The results presented in this chapter also demonstrate that a three day consecutive exposure to -0.5 MPa and -0.75 MPa osmotic stress significantly reduced grain set and pollen viability, whilst lower levels of osmotic stress at -0.25 MPa did not significantly affect these parameters. Therefore the osmotic level of -0.5 MPa, which has been regarded as a mild stress level in some reports (Cabuslay et al., 2002), could be considered as a critical point and hence will be applied to all other experiments hereafter unless otherwise stated. Moreover, the strong correlation between grain set, pollen viability and microspore viability again proved that fertility of rice plants under water deficit stress could be assessed at the early stage using a young microspore viability index which could be determined immediately after removal of the water deficit stress.
CHAPTER 4: WATER DEFICIT AFFECTS SUGAR METABOLISM IN ANTHERS

4.1. Introduction

Many investigators have focussed on the effects of environmental stresses such as chilling and drought on carbohydrate metabolism in rice anthers (Sheoran & Saini, 1996; Oliver et al., 2005; Alfred, 2006) with particular interest in the role of acid invertases. As discussed in Chapter 1, abnormally high levels of non-reducing sugars (mainly sucrose) and reducing sugars (mainly glucose and fructose) from anthers of plants subjected to water and chilling stress have been proposed to be the result of impairment of vacuolar and cell wall acid invertases (Ito, 1978; Dorion et al., 1996; Sheoran & Saini, 1996; Koonjul et al., 2005; Oliver et al., 2005). Transcriptional down regulation of cell wall invertases has been claimed to cause the blockage of sugar supplies to developing microspores, consequently leading to a failure of starch deposition in unviable pollen (Koonjul et al., 2005; Oliver et al., 2005; Alfred, 2006). However, there is little information in regard to the immediate effects of stresses on sugar metabolism in anthers. Results from dual staining of early microspores with FDA and PI (Chapter 3) illustrated that water deficit imposed for three consecutive days caused a significant impairment of early microspore development. Dead microspores might directly result from improper functioning of respiratory pathways such as glycolysis, TCA cycle and oxidative phospholylation, which are considered as central energy generators for growing anthers.

Moreover, morphological and cytological studies of male gametophyte development of rice anthers have shown that plasmodesmatal connections between the tapetum and other cell layers of the anther wall are not detectable post meiosis, and formation of the callose wall (β-1, 3 glucan) segregates the meiocytes from the anther
walls (Raghavan, 1988; Li, 2005; Mamun et al., 2005b). The tapetum layer plays a critical role in controlling pollen development and ultimately plant fertility, by supplying nutrients to the developing pollen and pollen wall (Pacini, 1990; Piffanelli & Murphy, 1998). Because of this symplastic isolation, carbohydrate supply to the tapetum cells and the developing microspores might require apoplastic transport (Clement & Audran, 1995; Truernit et al., 1999; Mamun et al., 2006). In other words, the importation of sugars from connective tissues into the anther locules to support developing microspores may involve the activity of several disaccharide and monosaccharide transporters. Active sugar carriers such as sucrose transporters and monosaccharide transporters have been known to play crucial roles during flowering and grain development in rice (Takeda et al., 2001; Lim et al., 2006) and other plants (Sauer, 2007). However, there is a lack of information regarding this mode of sugar unloading and transport in anthers. Some models of sugar unloading and transport in Lilium and rice anthers have been proposed (Clement & Audran, 1995; Alfred, 2006), however they all remain speculative and incomplete.

This chapter will therefore extend previous work by further exploring the effects of water deficit stress on carbohydrate metabolism in rice anthers. New findings that supplement the existing models of sugar unloading and transport in the anther are presented. Moreover, the association of carbohydrate metabolism in rice anthers with pollen abortion under drought is also discussed in detail.

4.2. Materials and methods

4.2.1. Plant materials, water stress treatment and anther collection

Plant culture and anther collection are carried out as described in Chapter 2 (Section 2.2.2 & 2.4). Mild water stress treatment at -0.5 MPa was initiated as described in Chapter 2 (Section 2.3.2). Plants were stressed for three consecutive days at
individual stages centring on the PMC, tetrad and microspore stage. Anthers from treated plants were collected immediately after the removal of the stress and distinguished as tetrad, microspore, and vacuolated microspore stage by light microscopy.

4.2.2. Carbohydrate extraction and measurement

Anther carbohydrates were extracted according to the method of Dorion et al. (1996). Anthers (15-25 mg of fresh weight) were ground in liquid nitrogen to a fine powder. Five hundred microlitres of 80% (v/v) ethanol was added and the homogenate was incubated in a water bath at 70°C for 10 minutes then allowed to cool to room temperature. After centrifugation at 10,000 rpm for 5 minutes, the supernatant was decanted to a fresh tube and the pellet was extracted another two times with ethanol as described above. The supernatants were pooled and aliquots were used to measure sugars.

4.2.2.1. Total soluble sugars

Total soluble sugars were determined using the anthrone method (Yemm & Willis, 1954). Four mL of anthrone solution (0.15 g anthrone in 100 mL 80% H₂SO₄) was added to 100 µL of the soluble sugar extract and incubated in a boiling water bath for 10 minutes and allowed to cool to room temperature. The absorbance of the samples was determined spectrophotometrically at 620 nm using glucose as the standard. The data are presented as D-glucose equivalents.

4.2.2.2. Reducing sugars

Reducing sugars were determined using the copper/arsenio-molybdate method (Nelson, 1944). After addition of 1 mL copper solution to 200 µL of the soluble sugar extract, the mixture was incubated in a boiling water bath for 20 minutes. Arseno-molybdate solution was added and incubated at room temperature for 10 minutes. The
absorbance of the samples was determined spectrophotometrically at 500 nm using glucose as the standard. The data are presented as D-glucose equivalents.

4.2.2.3. Sucrose and hexoses

Sucrose and hexoses were determined enzymatically using a Sucrose, D-Fructose and D-Glucose assay kit (Megazyme International Ireland Limited). In brief, a 0.1 mL aliquot of the extract was mixed with 2.42 mL buffer, which contained 2.1 mL distilled water, 0.1 mL imidazole buffer, 0.1 mL NADP+/ATP, and 0.02 mL HK/G6P-DH (all unit definitions are according to the supplier Megazyme) to convert D-glucose to glucose-6-phosphate, which is then oxidised by NADP+ to gluconate-6-phosphate. NADPH production by this reaction was measured at 340 nm and was used as a measure for the amount of D-glucose in the sample. Phosphosglucose isomerase (PGI) (0.02 mL) was then added to the reaction to convert fructose-6-phosphate to glucose-6-phosphate. The glucose-6-phosphate thus formed reacts in turn with NADP+ forming gluconate-6-phosphate and NADPH, leading to a further rise in absorbance that accounts for the amount of D-fructose. The sucrose in the extract was first hydrolysed by adding 0.2 mL β-fructosidase to the reaction buffer and the total D-glucose in the sample following the hydrolysis of sucrose was determined as described above. The sucrose content is calculated from the difference in D-glucose concentrations before and after hydrolysis by β-fructosidase.

All measurements were carried out on three extracts prepared from different sets of plants, grown at different times and treated under the same conditions, unless otherwise stated.

4.2.3. RNA extraction and real time qRT-PCR

Total RNA extraction and quantification are as described in Chapter 2 (Section 2.5 & 2.6). Table 4.1 shows specific primers of the targeted genes. Initial conventional
RT-PCRs for detection and cDNA sequencing to confirm homology of amplified products and transcripts of interest were carried out as described in Chapter 2 (Section 2.6). Rice OsTuba1 gene (Genebank ID AF182523) was used as a reference control in the real-time qRT-PCR as described in Chapter 2 (Section 2.6). The reaction mixture using gene specific primers is as described in Chapter 2 (Section 2.6).

<table>
<thead>
<tr>
<th>Protein (gene) name</th>
<th>Genebank ID</th>
<th>Primer sequence (5’-3’)</th>
<th>cDNA size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoplastic invertase (OsCIN4)</td>
<td>AY578161</td>
<td>[F] GATCAAGGGAACTGATACTTTACA</td>
<td>195</td>
</tr>
<tr>
<td>Sucrose transporter (OsSUT5)</td>
<td>AB091674</td>
<td>[F] CTAGTGCGAAACTCCATCAA</td>
<td>249*</td>
</tr>
<tr>
<td>Monosaccharide transporter (OsMST7)</td>
<td>AY643749</td>
<td>[F] GTGCTGTCAAGACCATCGG</td>
<td>184</td>
</tr>
<tr>
<td>Fructokinase (OsFKI)</td>
<td>AF429948</td>
<td>[F] CAGGGATTTCGCGAGCCTGT</td>
<td>134</td>
</tr>
<tr>
<td>Hexokinase (OsHXK3)</td>
<td>DQ116385</td>
<td>[F] CACTACTGAGGGTTTATTCAACTT</td>
<td>178</td>
</tr>
</tbody>
</table>

**Table 4.1:** Target genes for analysis of expression profiles. (*) Primer sequence adapted from Aoki *et al.* (2003). F forward primer, R reverse primer.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Denaturing</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Optional step</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsCIN4</td>
<td>94°C – 20secs</td>
<td>61°C – 30secs</td>
<td>72°C - 25secs</td>
<td>Not applied</td>
</tr>
<tr>
<td>OsSUT5</td>
<td>94°C – 15secs</td>
<td>55°C – 30secs</td>
<td>68°C - 20secs</td>
<td>79°C – 15secs</td>
</tr>
<tr>
<td>OsMST7</td>
<td>94°C – 20secs</td>
<td>60°C – 30secs</td>
<td>68°C - 15secs</td>
<td>79°C – 15secs</td>
</tr>
<tr>
<td>OsFKI</td>
<td>94°C – 20secs</td>
<td>63°C – 30secs</td>
<td>72°C - 25secs</td>
<td>Not applied</td>
</tr>
<tr>
<td>OsHXK3</td>
<td>94°C – 20secs</td>
<td>62°C – 30secs</td>
<td>68°C - 25secs</td>
<td>Not applied</td>
</tr>
</tbody>
</table>

**Table 4.2:** Cycling profiles for real time qRT-PCR reactions.
In general, qRT-PCR consisted of a reverse transcription step at 50°C for 20 minutes, and then initial denaturation at 94°C for 5 minutes following 35-40 cycles, was applied to duplicate samples of all genes under investigation. Variations in temperature and steps during the cycles of individual genes are presented in Table 4.2. Specificity of amplified products was monitored by an optional step and a melt curve at the end of RT-PCR reactions. Fluorescent signals were acquired at the last step during cycles. The real-time amplification data were statistically analysed as described in Chapter 2 (Section 2.6).

4.2.4. In situ hybridisation

4.2.4.1. Maintenance of an RNase free environment

It is crucial to maintain an RNase free environment for the whole process of in situ hybridisation. All equipment e.g. glassware, slide holders, etc. used for the procedure were pre-soaked in 2% Pyroneg overnight, cleaned thoroughly with double distilled water and autoclaved at 121°C for 20 minutes. A final rinse was carried out with 0.1% diethylene pyrocarbonate (DEPC) treated water shortly before use to ensure complete elimination of RNase (Sambrook & Russell, 2001). All solutions were made using DEPC treated water. Bench surfaces and fumehood areas were soaked with RNase Away (Sigma-Aldrich, Australia) and wiped off using RNase free towels before use.

4.2.4.2. Paraffin embedded section preparations

Paraffin embedded sections were prepared as described in Chapter 2 (Section 2.7).

4.2.4.3. Single stranded RNA probes

Because of its higher effectiveness and sensitivity compared to double stranded RNA or nick translated DNA probes (Cox et al., 1984), single stranded RNA probes
were chosen for this *in situ* hybridisation experiment. cDNAs of targeted genes were cloned into a pGEM-T-easy vector (Promega, Australia), which contains restriction sites within the multiple cloning regions, according to the manufacturer's instructions. In brief, 1 µL of cDNA fragment was ligated with 1 µL pGEM-T-easy vector in a reaction mixture including 2 µL sterilised distilled water, 5 µL 2X rapid ligation buffer, and 1 µL T4 ligase enzyme. After a 60 minute incubation at room temperature, the recombinant vector was subsequently transformed into JM109 high efficiency competent cells by heat shock at 42°C for 45 seconds and further incubated in 950 µL of SOC medium (Appendix I) at 37°C with shaking for 1.5 hours. 100 µL of the transformation mix was evenly spread on an ampicillin Luria-Bertani (LB) agar plate (Appendix I) which contained 20 µL of 50 mg/mL X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) (Sigma-Aldrich, Australia) and 100 µL of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Australia). The plates were then incubated upside down overnight at 37°C.

PCR screening of recombinant plasmids was carried out by the method of Lee & Cooper (1995) the following day. The white bacterial colonies, which often carry cDNA inserts, were individually inoculated onto fresh ampicillin LB plates and incubated at 37°C. Part of this same colony was also resuspended in a 1.5 mL microcentrifuge tube containing 100 µL of sterile double distilled water and incubated at 95°C for 5 minutes. The suspension was centrifuged at 13,000 rpm for 5 minutes to remove cell debris. The supernatant was used for subsequent PCR. The PCR mixture of 10 µL contained: 1X PCR buffer, 200 µM dNTPs, 1.6 mM MgCl₂, and 200 nM gene specific primers. The PCR profile was similar to the RT-PCR described in Chapter 2 (Section 2.6.2) with the exception that the RT cycle was omitted. Amplified products were electrophoresed in a 1% agarose gel for 1.5 hours at 90 V. The agarose gel was post stained in a water bath
containing ethidium bromide and visualised under a UV light. The bacterial colonies, which yielded PCR products with a similar band size to the cDNA from the RT-PCR reaction of the same gene, were considered positive and subcultured again.

Plasmid DNA was isolated from the screened bacterial colonies using Wizard® Plus SV Minipreps DNA purification kit (Promega, Australia) following the manufacturer’s instructions with some modifications. Briefly, the subcultured cells were directly resuspended in a 1.5 mL microcentrifuge tube containing 250 µL of cell resuspension solution and vortexed. A subsequent 250 µL of cell lysis solution was added and gently mixed by inverting the tube. The mixture was further incubated with 10 µL of alkaline protease solution for 5 minutes at room temperature. The bacterial lysate was centrifuged for 10 minutes at 13,000 rpm after adding 350 µL of neutralisation solution. The cleared lysate was decanted to a spin column and centrifuged at 13,000 rpm for 1 minute at room temperature. The column was washed twice with column wash solution containing 95% ethanol. The plasmids were eluted by adding 50 µL of nuclease-free water and centrifuged for 1 minute at 13,000 rpm.

The orientation of the DNA insert within the pGEM vector was confirmed by sequencing analysis so that the appropriate RNA polymerase (T7/SP6) could be applied to generate either sense or antisense probes. The procedure used to prepare plasmids for sequencing analysis was similar for cDNA as described in Chapter 2 (Section 2.6.2).

Plasmids containing DNA inserts of interest were linearised with either NcoI or SpeI restriction enzyme which generates DNA fragments with 5’ protruding ends (Schenborn & Mierendorf, 1985). Linearised plasmids were purified using a QIAquick PCR purification kit (QIAGEN, Australia) as described in Chapter 2 (Section 2.6.2). RNA probe synthesis was carried out in vitro using a DIG RNA labelling kit (SP6/T7) (Roche, Australia) according to the manufacturer’s instructions. Briefly, 1 µg of
purified template DNA was incorporated in 13 µL labelling mix containing 2 µL 10X NTP labeling mixture, 2 µL 10X transcription buffer, 2 µL protector RNase inhibitor and 2 µL appropriate RNA polymerase. The mixture was centrifuged briefly and incubated at 37°C for 2 hours. 2 µL of DNA I RNase free was added to remove template DNA and further 15 minute incubation was conducted. The reaction was stopped by adding 2 µL 0.2 M EDTA (pH 8.0).

Newly transcribed RNA probes were analysed by formaldehyde agarose (FA) gel electrophoresis. A mini FA gel (1% agarose) was prepared by melting 0.3 g ultra pure agarose in 27 mL DEPC treated H2O. After cooling to 65°C, the gel mixture was further supplemented with 3 mL 10X 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.0), 0.3 mL of 37% ultra pure formaldehyde (PCR grade) and 2 µL of a 10 mg/mL ethidium bromide solution. The gel was then poured and allowed to set for around 40 minutes. About 2 µL of RNA marker (Sigma, Australia) and 6 µL of probes were individually mixed with RNA loading buffer [(62.5% (v/v) deionised formamide, 1.14 M ultrapure formaldehyde, 1.25X MESA buffer (50 mM MOPS, 12.5 mM sodium acetate, and 1.25 mM EDTA, pH 7.0), 200 µg/mL bromphenol blue, 200 µg/mL xylene cyanole FF)], incubated at 65°C for 5 minutes, then immediately chilled on ice and subsequently loaded onto the gel. The gel was electrophoresed for 1 hour at 90 V in 1X MOPS buffer (pH 7.0) with 1% ultra-pure formaldehyde solution, then visualised and photographed under a UV light. Care was taken to prevent RNase contamination during the electrophoresis step. All gel apparatus was soaked in a 0.2% SDS solution overnight and thoroughly washed with tap water and then DEPC water.

4.2.4.4. Hybridisation

The hybridisation procedure was carried out following the method of Komminoth (2002) with some modifications. In brief, 8 µm thick paraffin embedded
sections were dewaxed twice in fresh xylene for 10 minutes each time, and rehydrated in a series of ethanol washes (100%, 95%, and 70%) once for 5 minutes each series and washed in DEPC treated water twice for 2 minutes each. The sections were then incubated in 0.2 M HCl containing 0.1% pepsin at 37°C for 15 minutes. After washing twice for 5 minutes in DEPC treated 1X PBS, the sections were further permeabilised for 30 minutes at 37°C with TE buffer containing 50 µg/mL RNase free Proteinase K. Proteinase K treatment was stopped by incubating sections for 1 minute in 2 mg/mL glycine in PBS followed by two 1 minute incubations in DEPC treated PBS. Post-fixing the sections was carried out for 5 minutes with 4% paraformaldehyde in PBS. Following fixation, the sections were washed twice for 5 minutes each with DEPC treated PBS. Acetylation of sections was carried out in 0.1 M triethanolamine (TEA) buffer at pH 8.0, containing 0.25% (v/v) acetic anhydride for 10 minutes with vigorous agitation on a shaker. After treatment with acetic anhydride, the sections were rinsed twice for 1 minute each in PBS buffer.

Hybridisation was carried out by incubating each section with 30 µL of hybridisation buffer containing 40% deionized formamide, 10% dextran sulfate, 1X Denhardt’s solution, 4X SSC, 10 mM DTT, 1 mg/mL yeast t-RNA, 1 mg/mL fish sperm DNA and 10 ng of RNA probe. Sections were then covered with hydrophobic hybridisation slips and incubated at 50°C overnight in a humidified chamber. Negative controls were carried out by substituting sense for antisense probes in the hybridisation solution. The following day, coverslips were removed from sections by immersing slides for 5 min in 2X sodium chloride sodium citrate buffer (SSC). Stringent washes for sections with 2X SSC were conducted in a shaking water bath at 37°C, twice for 15 minutes each. To eliminate any single-stranded RNA probe, sections were incubated for 30 minutes at 37°C in NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0)
containing 20 µg/mL RNase A. Two final washes with 0.1X SSC were also carried out in a shaking water bath at 37°C for 15 minutes each.

4.2.4.5. Immunological detection

Immunological detection of DIG labelled RNA probes was carried out using a DIG nucleic acid detection kit (Roche, Australia) following the manufacturer’s instructions. In brief, following the 0.1X SSC wash, the sections were incubated twice in washing buffer which contains 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween 20, for 5 minutes each at room temperature. Afterwards, the sections were incubated with blocking solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 1% blocking reagent) for 30 min at room temperature. Further incubation was performed on sections in a humidified chamber for 2 hours at 25°C with anti-DIG-AP in blocking solution (ratio 1:200). Two wash steps were carried out afterwards with washing buffer for 5 minutes each. After 10 minutes equilibration with detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5), each section was covered with 50 µL colour substrate solution containing nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (NBT/BCIP) stock solution and detection buffer at a ratio of 1:20 and incubated in a humidified chamber overnight at 25°C in the dark. When colour development was clearly visible, the colour reaction was stopped by incubating the slides in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Sections were dehydrated briefly through a series of ethanol washes (70%, 95%, and 100%), covered and sealed with nail polish, and allowed to air dry for 5 minutes. The slides were then directly viewed under a light microscope (Olympus BX51, Japan) using bright field and images were captured for further analysis.
4.3. Results

4.3.1. Changes in the level of carbohydrate

In unstressed anthers, the level of total soluble sugars did not vary remarkably from the tetrad to vacuolated microspore stage (Figure 4.1) whilst reducing sugars increased steadily over the period (Figure 4.2). Anthers of plants subjected to three day water stress treatment at -0.5 Mpa have increased levels of both total (Figure 4.1) and reducing sugars (Figure 4.2). These results indicate that sugars were abnormally accumulated in rice anthers after drought treatment. In drought stressed anthers at the tetrad stage, the levels of total and reducing sugars was more than twice that of the untreated controls (P<0.001; Figures 4.1 & 4.2). The total sugar at the vacuolated microspore stage was almost three times higher in stressed anthers than in the control (Figure 4.1).

The level of sucrose in the anthers under normal growing conditions fluctuated from 96 -132 µmol g⁻¹ fresh weight (FW) (P<0.001; Figure 4.3), whilst the level of glucose and fructose increased steadily throughout the growing period from the tetrad to vacuolated microspore stage (P<0.001; Figures 4.4 & 4.5). This suggests an important role of hexoses during anther development. Results from the drought stressed anther assays showed that the level of sucrose, glucose and fructose all increased after the water stress (Figures 4.3, 4.4 & 4.5). On average, the level of sucrose by the post water stress period was 1.4 times higher (Figure 4.3) whilst it was 1.6 fold higher for glucose and fructose after stress (Figures 4.4 & 4.5). Intriguingly, stressed anthers at the vacuolated stage did not show a significant increase in sucrose (Figure 4.3). Although the pattern of change in sucrose, glucose and fructose levels (Figures 4.3, 4.4 & 4.5) were the same as those of total and reducing sugars (Figures 4.1 & 4.2), the level of the former group exceeds that of the latter group at all three stages. This inconsistency may
be a result of the sample variation and the difference in the techniques used in the two cases. The total and reducing sugars were measured indirectly based on amount of glucose equivalents standard, while measurement of sucrose, glucose and fructose were quantified individually based on enzymatic reactions. Similar discrepancies have been reported in other carbohydrate assays (Dorion et al., 1996; Oliver et al., 2005).

Figure 4.1: Effect of water deficit treatment on total sugar levels in rice anthers treated at different stages. Total carbohydrate was measured using the anthrone method. Vertical bars are standard errors (SE). Data are the mean of three replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by ANOVA (P<0.001), LSD_{0.05} = 28.32. FW: fresh weight.
**Figure 4.2:** Effect of water deficit treatment on reducing sugar levels in rice anthers treated at different stages. Reducing sugars were determined using the copper/arsenomolybdate method. Vertical bars are standard errors (SE). Data are the mean of three replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by ANOVA (P<0.001), LSD_{0.05}= 5.41. FW: fresh weight.

**Figure 4.3:** Effect of water deficit treatment on sucrose levels in rice anthers treated at different stages. Vertical bars are standard errors (SE). Data are the mean of three replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by ANOVA (P<0.001), LSD_{0.05}= 29.1. FW: fresh weight.
**Figure 4.4:** Effect of water deficit treatment on glucose levels in rice anthers treated at different stages. Vertical bars are standard errors (SE). Data are the mean of three replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by ANOVA (P<0.001), LSD$_{0.05}$ = 8.8. FW: fresh weight.

**Figure 4.5:** Effect of water deficit treatment on fructose levels in rice anthers treated at different stages. Vertical bars are standard errors (SE). Data are the mean of three replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by ANOVA (P<0.001), LSD$_{0.05}$ = 10.29. FW: fresh weight.
4.3.2. Expression of the cell wall acid invertase gene OsCIN4

To investigate transcriptional regulation of cell wall acid invertase during anther development, anthers at three stages were selected from both control and water stressed plants. Temporal expression of the cell wall acid invertase gene OsCIN4 in control anthers remained almost unchanged at early stages (tetrad, microspore) (Figure 4.6). This transcript was less abundant at the vacuolated microspore stage (Figure 4.6). Three days of water deficit treatment resulted in partial repression of expression of this gene only at the vacuolated microspore stage (P<0.05; Figure 4.6). On average, a 6-fold reduction of this mRNA compared to the non-treated control was recorded at this stage (Figure 4.6). Spatial expression of this gene was further localised within the anther locule by in situ hybridisation (Figure 4.7). Hybridisation with antisense labelled probes showed strong expression of OsCIN4 in the growing microspore and tapetum (Figures 4.7A & 4.7C). Reduced expression of OsCIN4 was also observed in the middle layer and expression of this mRNA was only minor in the vascular bundle (Figures 4.7A & 4.7C). Images taken at a higher magnification clearly show the position of OsCIN4 within the anther locule i.e. in the young microspore, tapetum and middle layer cells (Figures 4.7E). Water deficit stress did not alter the spatial expression of OsCIN4 in the anther (Figure 4.7B & 4.7D). Negative controls for the hybridisation procedure with sense probe did not show any DIG positive signals (Figure 4.7F).
Figure 4.6: Relative expression of the cell wall acid invertase gene (OsCIN4) at different developmental stages. Vertical bars are standard errors (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by randomisation test (P<0.05).
Figure 4.7: Localisation of the cell wall acid invertase gene OsCIN4 within transverse sections of anther by in situ hybridisation. ml middle layer; t tapetum; lo locule; vb vascular bundle; mi microspore. Scale bars: 20 µm.

Hybridisation using antisense probes: (A) Section from a control plant at the microspore stage. Strong expression was observed in tapetum layer and microspores (black arrows). Weak positive signals were also found at the vascular bundle. (B) Section from a stressed plant at the microspore stage. OsCIN4 expressed strongly in the tapetum layer and microspores and to a much lesser extent in the vascular bundle (black arrows). (C) and (D) Sections from control and stressed plants respectively at the vacuolated microspore stage. Expression of OsCIN4 at this stage was similar to the microspore stage: strongly expressed in the tapetum and microspores and weakly expressed in the vascular bundle. (E) Close up image of an anther locule shows DIG positive signal mainly found in the tapetum, microspores and to a lesser extent in the middle layer.

Hybridisation using sense probes as negative control. (F) No positive DIG signal was observed.
4.3.3. Expression of the sucrose transporter gene OsSUT5

Expression of the sucrose transporter gene OsSUT5 was also analysed using qRT-PCR and in situ hybridisation. qRT-PCR assays illustrated a gradually increasing level of this transcript in control anthers during the course of development from the tetrad to the vacuolated microspore stage (Figure 4.8). Approximately 9 times the amount of this mRNA was recorded in untreated plants in this period (Figure 4.8). Three days of water deficit treatment did not influence the relative expression of OsSUT5 at the tetrad stage. However, the transcriptional regulation of this gene was dramatically stimulated at the microspore and vacuolated microspore stage (Figure 4.8).

![Figure 4.8: Relative expression of the sucrose transporter gene (OsSUT5) at different developmental stages. Vertical bars are standard errors (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by randomisation test (P<0.05).](image)

On average, the relative expression of OsSUT5 in stressed anthers was four fold and ten fold higher at the microspore and vacuolated microspore stages in comparison to the respective control (Figure 4.8). In order to understand the mechanism of this
transcriptional up-regulation, spatial expression of this gene was further explored by in situ hybridisation. As early as the microspore stage of anther development, OsSUT5 was expressed strongly in the tapetum layer and the young microspores (Figure 4.9A). No clear hybridisation signal was seen in the vascular bundle at the microspore stage (Figures 4.9A & 4.9B). However, expression of this transcript has been found at low levels in the vascular bundle tissues at the vacuolated microspore stage (Figures 4.9C, 4.9D).

Although this is a qualitative assay, in situ hybridisation also suggested an increasing expression of the gene in the later stages of development compared to earlier stages (Figures 4.9A & 4.9C). This result is consistent with the data obtained using the qRT-PCR assay (Figure 4.8). Similar to the spatial expression of OsCIN4 presented above, water deficit treatment did not alter the positional expression of OsSUT5 (Figures 4.9B & 4.9D). Close up images of in situ hybridisation ascertained the position of OsSUT5 within the anther locule (Figure 4.9E). The hybridisation signal of OsSUT5 was found in the young microspores, and the inner tapetal and middle layers of the anther (Figure 4.9E). The negative control did not show any evidence of DIG labelling within the section (Figure 4.9F).
**Figure 4.9:** Localisation of the sucrose transporter gene OsSUT5 within anther sections by *in situ* hybridisation. ml middle layer; t tapetum; lo locule; vb vascular bundle. mi microspore. Scale bars: 20 µm.

Hybridisation using antisense probes: (A) and (B) Sections from control and stressed plants respectively at the microspore stage. Strong expression was observed in the tapetum layer and microspores (black arrows). Little or no expression was found in the vascular bundle. (C) and (D) Sections from control and stressed plants respectively at vacuolated microspore stage. Strong expression of OsSUT5 was observed at the tapetum; microspores and to a lesser extent at the vascular bundle. (E) Close up image of an anther locule shows DIG positive signal distinctively in tapetum, middle layer and microspores (black arrows).

Hybridisation using sense probes as negative control (F) No positive DIG signal was observed.
4.3.4. Expression of the monosaccharide transporter gene OsMST7

Monosaccharide transporters play an important role as active carriers for transportation of hexoses e.g. glucose, fructose, etc, within living cells. To elucidate the contribution of these proteins to anther development, temporal and spatial expression of a monosaccharide transporter gene (OsMST7) was investigated. The data showed that expression of this gene was at low levels as early as the tetrad stage of anther development (Figure 4.10, inset). During the course of development, the transcriptional regulation of OsMST7 became more abundant from the microspore to the vacuolated microspore stage (Figure 4.10).

![Figure 4.10](image)

**Figure 4.10**: Relative expression of the monosaccharide transporter gene (OsMST7) at different developmental stages. Vertical bars are standard errors (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by randomisation test (P<0.05).
Expression of OsMST7 appeared up-regulated in the post-stress period at the tetrad stage (Figure 4.10). However, this up-regulation was not statistically significant because of high variation between the samples (P>0.05) (Figure 4.10 & Table 4.3). Nevertheless, water deficit effectively induced expression of this gene at the microspore and vacuolated microspore stage (P<0.05) (Figure 4.10). OsMST7 expression was about 60 and 30 fold more abundant in stressed anthers at the microspore and vacuolated microspore stage respectively in comparison to the non-treated control (P<0.05) (Figure 4.10).

Localisation of this gene within the anthers is presented in Figure 4.11. During development from the microspore to vacuolated microspore stage, expression of this gene was found at high levels in the young microspores, tapetal cells and to a much lesser extent in the vascular bundle as well as the middle layer (Figures 4.11A, 4.11C & 4.11E). OsMST7 expression did not change position post water stress (Figures 4.11B & 4.11D). *In situ* hybridisation results also indicated that this gene was up-regulated after the three day water deficit stress compared to the control during the same developmental period (Figure 4.11B & 4.11D). These data agree with results from the qRT-PCR assay presented above (Figure 4.10).
Figure 4.11: Localisation of the monosaccharide transporter gene OsMST7 within anther sections by in situ hybridisation. ml middle layer; t tapetum; lo locule; vb vascular bundle. mi microspore. Scale bars: 20 µm.

Hybridisation using antisense probes: (A) and (B) Sections from control and stressed plants respectively at the microspore stage. Strong expression was observed in the tapetum layer and microspores (black arrows). Little expression was found in the vascular bundle. (C) and (D) Sections from control and stressed plants respectively at the vacuolated microspore stage. Strong expression of OsMST7 was observed in the tapetum; microspores and to a lesser extent in the vascular bundle. (E) Close up images of an anther locule show strong DIG positive signal in the tapetum, microspores and to a lesser extent in the middle layer (black arrows).

Hybridisation using sense probes as negative control (F) No positive DIG signal was observed.
4.3.5. Expression of the fructokinase gene OsFKI

To unveil the role of fructokinase enzymes during anther development, the expression of a fructokinase gene (OsFKI) was investigated in this study. The transcriptional regulation of OsFKI gradually decreased from the tetrad to the vacuolated microspore stage in control anthers (Figure 4.12). Water deficit treatment for three consecutive days however, did not significantly affect its expression at any of the three stages (P<0.05) (Figure 4.12).

![Figure 4.12](image_url): Relative expression of the fructokinase gene (OsFKI) at different developmental stages. Vertical bars are standard errors (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by randomisation test (P<0.05).

4.3.6. Expression of the hexokinase gene OsHXK3

Transcriptional regulation of a hexokinase enzyme is also included in this study. Temporal expression of a hexokinase gene (OsHXK3) during early stages of anther development was analysed. The relative expression of OsHXK3 gradually increased from the tetrad to the vacuolated microspore stage in control anthers (Figure 4.13).
Figure 4.13: Relative expression of the hexokinase gene (OsHXK3) at different developmental stages. Vertical bars are standard errors (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences between the treatment and control for each stage by randomisation test (P<0.05).

A three day water deficit treatment did not affect expression of this gene at the tetrad and microspore stage (Figure 4.13). However, water deficit occurring at the vacuolated microspore stage significantly repressed expression of this gene by 3-fold compared to the control anthers (Figure 4.13).

To ascertain the role of the aforementioned genes during anther development, the abundance of their transcripts during time course was compared with the housekeeping gene OsTuba1 (see Chapter 2) using cycle crossing point (CP) values derived from qRT-PCR reactions. The more abundant the transcripts, the fewer PCR cycles it takes to reach the CP. Results illustrate that the CP of OsTuba1 is around the 15th cycle (Table 4.3) which is considered to be abundantly expressed (Oliver et al., 2005). The expression of OsCIN4, OsFKI and OsHXK3 may be considered high in this context (Table 4.3). Nevertheless, expression of OsSUT5 and OsMST7 was lower at the early stage (tetrad) and increased steadily at later stages (Table 4.3). Moreover, the data
presented in Table 4.3 again confirmed that expression of OsTuba1 was consistent for non-treated control and water stressed samples at all stages of investigation.

<table>
<thead>
<tr>
<th>Gene/Stage</th>
<th>Control samples</th>
<th>Stressed samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tetrad</td>
<td>MS</td>
</tr>
<tr>
<td>OsTuba1</td>
<td>14.7 ± 0.5</td>
<td>14.9 ± 0.4</td>
</tr>
<tr>
<td>OsCIN4</td>
<td>15.2 ± 0.2</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>OsSUT5</td>
<td>24.1 ± 0.2</td>
<td>23.4 ± 1.3</td>
</tr>
<tr>
<td>OsMST7</td>
<td>30.3 ± 0.7</td>
<td>26.3 ± 1.3</td>
</tr>
<tr>
<td>OsFKI</td>
<td>14.6 ± 0.3</td>
<td>15.2 ± 0.1</td>
</tr>
<tr>
<td>OsHXK3</td>
<td>16.1 ± 0.3</td>
<td>14.6 ± 0.1</td>
</tr>
</tbody>
</table>

Table 4.3: Mean cycle crossing point (CP) (n=4) ± standard error (SE) of target genes. MS microspore, VM vacuolated microspore.

4.4. Discussion

4.4.1. Understanding carbohydrate unloading and transport in anthers

To identify the role of carbohydrate metabolism during anther development and how it is affected by water deficit, it is critical to understand the mechanism of sugar unloading and transport within the cellular complex of anthers. Because of its ability to travel over a long distance and its stability from degrading enzymes, sucrose is the main form of photoassimilate for transportation in most eukaryotic plants (Kuhn et al., 1999; Sauer, 2007). In the present study, the presence of high levels of sucrose compared to hexoses (Figures 4.3, 4.4, & 4.5) suggested that sucrose is the common substrate of soluble carbon flux in the rice anther.

Sucrose synthesised in the source organs could be transported to the sink via long distance phloem transport (Patrick, 1997; Oparka & Cruz, 2000). In sink cells like
those in anthers, this disaccharide could be unloaded at the connective tissue, which is continuous with the epidermis and endothecium and further exported to inner layers and locules by different mechanisms (Lalonde et al., 2003). The presence of OsCIN4, OsSUT5 and OsMST7 at the vascular bundle (Figures 4.7AC, 4.9C & 4.11AC) suggests that these proteins may be involved in the apoplastic unloading of sugar from the sieve element – companion cell complex (Lalonde et al., 1999; 2003). Nevertheless, Alfred (2006) demonstrated that symplastic unloading of sugar is also potentially active. Perhaps, both symplastic and apoplastic pathways of sugar unloading are working concurrently in the anther.

As mentioned in Chapter 1, sucrose unloaded in the anther connective tissue could either be effluxed directly to the inner layers and locules via sucrose transporters or be first hydrolysed by cell wall invertases and then newly formed hexoses would be imported to the locules by monosaccharide transporters (Lalonde et al., 1999; Sturm & Tang, 1999). Before meiosis, the supply of sugar from the connective tissue to the tapetum and locules might not involve apoplastic pathways due to the availability of the plasmodesmal connection between most cell layers in rice anthers (Li, 2005; Mamun et al., 2005a). Transported sugar from the source leaves could discharge at the vascular bundle and diffuse to anther wall layers and locules via plasmodesmata. In addition, as the meiocytes are connected by cytoplasmic channels that disappear concurrently with deposition of the callose wall (Li, 2005; Mamun et al., 2005a), the meiocytes may absorb nutrient and sugar directly from the locular fluid or from adjacent cells by a simple passive diffusion. The advent of apoplastic transport is generally signalled by low plasmodesmatal frequency (Turgeon, 1996) and enhanced activity of its crucial facilitators such as sucrose hydrolytic enzymes and active sugar carriers. However, expression of cell wall acid invertase (OsINV4) and monosaccharide transporter
(OsMST8) genes has not been detected until the tetrad stage in rice anthers (Oliver, 2004; Alfred, 2006). Consistent with these reports, data in this study show that transcripts of another monosaccharide transporter (OsMST7) and a sucrose transporter (OsSUT5) were not abundant in the anther at the tetrad stage (Figures 4.8, 4.10 & Table 4.5). These data might again confirm that symplastic unloading of sugar is the most likely mechanism before the meiotic stage of anther development. It is possible that apoplastic transport of sugar only occurs from the tetrad stage onwards because the newly formed tetrad microspores are symplastically isolated from the anther wall layers i.e. tapetum, middle layer and callose walls (Li, 2005; Mamun et al., 2005b) and are coupled with gradually increased activity of sugar carriers such as OsMST8 (Alfred, 2006), OsSUT5 and OsMST7 (Figures 4.8, 4.10 & Table 4.5). Although the tapetum starts to degenerate at the vacuolated microspore stage, the middle layer remains unchanged (Clement & Audran, 1995; Li, 2005), therefore the apoplastic pathway may continue to remain a crucial and sole mode for sugar transport from the middle layer to the locules during development of microspores from the tetrad stage through to mature pollen.

There are some proposed models of sugar unloading and transport within the anther locule in the literature. Clement and Audran (1995) hypothesised a model of sugar unloading in the anther of *Lilium* where sugars could be discharged at the middle layer and apoplastically transported to tapetal cells and finally to the locules. Likewise, the uptake of sugars from the developing microspore could also be carried out via apoplastic carriers. Alfred (2006) observed a monosaccharide loading into rice anthers under laser confocal microscopy using the fluorescent sugar analogue probe 6-deoxy-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoglucose (6NBDG). The author assumed that the 6NBDG could be transported via diffusion from the connective tissue into the
locular fluid and into the cytoplasm of the developing microspores via a monosaccharide transporter (OsMST8) (Alfred, 2006). Finally a model of sugar transport combining information from spatial expression of OsINV4, OsMST8 and 6NBDG in rice anther at different stages of development was proposed (Figure 4.15) (Alfred, 2006). Nevertheless, the involvement and actual role of sucrose transporters have not been experimentally determined.

The results from the present study, using the in situ hybridisation technique, agree with the findings of Alfred (2006) and clearly demonstrate the presence and strong expression of a monosaccharide transporter gene within the anther i.e. OsMST7 (Figure 4.11). The sucrose being transported might be first hydrolysed into hexoses by a cell wall acid invertase OsCIN4 (Figure 4.7) that was coordinately expressed with the OsMST7 (Figure 4.11). Spatially, these two genes were detected in mainly the young developing microspores, tapetum, and to a much lesser extent in the middle layer and vascular bundle (Figures 4.7 & 4.11). The expression pattern of these genes indicates a distinct mode of sugar transport from the anther walls to the locules and young microspores. However, as also reported in the above-mentioned model, carbohydrate present in the anther locule and young microspores is not restricted to monosaccharides alone. The existence of cell wall acid invertases OsCIN4 (Figure 4.7) and OsINV4 (Oliver et al., 2005), in young microspores logically infers the presence of soluble sucrose in the anther locule. In addition to results presented in previous reports (Alfred, 2006), the in situ hybridisation results in the present experiments have indicated that there was participation of a sucrose transporter, OsSUT5, in the apoplastic transport of sucrose (Figure 4.9) in the anther. Similarly to OsCIN4 and OsMST7, OsSUT5 was found to be abundantly expressed in young microspores, the tapetum, and middle layer and to a much lesser degree in the vascular bundle (Figures 4.9A & 4.9C). Strong
expression of OsSUT5 in the middle layer suggests that this layer is likely to be the site for sucrose retrieval, which will be then transported to the tapetum and anther locules without prior cleavage to hexoses by cell wall acid invertase (Figure 4.9E). Indeed, another isoform of OsSUT5, the sucrose transporter OsSUT2, has been known to be expressed in developing pollen (Takeda et al., 2001). Kong et al. (2007) confirmed the important role of a sucrose transporter gene OsSUT3 during anther development in CMS and fertile lines. Moreover, a recent publication by Castro & Clement (2007) has proved the presence of sucrose in the anther locule of Lilium. The authors reported that high levels of sucrose have been found in locular spaces and to a lesser extent in the anther walls and young microspores during early stages of anther development.

In summary, there is strong evidence of sucrose unloading in the vascular bundle followed by symplastic transport to the middle layer. The symplastic transport of sucrose from the middle layer to inner sites could be continued until meiosis. Due to the formation of the callose wall around the pollen mother cells, and compression and degeneration of the middle layer, the transport of sucrose from the middle layer inwards switches from the symplastic to apoplastic mode during meiosis. Co-existence of OsCIN4, OsSUT5 and OsMST7 in the middle layer, tapetum and young microspores from the tetrad stage onwards supports this hypothesis (Figures 4.7E, 4.9E & 4.11E).
Figure 4.14: Proposed model of sugar transport at different stages of anther development. Adapted from Alfred (2006).
4.4.2. Sugar modulated gene expression in anther under drought

The present results clearly illustrate that anthers from plants experiencing mild water stress for three consecutive days accumulated total soluble sugars (Figure 4.1). The accumulation of the total soluble sugars, however, was not restricted to any particular stage, but applied to all developmental stages i.e. water stress imposed at any time during anther development from the pollen mother cell to vacuolated microspore stage caused a statistically significant accumulation of sugar (P<0.05) (Figure 4.1). The data also show that the level of reducing sugars, mainly glucose and fructose, was also remarkably increased after stress (P<0.05) (Figure 4.2). Significantly increased levels of individual sugars such as sucrose, glucose and fructose were also observed in anthers from stressed plants (P<0.05) (Figures 4.3, 4.4 & 4.5).

Earlier work by Dorion et al. (1996) and Sheoran & Saini (1996) has illustrated that severe water deficit stress occurring during reproductive phases can cause an accumulation of total sugar and non-reducing sugars in wheat and rice anthers, while the level of reducing sugars fluctuated. Accumulation of non-reducing sugars (mainly sucrose) was predicted to be the result of impairment of the vacuolar and cell wall acid invertase (Saini & Westgate, 2000).

Similar to the rising level of non-reducing sugars, the accumulation of reducing sugars e.g. glucose and fructose, in stressed anthers may also imply that other downstream pathways of hexose utilisation e.g. glycolysis, might have been disrupted. It could be proposed that the accumulation of reducing sugars might be partly the result of down regulation of enzymes involved in glycolysis in the anthers after water stress. Down regulation of these enzymes might potentially hamper input supplies such as pyruvate for the TCA cycle in mitochondria, which subsequently affects ATP production in the cell.
Of the enzymes involved in this pathway, fructokinase and hexokinase are the most important enzymes for initial steps in hexose phosphorylation in sink tissues (Quick & Schaffer, 1996). Fructokinases play a critical role in sucrose metabolism to maintain hexose flux in the sink cells. They phosphorylate hexoses, especially fructose, to provide energy and input for the tricarboxylic acid cycle (TCA). Fructokinase protein has been shown to be present in rice anthers (Kerim et al., 2003). Hexokinases are also important enzymes that catalyse the initial step of sugar phosphorylation in sink cells. Along with fructokinases, they utilise hexoses as substrates to provide energy and input for the TCA cycle. Activity of these two enzymes has been shown to be affected under environmental stress. Karni & Aloni (2002) demonstrated that fructokinase activities were decreased by approximately 50% and 30% in pollen grains and developing anthers respectively in Bell pepper (Capsicum annuum L.) after heat stress. Likewise, the authors also proved that heat stress caused about 50% reduction of hexokinase activity in developing anthers.

Intriguingly, qRT-PCR data illustrated that a water deficit of -0.5 MPa osmotic potential did not significantly affect expression of OsCIN4 at early stages i.e. the tetrad and microspore stage (P<0.05) (Figure 4.6). Likewise, expression of OsFKI in the stressed samples did not change significantly when compared to the control after water deficit treatment in all stages (P<0.05) (Figure 4.12). Similarly, expression of OsHXK3 remained unchanged at the tetrad and microspore stage (P<0.05) (Figure 4.13). A relatively high expression of these mRNAs compared to the housing keeping gene, OsTuba1 (Table 4.5), suggests that their corresponding proteins might be functionally very active during the early stages of anther development. These results refute that glycolysis, or the down regulation of enzymes involved in glycolysis, are the cause for soluble sugar accumulation. These results pose an interesting question; are changes in
gene expression caused by sugar accumulation after water deficit treatment or vice versa? As mentioned above, down regulation of some sucrose hydrolytic enzymes has been assumed as the reason for sugar accumulation in rice and wheat anthers post stress (Dorion et al., 1996; Sheoran & Saini, 1996; Oliver et al., 2005). However, it is reasonable to expect that abnormally high levels of sugar might repress or induce sugar-regulated genes and hence influence their transcription under water deficit conditions (Koch, 1996; Roitsch, 1999; Ho et al., 2001; Ramanjulu & Bartels, 2002; Rolland et al., 2006; Huang et al., 2007). Indeed, transcription and post-transcription of two acid invertase genes Ivr1 and Ivr2, which are expressed abundantly in pollen and anthers of maize (Zea mays L), was found to be differentially modulated by either sucrose or glucose application; high concentrations of sugars significantly repressed Ivr1, whereas they enhanced Ivr2 (Xu et al., 1996). Likewise, expression of a sink specific cell wall acid invertase gene, Lin6 in tomato (Lycopersicon esculentum cv Moneymaker), has been shown to be induced by glucose (Godt & Roitsch, 1997). Moreover, sugar modulates not only transcriptional expression of metabolic enzymes but active sugar carriers and their transport activity as well. Application of exogenous sugar (either sucrose or glucose) at high concentrations effectively decreased expression of both sucrose transporter, VfSUT1, and monosaccharide transporter, VfSTP1, in fava bean cotyledons (Weber et al., 1997). Similarly, high levels of exogenous sucrose have repressed transcription of a sucrose transporter gene, BvSUT1, and its functional protein in sugar beet leaves (Beta vulgaris Linnaeus) (Chiou & Bush, 1998; Vaughn et al., 2002). In contrast, increased levels of endogenous sugar have been known to induce transcriptional expression of OsSUT1 in the rice embryo (Matsukura et al., 2000). Different glucose concentrations in the cell suspension culture medium have been shown to regulate the expression of a monosaccharide transporter VvHT1, its protein
and sugar transport activity in grape (*Vitis vinifera*) (Conde *et al.*, 2006). High concentrations of exogenous glucose reduced VvHT1 transcription and glucose uptake, whereas low glucose concentrations induced VvHT1 expression and glucose consumption of suspension cultured cells (Conde *et al.*, 2006).

Consistent with those reports, drought-induced accumulation of sugar might have down-regulated expression of OsCIN4 (Figure 4.6) and OsHXK3 (Figure 4.13) in the late stages, while it had no effect on OsFKI (4.12) in the present study. In contrast, the expression of OsSUT5 (Figures 4.8) and OsMST7 (Figure 4.10) were strongly induced from the microspore to vacuolated microspore stage. It is pertinent to remember that water stress affected expression of OsCIN4 and OsHXK3 in the same manner i.e. no alteration at the tetrad and microspore stages, and sharp down-regulation at the vacuolated microspore stage (P<0.05) (Figures 4.6 & 4.13), while at the same time, stress affected expression of OsSUT5 and OsMST7 showed the reverse trend i.e. no change at the tetrad stage, and gradually becoming up-regulated from the microspore to vacuolated stage (P<0.05) (Figures 4.8 & 4.10). In addition, the level of total sugars (Figure 4.1) and hexoses (Figures 4.4 & 4.5) were shown to reach the highest level at the vacuolated stage compared to the tetrad and microspore stage under water deficit conditions. It may be that this very high level of sugar at the vacuolated microspore stage suppressed expression of OsCIN4 and OsHXK3 in the present study (P<0.05) (Figures 4.6 & 4.13). Similarly, the accumulation of sugar induced the expression OsSUT5 and OsMST7 at the microspore and vacuolated stage (P<0.05) (Figures 4.8 & 4.10). Perhaps, accumulation of sugar at the tetrad stage (Figures 4.1, 4.4 & 4.5) was not high enough to up-regulate OsSUT5 and OsMST7 genes in the stressed anther compared to the controls.
4.4.3. Association of carbohydrate metabolism with abortion of young microspores post water stress

As discussed in the introduction to this chapter, the most important question remains as to whether the disturbance of sugar metabolism in rice anthers is the cause of, or at least associated with, the failure of young microspore development after water deficit stress.

Some studies suggested that transcriptional down-regulation of sugar transporters and sucrose hydrolytic enzymes (e.g. vacuolar and cell wall acid invertases) might have resulted in a blockage of sugar supply to the developing microspore, which later caused pollen sterility in rice under chilling stress (Oliver et al., 2005; Alfred, 2006) and in wheat under drought stress (Koonjul et al., 2005). However, this may not be the case in the current experiments. As discussed in the previous sections, temporal expression of OsSUT5 and OsMST7 increased gradually from the tetrad to the vacuolated microspore stage suggesting that they play an important role during anther development under normal growing conditions (Figures 4.8 & 4.10). Spatially, these two isoforms are expressed mainly in young microspores, the tapetum and middle layer and to a much lesser extent in the vascular bundle of the anther (Figures 4.9A, 4.9C, 4.11A & 4.11C), which may imply that they facilitate the inward flux of sugar to the tapetal cells and the locules, which become symplastically isolated by the end of meiosis (Li, 2005; Mamun et al., 2005b). Three-day water deficit induced expression of OsSUT5 and OsMST7 gradually from the microspore to the vacuolated microspore stage with no changes of spatial expression of these genes (Figures 4.9B, 4.9D, 4.11B & 4.11D). These data suggest that the level of sugar e.g. sucrose and hexoses was not depleted in the tapetum and anther locules after water deficit treatment; in contrast it probably increased substantially. The down-regulation of OsCIN4 (P<0.05) (Figure
4.6), which is mainly expressed in the tapetum and microspore (Figure 4.7) and OsHXK3 (P<0.05) (Figure 4.13) at the vacuolated microspore stage, may be caused by this very high concentration of sugar in the tapetum and microspore.

Hence, it could be speculated that the reduction of male fertility under drought was attributed to the failure of downstream sugar utilisation during respiration which caused a lack of energy for the developing microspores. Indeed, sterile anthers under chilling stress have been reported to be closely correlated with considerably lowered respiratory activity (Toriyama & Hinata, 1984). Respiratory pathways in plants can be classified into three main categories: glycolysis, the TCA cycle which is in the mitochondrial matrix and the electron transport chain which is in the inner mitochondrial membrane, and all are essential sources of energy and other physiological functions (Fernie et al., 2004). Although the pentose phosphate pathway cannot produce ATP directly from glucose like glycolysis, it plays an important role in producing intermediates e.g. amino acids, nucleotides and NADPH (Lambers et al., 1998b). Glycolysis which occurs in both the cytosol and the plastid oxidises hexoses to generate ATP and pyruvate, an important fuel of the TCA cycle (Plaxton, 1996). Nevertheless, the qRT-PCR data in this study show that water deficit did not affect expression of OsFKI (P<0.05) (Figure 4.12) nor OsHXK3 at least at the tetrad and microspore stage (P<0.05) (Figure 4.13). These findings suggest that the problem might not be due to glycolysis in this case.

There are two assumptions to consider at this point. Firstly, even though phosphorylated sugar is made available to feed downstream processes, respiratory pathways (e.g. the TCA cycle and oxidative phosphohylation) in mitochondria, might have failed to utilise this resource leading to a shortage of energy and hence causing young microspore death. Secondly, water deficit might have induced production of
lethal sporocides such as reactive oxygen species (ROS) that in turn cause direct damage to the cell. Thus there might have been accumulation of sugar in order to protect young microspores from this damage. Either of these assumptions might be possible in this case.

On the one hand, accumulation of osmolytes like sugars in the cytoplasm has been reported to provide a better osmotic balance under dehydration conditions while not interfering with normal metabolic pathways (Bray, 1997; Bray et al., 2000; Bartels & Sunkar, 2005). The ability to maintain osmotic adjustment has been proposed as an important agronomic criterion for improving drought tolerance in rice (Jongdee et al., 2002). Higher levels of sugar observed in the present work might have conferred a protection to the young microspores rather than causing sterility as male fertility was less affected (Chapter 3) when the level of sugars was rising under drought (Figures 4.1, 4.2, 4.3, 4.4 & 4.5). Indeed, increasing levels of endogenous soluble sugar have been shown to better maintain osmotic adjustment for water stressed medicinal plants (Tan et al., 2006). Accumulation of trehalose, a non-reducing disaccharide of glucose, has been illustrated to provide a better protection for transgenic rice under abiotic stress such as salinity, drought and chilling conditions (Garg et al., 2002). Similarly, high levels of other sugars such as raffinose and galactinol have been shown to improve drought tolerance during seed development in transgenic Arabidopsis (Taji et al., 2002). Possible molecular mechanisms for the protection provided by sugar against drought will be further discussed in Chapter 6.

On the other hand, it has been well documented in the literature that mitochondrial respiration plays an important role in energy generation and carbon metabolism of plant cells (Siedow & Day, 2000). Possibly, mitochondrial respiration has failed to generate sufficient energy for the developing microspores. Concurrently
the improper respiration could lead to excessive production of ROS during electron transport, at the same time resulting in oxidative damage. Failure of mitochondrial functions has also been reported to be associated with an excessive generation of ROS, depletion of the ATP pool, and pollen failure in CMS rice (Li et al., 2004; Wan et al., 2007) and these events will be the topic for further investigation in Chapter 5.

4.5. Conclusion

This chapter has provided a comprehensive review of sugar metabolism in rice anthers. The level of carbohydrates and the expression of the cell wall acid invertase OsCIN4, the sucrose transporter OsSUT5, the monosaccharide transporter OsMST7, and the two genes of glycolytic pathways, OsFKI and OsHXK3 have been presented under water deficit stress. Supplemental data to support the existing model of carbohydrate unloading and transport within the rice anther (Alfred, 2006) has been postulated and discussed. Water deficit-induced accumulation of sugars has been shown to be correlated with repressed expression of OsCIN4, OsHXK3 and with the up-regulation of OsSUT5 and OsMST7. Up-regulation of OsSUT5 and OsMST7 seems to provide a better sugar supply to the tapetum and anther locules and this corresponds to the alleviation of male fertility reduction under drought (Chapter 3). Although the present work only looked at transcriptional regulation of sugar metabolism in rice anthers, the results indicate that water deficit might not cause sugar starvation for developing microspores as previously thought, nor inhibit the initial steps of sugar utilisation e.g. glycolysis. Nevertheless, it remains to be identified whether water deficit has disturbed respiratory pathways in mitochondria, which would result in an insufficient supply of energy and possible production of sporocides such as ROS.
CHAPTER 5: WATER DEFICIT INDUCED PROGRAMMED CELL DEATH AND OXIDATIVE STRESS IN RICE ANTHERS

5.1. Introduction

As demonstrated in the Chapter 3, a water deficit treatment of equal to or less than -0.5 MPa osmotic potential imposed for three consecutive days during reproductive development significantly inhibited microspore development and reduced grain set. The decreased number of viable mature pollen leading to unsuccessful or poor pollination at flowering is also a well-known feature of cytoplasmic male sterility (CMS) phenotypes (Schnable & Wise, 1998). CMS is a maternally inherited trait and is characterised by the inability to produce functional pollen, although vegetative development is unaffected (Duviek, 1965; Edwardson, 1970). CMS is believed to be a consequence of the inability of mitochondria to meet the energy demands of male gametophyte development. Studies on CMS rice illustrated that pollen abortion of the sterile rice plants was attributed to programmed cell death (PCD) at meiosis and chronic oxidative stress in mitochondria during microspore development (Li et al., 2004; Wan et al., 2007).

As introduced in Chapter 1, PCD, which is an active and inducible process, plays a critical role in the normal development of angiosperms, as well as a response to a variety of diseases, by controlling cell numbers or strategically eliminating infected, damaged cells in defence (Greenberg, 1996, 1997; Vaux & Korsmeyer, 1999). Molecular characteristics often observed in PCD include the release of cytochrome c, activation of cysteine proteases, changes in calcium flux, decreases in the ATP pool, excessive production of ROS, and decreased activity of antioxidant enzymes (Saraste & Pulkki, 2000; Moller, 2001; Tiwari et al., 2002; Moller et al., 2007; Ryter et al., 2007).
During male reproductive development, improper functioning of PCD can lead to abnormal pollen development and subsequent sterility (Sanders et al., 2000; Wu & Cheung, 2000; Ku et al., 2003). Water deficit stress has been shown to cause oxidative stress in plants as a response (Flexas et al., 2006; Zhou et al., 2007) which in turn can stimulate a PCD (Slater et al., 1995; Dumont et al., 1999; Tiwari et al., 2002).

Moreover, signaling proteins associated with PCD events such as 14-3-3 have attracted much attention for several decades. 14-3-3 protein is assumed to act as a dimer binding with other functional proteins and to be involved in many biochemical processes (Fu et al., 2000). In plants, this ubiquitous protein has been shown to be relevant to not only PCD but also a variety of metabolic activities. Several studies have suggested that 14-3-3 proteins can play an anti-apoptotic function (Zhang et al., 1999; Fu et al., 2000; Xing et al., 2000). 14-3-3 proteins also play a fundamental role in the regulation of starch synthesis, ATP production, peroxide detoxification, and participate in modulation of several other important biochemical pathways as an intermediate of ABA signal transduction, gene regulation and environmental stress response (Ferl, 1996; Fulgosi et al., 2002; Schoonheim et al., 2007).

However, there is no information regarding association of PCD and relevant events with pollen sterility under water deficit stress to date. A recent study reported that chilling stress during male reproductive development induced PCD that was associated with pollen sterility in rice anthers (Li, 2005) suggesting a similar symptom might occur in drought-stressed rice. This chapter will present findings about PCD, DNA laddering and related events such as ATP, ROS and the antioxidant system in rice anthers under drought stress. Moreover, expression of a 14-3-3 gene that is claimed to be associated with apoptotic events in mammal and plant tissues is also considered.
5.2. **Materials and methods**

5.2.1. **Plant materials and water stress treatment**

Rice plants were grown hydroponically as described in the Chapter 2, Section 2.2.2. Water stress exposure was conducted as described in the Chapter 2, Section 2.3.2.

5.2.2. **TdT-mediated dUTP nick-end labelling (TUNEL) assay**

Transverse sections of florets embedded in paraffin for immunological localisation were prepared as described in Chapter 2, Section 2.7. After de-paraffinisation and rehydration, sections were pretreated with Proteinase K working solution (20 µg/mL in 10 mM Tris-HCl, pH 7.5) at room temperature for 10 minutes. Sections were then washed in PBS buffer, pH 7.2 (160 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄) twice for two minutes each time.

*In situ* nick-end labelling of nuclear DNA fragmentation was performed in a humidity chamber for 1 h in the dark at 37°C with an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Australia) according to the manufacturer’s instructions. In brief, a 50 µL TUNEL reaction mixture containing 10 µL of terminal transferase enzyme solution and 40 µL of label solution was applied directly onto sections. Sections used for positive control were pretreated with 200 U/mL recombinant DNase I (50 µL DNase I stock solution, 1 mg bovine serum albumin (BSA) and 950 µL of 50 mM Tris-HCl, pH 7.5) for 10 min at 15-25°C to induce DNA strand breaks, prior to labelling procedures. Sections used for negative controls were incubated in 50 µL label solution without enzyme. Labelled sections were immediately observed under a fluorescence microscope (Olympus BX51, Japan) and images were captured for further analysis.
5.2.3. DNA extraction and electrophoresis to detect DNA fragmentation

Anthers were collected for genomic DNA extraction as described in Chapter 2, Section 2.4. DNA was extracted using cetyl trimethylammonium bromide (CTAB) (Sambrook & Russell, 2001). Approximately 30 mg of frozen anthers were ground in liquid nitrogen and 450 µL of extraction buffer containing 50 mM Tris-HCl, 0.7 M NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) CTAB, 1% β-mercaptoethanol, pH 8.0 was added. After vigorous mixing, samples were incubated at 65°C for 30 minutes in a water bath with occasional mixing of the contents. The mixture was incubated with 6 µL of Proteinase K (20 mg/mL) at 37°C for 90 minutes and then 900 µL of chloroform: isoamyl alcohol (24:1) was added and emulsified by shaking. Extracts were centrifuged for 5 minutes at 11,000 rpm at room temperature and the top aqueous layer was transferred to a clean 1.5 mL microcentrifuge tube. The interface between the top and bottom layer in the original tube was washed with a further 40 µL of extraction buffer, centrifuged as before and pooled with the previous extracted aqueous solution. DNA in the aqueous solution was precipitated by adding 400 µL of cold isopropanol and centrifuging for 2 minutes at 11,000 rpm. The DNA pellet was dissolved in 1 mL of cold 70% ethanol and precipitated by centrifugation at 11,000 rpm for 10 minutes. After air drying for 60 minutes in a fume hood, the pellets were re-suspended in 50 µL of TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) and stored at -20°C until required.

DNA samples were quantified using a NanoDrop® ND-1000 spectrophotometer (Biosciences, Australia). Control and stressed samples containing 1 µg of DNA from different stages were electrophoresed in a 1.5% agarose gel at 90V for 1.5 hours. The agarose gel was post-stained in a water bath containing 1.5% ethidium bromide and
visualised under a UV light. The image of the gel was captured by a Gel Doc (Bio-Rad, Australia) digital imaging system.

### 5.2.4. RNA extraction and real-time qRT-PCR

Total RNA extraction and quantitation are as described in Chapter 2, Section 2.5. Table 5.1 shows specific primers of the targeted genes. Initial conventional RT-PCRs for detection and cDNA sequencing to confirm homology of amplified products and transcripts of interest were carried out as described in Chapter 2, Section 2.6. Rice OsTuba1 gene (Genebank ID AF182523) was used as a reference control in the real-time qRT-PCR as described in Chapter 2, Section 2.6. The reaction mixture using gene specific primers is as described in Chapter 2, Section 2.6.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genebank ID</th>
<th>Primer sequence (5'-3')</th>
<th>cDNA size (bp)</th>
</tr>
</thead>
</table>
| 14-3-3              | AJ276594    | [F] CCACTGAGAAAAACACAAACAGT  
[R] TCAGAGTCAACTGTCTTAGCAAC | 152            |
| Catalase            | X61626      | [F] CTCTCCCAGTGTGATGATGATCGTT  
[R] TGCATCTGACATTGTCTGGCCTT | 154            |
| Ascorbate Peroxidase | D45423     | [F] TTACTTCACGGAACCTTATCGTTGAGT  
[R] GAAAGCCTTCTCATCTGAGCAT | 127            |
| Dehydroascorbate Reductase | AY074786 | [F] TGAGTATGATCAGTGAGGATCAA  
[R] ATAAAGGCGGATGAGCTTTCAG | 144            |

**Table 5.1:** Target genes for analysis of expression profiles. F forward primer; R reverse primer.

In general, duplicate qRT-PCR reactions consisting of a reverse transcription step at 50°C for 20 mins, then initial denaturation at 94°C for 5 mins following 35-40 cycles were applied to all genes under investigation. Variations in temperature and steps during the cycles of individual genes are presented in Table 5.2. Specificity of amplified products was monitored by an optional step and a melt curve at the end of qRT-PCR.
reactions. Fluorescent signals were acquired at the last step during cycles. The real-time amplification data were statistically analysed as described in Chapter 2, Section 2.6.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Denaturing</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Optional step</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3</td>
<td>94°C – 20secs</td>
<td>61°C – 30secs</td>
<td>72°C - 25secs</td>
<td>77°C – 15secs</td>
</tr>
<tr>
<td>Catalase</td>
<td>94°C – 20secs</td>
<td>64°C – 30secs</td>
<td>72°C - 25secs</td>
<td>not applied</td>
</tr>
<tr>
<td>Ascorbate Peroxidase</td>
<td>94°C – 20secs</td>
<td>62°C – 30secs</td>
<td>68°C - 25secs</td>
<td>not applied</td>
</tr>
<tr>
<td>Dehydroascorbate Reductase</td>
<td>94°C – 20secs</td>
<td>62°C – 30secs</td>
<td>68°C - 25secs</td>
<td>not applied</td>
</tr>
</tbody>
</table>

**Table 5.2:** Cycling profiles for real time qRT-PCR reactions.

### 5.2.5. Adenosine triphosphate (ATP) assay

ATP was extracted from anther samples according to the method of Botrel and Kaiser (1997) with some modifications. Briefly, frozen anthers (20 mg) were ground in liquid nitrogen. 750 µL of 4.5% HClO₄ was added to the frozen tissue powder in a microcentrifuge tube and vortexed vigorously. After thawing, 18 µL Tris (2 M) was added and the samples were incubated on ice for 30 minutes. The suspension was clarified by centrifugation at 11,000 rpm for 1 min. After removal of the precipitate, the supernatant was adjusted with 5 M K₂CO₃ to pH 7.8 (about 90 µL 5 M K₂CO₃) and centrifuged at 11,000 rpm for 2 minutes. The supernatant was transferred to a new microcentrifuge tube and diluted to 1 mL with sterile water and stored at -80°C until required. ATP concentration was quantified luminometrically by a luciferin-luciferase method (Deluca & McElroy, 1978) following the manufacturer’s protocol included in the ATP Bioluminescent assay kit (Sigma-Aldrich, Australia) with some modifications. In brief, 75 µL of samples was pipetted into each well of a Microlite™ microwell plate (Pathtech, Australia) designed for luminescent assays. A 50 µL ATP assay mix which includes luciferase, luciferin, MgSO₄, DTT, EDTA, bovine serum albumin and tricine
buffer salts was added to each well using a multichannel pipettor. The mixture was then immediately measured in a Fluoroskan Ascent® FL microplate reader (Thermo Labsystems, Australia) using the luminometric function. The same procedure was applied to an ATP standard curve of known concentrations. The ATP content of samples was calculated using this standard curve.

5.2.6. Hydrogen peroxide assay

About 50 mg of frozen anthers were ground in liquid nitrogen and homogenised in 800 μL of ice cold 5% (w/v) trichloroacetic acid (TCA) (Warm & Laties, 1982). The mixture was then vortexed vigorously for 1 minute and incubated on ice for 15 minutes. The cell lysate was subsequently centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was decanted and made to 1 mL with ice cold 5% TCA. Samples were stored at -80°C if not used immediately. Hydrogen peroxide levels in stressed and control samples were measured using the QuantiChrom™ peroxide assay kit (BioAssay Systems, Australia) following the manufacturer’s instructions. This measurement is based on the oxidation of ferrous ion with hydrogen peroxide in the presence of xylenol orange as an indicator (Wolff, 1994). Briefly, a mixture consisting of 20 μL of TCA extract from treated and control samples and 100 μL of working reagent containing sulfuric acid, ferrous ammonium sulfate and xylenol orange was pipetted into clear-bottom 96-well microtitre plates. A similar mixture with 100 μL working reagent containing sulfuric acid and xylenol orange was used as a control blank. The mixtures were incubated for 30 min at room temperature and absorbance was read at 585 nm using a Multiskan Ascent microplate reader (Thermo Labsystems, Australia). The sample peroxide content was calculated by comparing the sample absorbance ΔOD and a calibration curve of known H₂O₂ concentrations.
5.2.7. Mitochondrial activity staining

The staining protocol for mitochondrial activity was adapted from the method of Mamun et al. (2005a) with modifications. Rhodamine 123 (Molecular Probes) (25 mg/L) was used to stain mitochondria. The dye was made up in an anther culture medium as described in Section 3.2.7, Chapter 3 with omission of sucrose. Additional components to the previous anther culture medium (Section 3.2.7, Chapter 3) were as follows: glutamine (500 mg/L), myo-inositol (100 mg/L), arginine (50 mg/L), asparagine (50 mg/L), cystine (50 mg/L), cytidine (50 mg/L), histidine (50 mg/L), isoleucine (50 mg/L), lysine (50 mg/L), methionine (50 mg/L), phenylalanine (50 mg/L), proline (50 mg/L), serine (50 mg/L), threonine (50 mg/L), tryptophan 50 (mg/L), uridine (50 mg/L), valine (50 mg/L), nicotinic acid (1 mg/L), pyridoxine HCl (1 mg/L), thiamine HCl (1 mg/L), maltose (14.4 g/L), and ATP (1 g/L) (Cantrill et al., pers. comm.).

Due to their highly hydrophobic nature and to facilitate the dye uptake, living anthers were dissected longitudinally along the mid-line of the anther filament, and incubated in the dye mixture for 1 h to allow sufficient dye penetration through the cut surface before capture of images. The photos of stained sections were taken using a Zeiss LSM 5 Pascal confocal microscope attached to a Zeiss Axiovert 200M (Carl Zeiss, Germany) illuminated with the 488 nm line of the argon laser.

5.2.8. Statistical analysis

All the experiments were conducted in triplicate unless otherwise stated; the values are expressed as means of three replicates plus standard error, and analysed by one way analysis of variance using Genstat (version 8.0). Least significant differences were calculated to compare significant effects at the 5% level.
5.3. Results

5.3.1. Nuclear DNA degradation caused by water deficit

To test whether water deficit induced a PCD in anthers, a TUNEL assay was performed on both stressed and control anthers. A range of developmental stages was taken into account including tetrad, microspore, and vacuolated microspore stages. To control the background level of TUNEL, both negative and positive control sections were included in the assay for every experiment. For the negative control, terminal transferase enzyme was omitted from the labelling mix and this resulted only in green fluorescent background staining of cell walls and microspore walls (Figure 5.1A). By contrast, the sections for the positive control were first incubated with DNase I to induce DNA strand breaks and then continued with the standard labelling procedure. TUNEL assay showed that pretreatment with DNase I caused an intensive staining of all cell nuclei within the section (white arrows) (Figure 5.1B). Clearly distinct stained nuclei (bright yellow to green spots) were found in single anther lobes, young microspores (Figure 5.1C), and the vascular bundle (Figure 5.1D). Due to the nature of paraffin embedded sections and fluorescence resolution, this staining technique could not accurately localise the signals within anther walls. However, it appears that different anther layers could be identified by TUNEL positive signals represented by concentric circles formed by bright green spots (Figure 5.1B). This information can be used to estimate the amount of positive signals, or the percentage of dead cells, within anther layers.

Detailed TUNEL staining of anthers at the tetrad stage is presented in Figure 5.2. The results indicate that no positive signal was found in control anthers (Figure 5.2A). In contrast, there was more positive staining with TUNEL in the stressed anthers (Figure 5.2B). Figure 5.2C and Figure 5.2D are close-up images showing a single anther.
lobe and vascular bundle, respectively, of an anther at the tetrad stage. Positive signals (white arrows) were found in different cell layers of the anther walls (Figure 5.2C) and vascular bundle (Figure 5.2C). Similarly, TUNEL staining of control anthers at the microspore stage did not show any positive signal (Figure 5.3A). However, a large number of TUNEL positive signals were observed in the stressed anthers (Figure 5.3B). TUNEL positive signals (white arrows) were found abundantly in the single lobe of anthers, the inner most layer (the tapetum), the young tetrads (Figure 5.3C) and vascular bundle (5.3D).
Figure 5.1: In situ fluorescence labelling of DNA cleavage by TUNEL staining in rice anthers (transverse sections).

mi microspore; lo anther lobe; vb vascular bundle. White arrows indicate TUNEL positive signals. Scale bars: 20 µm.

A. Negative control: section labelled without terminal transferase enzyme: only green fluorescence was observed. There was no TUNEL positive signal.

B. Positive control: section was pre-treated with DNase I and labelled following standard procedure. Bright green spots indicate TUNEL positive signals.

C. Positive control: detailed TUNEL staining for single lobe.

D. Positive control: close-up image of TUNEL staining for vascular bundle.
**Figure 5.2:** DNA damage in rice anthers at the tetrad stage. DNA breaks in the nuclei were detected by TUNEL staining. 

mi microspore; lo anther lobe; vb vascular bundle. **White arrows** indicate TUNEL positive signals. Scale bars: 20 μm.

A. Control anthers: only green fluorescence of the background was observed; no TUNEL positive signals were seen.

B. Water stressed anthers: many TUNEL positive signals were observed.

C. Detailed TUNEL staining for single lobe of water stressed anthers.

D. Close-up image of TUNEL staining for vascular bundle of water stressed anthers.
**Figure 5.3:** DNA damage in rice anthers at the microspore stage. DNA breaks in the nuclei were detected by TUNEL staining.

**mi** microspore; **lo** anther lobe; **vb** vascular bundle. **White arrows** indicate TUNEL positive signals. Scale bars: 20 µm.

**A.** Control anthers: only green fluorescence of the background was observed; no positive signals were seen.

**B.** Water stressed anthers: many TUNEL positive signals were observed.

**C.** Detailed TUNEL staining for single lobe of water stressed anthers.

**D.** Close-up image of TUNEL staining for vascular bundle of water stressed anthers.
**Figure 5.4:** DNA damage in rice anthers at the vacuolated microspore stage. DNA breaks in the nuclei were detected by TUNEL staining.

mi microspore; lo anther lobe; vb vascular bundle. **White arrows** indicate TUNEL positive signals. Scale bars: 20 µm.

A. Control anthers: only green fluorescence of the background was observed; no positive signals were seen.

B. Water stressed anthers: many TUNEL positive signals were observed.

C. Detailed TUNEL staining for single lobe of water stressed anthers.

D. Close-up image of TUNEL staining for vascular bundle of water stressed anthers.
Figure 5.4 illustrates TUNEL staining results for the control and water stressed anthers at the vacuolated stage. As visualised for the control anthers at the tetrad and microspore stage, only a green fluorescent background was observed in the control anthers indicating no TUNEL positive signals (Figure 5.4A). Some positive signals (white arrows) were detected in the stressed anthers (Figure 5.4B). TUNEL signals (white arrows) were found in anther walls within a single lobe (Figure 5.4C) and vascular bundle (Figure 5.4D).

The results of the TUNEL assay clearly indicate that no positive signal was found in control anthers at any of the three stages at the light microscope level (Figures 5.2A, 5.3A, & 5.4A). Stressed anthers, in contrast showed positive staining with TUNEL (Figures 5.2B, 5.3B, & 5.4B). These data illustrate that water deficit imposed at any stage during anther development has caused damage to cellular DNA shown by the positive staining with TUNEL. Although the TUNEL assay remains only a qualitative method, it appears that the number of dead cells gradually decreased in developmental order from the tetrad to the vacuolated microspore stage (Figures 5.2B, 5.3B, & 5.4B). This suggests that the level of cellular DNA damage caused by water deficit stress varies depending on the stages of anther development.

To further identify the nature of cellular DNA damage, 1 µg of isolated genomic DNA was electrophoresed in 1% agarose gel. Intriguingly, results shown in Figure 5.5 indicate that no pattern of DNA laddering was found in either the control or stressed samples at different stages of anther development compared to the reference DNA marker. There are only some smear-like patterns in the lanes loaded with DNA from stressed samples (Figure 5.5). The size indicates the smear might be nucleotides. These results suggest that water deficit did not completely fragment the DNA strand. However, it might have caused some nicked DNA damage.
Figure 5.5: Agarose gel analysis of DNA in anthers from control and stressed plants. Total anther genomic DNA (1 µg) was loaded in each lane and electrophoresed using a 1% agarose gel stained with ethidium bromide. M DNA marker 1 kb plus (Invitrogen, Australia); CT control; TM treatment.

5.3.2. ATP level in rice anthers

Possible causes of water deficit-induced PCD were further explored by measuring the concentration of the ATP pool in rice anthers. In control anthers, the level of ATP increased from the tetrad stage (5.29 ng/mg FW) to the microspore stage (7.54 ng/mg FW), and then decreased at the vacuolated microspore stage (4.89 ng/mg FW) (Table 5.3). High levels of ATP during the tetrad and microspore stage indicate essential energy demand for early microspore formation. Interestingly, ATP levels dropped almost by half in stressed anthers compared to the controls at all stages (P<0.001). The decline of this important metabolite might signal adverse effects for
ATP-dependent processes in the cells following water stress. Moreover, the level of ATP from stressed samples did not vary significantly amongst stages of development suggesting constant effects resulting from osmotic stress caused by PEG (Table 5.3).

<table>
<thead>
<tr>
<th>ATP level (ng/mg FW anthers)</th>
<th>Tetrad</th>
<th>Microspore</th>
<th>Vacuolated microspore</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.29 ± 1.09a</td>
<td>7.54 ± 0.18b</td>
<td>4.89 ± 0.24a</td>
</tr>
<tr>
<td>Treatment</td>
<td>2.96 ± 0.72c</td>
<td>3.51 ± 1.99ac</td>
<td>2.47 ± 0.51c</td>
</tr>
</tbody>
</table>

Table 5.3: ATP content in control and water deficit treated samples at different stages of anther development. Data are the mean of three replicates ± standard error (SE). Data showing the same letter are not significantly different (P<0.001), LSD_{0.05} = 2.055 as determined by ANOVA. FW = fresh weight.

5.3.3. Hydrogen peroxide content

The content of hydrogen peroxide during microspore development in control anthers was almost constant in the range of 20.40 - 24.21 ng/mg FW (Table 5.4). This result indicates the ability of the cells to maintain a redox balance throughout development from the tetrad to the vacuolated microspore stage under normal conditions (Table 5.4). The balance appears to be violated by water deficit stress. An approximately 1.5 fold greater concentration of this species was observed in water stressed anthers compared to the control at different stages (P<0.004). Similar to the depleted level of ATP, the increased hydrogen peroxide amongst the water stressed anthers was almost the same at all stages and did not show any statistically significant difference (P<0.004) (Table 5.4). Perhaps the constant level of osmotic stress induced by PEG at different stages has resulted in an equal amount of hydrogen peroxide accumulation in the anther.
<table>
<thead>
<tr>
<th></th>
<th>Tetrads</th>
<th>Microspore</th>
<th>Vacuolated microspore</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.34 ± 1.54a</td>
<td>20.40 ± 3.30a</td>
<td>24.21 ± 3.38ab</td>
</tr>
<tr>
<td>Treatment</td>
<td>30.57 ± 1.45bc</td>
<td>30.51 ± 7.43bc</td>
<td>34.87 ± 2.90c</td>
</tr>
</tbody>
</table>

**Table 5.4:** Content of hydrogen peroxide in the control and water treated anthers. Data are the mean of three replicates ± standard error (SE). Data showing the same letter are not significantly different (P<0.004), LSD_{0.05} = 6.9 as determined by ANOVA. FW = fresh weight.

5.3.4. **Expression of the 14-3-3 gene**

To investigate events associated with PCD at a molecular level, qRT-PCR was conducted on a 14-3-3 gene. As shown in Figure 5.6, the expression of this 14-3-3 gene during microsporegenesis is differentially regulated. In control anthers, the level of expression of the 14-3-3 gene reduced gradually during development from the tetrad to the vacuolated microspore stage (P<0.05) (Figure 5.6). Significant down regulation of this gene from stressed anthers was recorded only at the tetrad stage which appears to have the highest level of dead cells (Figure 5.2), while it remained unchanged at the microspore and vacuolated stage (P<0.05) (Figure 5.6).
Figure 5.6: Expression of the 14-3-3 gene. Vertical bars are standard error (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences by randomisation test (P<0.05).

5.3.5. Expression of antioxidant genes

To get an insight into the response of the antioxidant system of developing anthers under water deficit, the transcriptional regulation of antioxidant genes was conducted and quantified by a real time qRT-PCR assay. Under normal growing conditions, the expression of the catalase gene (CAT) increased from the tetrad to early microspore stage and remained unchanged until the vacuolated microspore stage in control samples (P<0.05) (Figure 5.7). On average, the expression of CAT at the tetrad stage was only half of that at the microspore and vacuolated microspore stages. The expression of CAT in stressed anthers was significantly reduced only at the tetrad stage and remained unaffected at the microspore and vacuolated stage (P<0.05) (Figure 5.7).
**Figure 5.7:** Expression of the catalase gene (CAT). Vertical bars are standard error (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences by randomisation test (P<0.05).

The expression pattern of the ascorbate peroxidase gene (APX) throughout microspore development (Figure 5.8) was the inverse to that of CAT in control anthers. The expression of APX gradually decreased, approximately by half in control samples, from the tetrad to the vacuolated microspore stage (P<0.05) (Figure 5.8). Water deficit appeared to keep APX expressed constantly at all three stages in stressed anthers. The APX gene was significantly down-regulated by roughly 50% at the tetrad stage while it was consistent at the microspore stage under water deficit (P<0.05). However its expression was significantly up-regulated at the vacuolated microspore stage in stressed samples (Figure 5.8).
Figure 5.8: Expression of the ascorbate peroxidase gene (APX). Vertical bars are standard error (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences by randomisation test (P<0.05).

Figure 5.9: Expression of the dehydroascorbate reductase gene (DHAR). Vertical bars are standard error (SE). Data are the mean of four replicates ± SE. The stages labelled with an asterisk (*) indicate significant differences by randomisation test (P<0.05).

Unlike the expression of CAT and APX, the expression of the dehydroascorbate reductase gene (DHAR) remained almost constant throughout the developmental stages studied in control anthers (Figure 5.9). Noticeably water deficit effectively repressed the expression of DHAR by approximately 60% at the tetrad stage (P<0.05). However
expression of this gene did not decline significantly between control and stressed samples at the microspore and vacuolated microspore stage (Figure 5.9).

These qRT-PCR results indicate that expression of all three antioxidant genes was significantly suppressed if water deficit stress occurred around meiosis, while their expression was differentially regulated if water stress took place at other stages.

5.3.6. Mitochondrial activity

Figure 5.10 shows staining results for active mitochondria in longitudinal optical sections of normal anthers. Functional mitochondria were stained with Rhodamine 123 resulting in bright green dots (clumps) within the section. High mitochondrial activity was observed at the pollen mother cell and tetrad stage (Figures 5.10A & 5.10B). At the pollen mother cell stage, active mitochondria appeared to be distributed with similar density within the tapetal and endothecial layer (white arrows) (Figure 5.10A). The middle layer was dark and thin at this stage and seemed to have no mitochondrial staining (Figure 5.10A). However, during the tetrad stage, the highest staining was observed in the tapetum layer compared to other layers within the anther locule (Figure 5.10B, right and left sides). The endothecium layer was difficult to resolve by confocal microscopy at the tetrad stage due to very densely stained areas in the tapetum (Figure 5.10B, left side). The activity of mitochondria seemed to decrease at the microspore to the vacuolated microspore stage compared to previous stages (Figures 5.10C & 5.10D). Noticeably, the activity of mitochondria in the tapetal layer appears to have been strongly reduced at the late stages e.g. the microspores and vacuolated microspore stage (Figures 5.10C & 5.10D).
Figure 5.10: Rhodamine 123 staining of active mitochondria in normal anthers.

ep epidermis; en endothecium; ml middle layer; t tapetum; pmc pollen mother cell; mi microspore; vmi vacuolated microspore. **White arrows** indicate separate layers. Bright green dots (clumps) are mitochondria. Scale bars: 20 µm.

A. Pollen mother cell stage.
B. Tetrad stage.
C. Microspore stage
D. Vacuolated microspore stage
5.4. Discussion

5.4.1. Water deficit induced PCD in anthers

PCD is a phenomenon associated with biochemical and physical changes in the cytoplasm, nucleus and plasma membrane. A hallmark often observed in PCD is the fragmentation of genomic DNA, sometimes called DNA laddering (Arends et al., 1990; Collins et al., 1997; Saraste & Pulkki, 2000). PCD can be analysed by the TUNEL assay which allows for in situ detection of nicked DNA and DNA breaks at the single cell level (Gavrieli et al., 1992) or by the typical DNA laddering that can be visualised using agarose gel electrophoresis (Hale et al., 1996). In this work, a TUNEL assay using anthers from three different stages of development illustrated that PCD did not occur in anthers growing under normal conditions during these investigated periods (Figures 5.2A, 5.3A & 5.4A). However many positive TUNEL signals were observed in water stressed samples (Figures 5.2B, 5.3B & 5.4B) indicating detrimental effects of water deficit on the cellular DNA within the anther and therefore suggesting that an induced PCD had occurred. TUNEL positive signals appear not to be locally restricted to certain cell types. Rather, they could be found in different sites within the transverse section of the anther such as the vascular bundle, tapetum, middle layer and even young microspores (Figures 5.2B, 5.3B & 5.4B). In the water stressed samples, the level of nuclear DNA damage appeared more serious at the tetrad stage (Figure 5.2B), which coincides with water deficit stress during meiosis, compared to the other stages such as the microspore and vacuolated microspore (Figures 5.3B & 5.4B). This symptom was concurrent with a serious reduction of grain set and viable young microspores at the tetrad stage (Chapter 3). These results are also consistent with findings of PCD in rice anthers induced by chilling stress (Li, 2005).
The nature of the PCD was further investigated using a DNA laddering assay (Figure 5.5). Surprisingly, agarose gel electrophoresis did not show orderly fragments of the total genomic DNA ranging from 180 to 200 base pairs, which is often observed in a normal apoptotic process (Bortner et al., 1995). There was no evidence for the formation of DNA ladders in this study (Figure 5.5). This discrepancy again confirmed that an induced PCD rather than a regular apoptosis might have occurred. Indeed, a positive TUNEL result has been known to indicate not only the presence of double-stranded DNA breaks but that of single-stranded DNA breaks with free 3’-OH terminals as well (Gavrieli et al., 1992). Moreover, positive TUNEL signals have been reported to be found in both necrotic cell death as well as apoptotic cell death (Grasl-Kraupp B et al., 1995; Yasuda et al., 1995). Fath et al. (1999) also reported a PCD in barley aleurone cells incubated with gibberellic acid. The treated cells showed nuclear DNA degradation resulting in positive TUNEL staining while no DNA laddering was observed using agarose gel electrophoresis. All of these findings suggest that water deficit directly contributes to cell nuclear DNA damage via PCD in rice anthers.

5.4.2. Molecular and biochemical events associated with PCD

Commonly, PCD takes place in conjunction with biochemical events such as ROS accumulation and reductions in the ATP pool. ATP is a coenzyme and one of the most important compounds in the cellular metabolism of all eukaryotic organisms. ATP is required to maintain the normal function and metabolism in cells. Depletion or inadequate supply of this biological energy source could adversely affect ATP-dependent processes and subsequently cause cell death (Carson et al., 1986; Eguchi et al., 1997; Leist et al., 1997). Therefore, possible causes of water deficit induced PCD were further explored by measuring the concentration of the ATP pool in rice anthers. The data presented illustrate that rice anthers experiencing water deficit at any stage for
three consecutive days showed a decrease in ATP (Table 5.3). Water stress has been known to perturb the ATP pool in plants for some time (Meyer & de Kouchkovsky, 1992; Tezara et al., 1999). This reduction in ATP might have potentially caused disturbance to some dependent biological pathways and consequently caused the cell death observed in the present study.

A decrease of ATP levels that leads to cell death, either via apoptosis or necrosis (a type of induced cell death), has been demonstrated to be dependent on the level of ATP reduction (Skulachev, 2006). Maintenance of the ATP pool is required for apoptosis while depletion of ATP favours necrosis, because apoptosis is an energy-dependent process but necrosis is not (Liu et al., 1996; Eguchi et al., 1997; Leist et al., 1997; Grusch et al., 2002; Troyano et al., 2003). As PEG treatment of -0.5 MPa only causes mild water deficit (Chapter 3), it is likely that PCD rather than necrosis has been induced in this case (Van Breusegem & Dat, 2006). Therefore, this might again confirm that water deficit stress has resulted in an induced PCD in the present study, which is consistent with the results from the DNA laddering assay (Figure 5.5). Microsporegenesis requires a high input of energy for development, therefore the shortage of this ATP will probably disturb downstream dependent processes and these may be the physiological causes for the loss of young microspores in rice under water deficit as discussed in Chapter 3.

*In vivo*, ATP is regenerated from ADP by the F type ATP synthase, which is present in mitochondria and chloroplasts (Sambongi et al., 1999; Stock et al., 1999). During oxidative phosphorylation, ATP synthase reversibly hydrolyses ATP to ADP and phosphate ions. Wasteful hydrolysis of ATP under low oxygen conditions is circumvented by a mechanism that allows IF1, a natural protein, to inhibit this hydrolytic activity (Cabezon et al., 2000). Bunney et al. (2001) described the role of a
novel protein, 14-3-3, as a regulator of mitochondrial and chloroplast ATP synthases via an interaction with the F1 β-subunit. Their experiments on isolated mitochondria and chloroplasts from barley plants illustrated that the 14-3-3 protein is an inhibitor of ATP synthase hydrolytic activities. Abnormal expression of the 14-3-3 gene will also impact adversely on other 14-3-3 regulated cell processes. The differential down-regulation of the 14-3-3 gene in this context (Figure 5.6) might have contributed to the depletion of the ATP pool (Table 5.3) and thus activation of a cell death programme (Figures 5.2B, 5.3B & 5.4B) (Richter et al., 1996).

5.4.3. Water deficit induced oxidative stress

Water stress could possibly stimulate oxidative conditions in rice anthers. This was signalled in the present study by the increased level of hydrogen peroxide in the stressed anthers at all stages of development in comparison to the respective controls (Table 5.4). As a by-product of respiratory metabolism, ROS have been known to increase under biotic and abiotic stresses in plants (Smirnoff, 1993; Tambussi et al., 2000; Tiwari et al., 2002). Failure to control ROS could result in their excessive accumulation and lead to oxidative stress (Foyer & Noctor, 2005a) which in turn decreases the ATP pool. Consistently higher levels of ROS have been shown to cause damage to various biological molecules such as cell membranes, lipoproteins, DNA, and eventually leads to PCD (Droge, 2002; Moller et al., 2007; Ryter et al., 2007). Excessive ROS has also been reported to induce PCD in soybean cells (Levine et al., 1994; 1996) and Arabidopsis suspension cultured cells (Desikan et al., 1998). Under normal growing conditions, plants produce a variety of antioxidants to cope with ROS generation to retain an appropriate redox status within the cells. Catalases and peroxidases are the two major systems for the enzymatic removal of hydrogen peroxide in plants. Catalases catalyse the decomposition of hydrogen peroxide to water and
oxygen. Ascorbate peroxidases are enzymes that detoxify peroxides such as hydrogen peroxide using ascorbate as a substrate. The reaction they catalyse is the transfer of an electron from ascorbate to peroxide producing dehydroascorbate and water as products. Dehydroascorbate reductase is also a critical enzyme playing an important role in maintaining an appropriate level of ascorbate in plant cells to overcome the possible problems of oxidation (Kato et al., 1997). All of these enzymes are responsible for maintaining a favourable redox status in the cells preventing oxidative damage. Indeed, oxidative stress occurring in rice panicles has been shown to be correlated with an inefficient antioxidant defence, which leads to pollen sterility under water stress (Selote & Khanna-Chopra, 2004). Similarly, oxidative damage in anthers of CMS rice resulted from changes in antioxidant pools (Li et al., 2004; Wan et al., 2007). Increased activity of antioxidant enzymes has been demonstrated to confer a better protection for rice from chilling damage (Kuk et al., 2003). In this current work, antioxidant transcripts such as CAT (Figure 5.7), APX (Figure 5.8) and DHAR (Figure 5.9) appeared to be expressed in a coordinated way throughout the development of anthers from the tetrad to vacuolated microspore stage under normal growing conditions. Differential down-regulation of these genes after three days of water deficit stress illustrates that some dysfunction of the antioxidant defence system has occurred. Significant down-regulation of all scavenging genes at the tetrad stage (Figures 5.7, 5.8, & 5.9) after water deficit stress might imply the vulnerability of the antioxidant system around this stage, which is in agreement with the serious decline of viable microspores and hence grain set (Chapter 3). By contrast, stable (Figures 5.7 & 5.9) or even up-regulated (P<0.05) (Figure 5.8) expression of these genes at the vacuolated microspore stage resulted in fewer detrimental effects to the anthers.
5.4.4. Role of the tapetal mitochondria

Staining results for mitochondrial activity in the present work clearly illustrated that there were a large number of functional mitochondria at the pollen mother cell to the tetrad stage that decreased gradually at later stages e.g. microspore and vacuolated microspore stage (Figure 5.10). These data suggest that there was probably a high-energy demand during the pollen mother cell and tetrad stage. Moreover, the presence of numerous active mitochondria in the tapetal layer at the tetrad stage compared to other stages indicates a special requirement of energy supply from the tapetum to young microspores at this stage (Figure 5.10B). Indeed, the tapetum has generally been accepted as the nourishing layer within the anther providing the nutrients and structural components for the developing microspores in the locules (Pacini, 1994; Raghavan, 1997). Morphologically, the number of mitochondria have been shown to increase 20 and 40 fold in pollen mother cells and tapetal cells, respectively, between the pre-callose and tetrad stages in maize anthers during development (Lee & Warmke, 1979). Similarly, a significantly increased number of mitochondria was observed in the tapetum layer after meiosis in rice by TEM techniques (Li, 2005; Mamun et al., 2005b) suggesting a role for tapetal mitochondria in providing energy for developing microspores.

Tapetal dysfunction has been shown to be associated with chilling induced male sterility in rice (Nishiyama, 1984, 1997). Striking similarities between chilling and drought induced pollen sterility in rice suggest similar events could occur in rice anthers under water deficit. In the present work, attempts were made to compare the number of functional mitochondria between the control and drought stressed anthers. However, mitochondrial staining with Rhodamine 123 failed to differentiate the number of active mitochondria in these two types of samples (data not shown) even though it is a specific
probe for localisation and measurement of mitochondrial membrane potential (Johnson et al., 1980; 1981; Chen, 1988). Possibly, Rhodamine 123 staining of mitochondria in living anthers and the laser scanning confocal microscopy technique did not have sufficient resolution to determine subtle changes in mitochondrial membrane potential under this mild water deficit condition (Cantrill, pers. comm.). Nevertheless, evidence from the literature and the current work suggests that the actual problem might reside with mitochondrial activity. For example mitochondrial respiration is major source of ATP synthesis and ROS generation. In addition, mitochondrial malfunction has been associated with ATP reduction and PCD in CMS rice (Li et al., 2004), and this was attributed to excessive accumulation of ROS causing damage to mitochondrial DNA (Wan et al., 2007). Further work is required to confirm if a similar problem does occur under water deficit conditions.

Although water deficit stress for three consecutive days resulted in similar levels of ATP and hydrogen peroxide for all stages of anther development (Tables 5.3 & 5.4), the final consequential effects of drought on the grain set were different among the stages (Chapter 3). This phenomenon is possibly associated not only with the antioxidant defence as discussed in above sections but also with varied allocation of mitochondria within cell layers and amongst stages of anther development. For example anthers at the tetrad stage suffered the most damage which coincides with the highest density of mitochondrial staining in the tapetal cells (Figures 10A & 10B), whereas a lower staining density of mitochondria in the tapetum during the microspore and vacuolated microspore stages (Figures 10C & 10D) was associated with milder water stress effects. This might also explain the tapetal dysfunction mentioned above. Nevertheless, this association needs to be further explored.
5.5. Conclusion

This chapter has attempted to draw a comprehensive and clear picture about impacts of water stress on anthers, which can directly or indirectly cause pollen sterility in rice plants. In summary, three days of mild water stress at -0.5 MPa enhanced ROS production and suppressed the expression, to some extent, of antioxidant genes in rice anthers. Significantly increasing levels of ROS may have caused damage to various cells and led to the depletion of the ATP pool. As a result, PCD was induced and DNA breaks were detected in various cell compartments of the anthers, including developing microspores. This process has also been signalled by differential expression of the 14-3-3 gene. All in all, there is strong evidence of oxidative damage, which is caused by water deficit in this study. Male reproductive development is a complicated and highly organised process that requires harmonious activities of different pathways in a system. Failure or improper functioning of one pathway or whole systems would possibly result in repression of microspore development and subsequent death. Water deficit might have induced oxidative stress in rice anthers which later resulted in PCD and repressed microspore development. This might be one of the main reasons for the reduction of viable microspores post stress, which has been discussed in Chapter 3.
CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1. Introduction

Water deficit occurring during reproductive development frequently results in a decline in grain set which is attributed to a decrease in male fertility (Saini & Westgate, 2000; Boyer & Westgate, 2004). This work has therefore attempted to elucidate the events associated with male fertility reduction under water deficit stress using comprehensive investigations covering different aspects from physiology, biochemistry, and molecular biology.

Initial work in this study involved designing and assessing an osmotic stress induction protocol, which aimed to develop a precise, controllable and easily reproducible drought-induction method. Several levels of osmotic stress were applied to determine the critical osmotic level that effectively influences male fertility represented by parameters such as pollen viability and grain set (Chapter 3). To further explore possible causes of male sterility, a significant part of this work was devoted to understanding carbohydrate metabolism in anthers under normal and water deficit conditions. New findings supplementing to existing models of sugar unloading and transport in anther have been presented and discussed (Chapter 4). Finally, the relationship between PCD, oxidative damage, antioxidant defences stress has been investigated in detail to determine if oxidative stress is the main cause of pollen sterility under water deficit (Chapter 5). This chapter will summarise main findings presented in this thesis and discuss them in a broader context.
6.2. Young microspore viability as a determinant of grain set

Undoubtedly, the final outcome of the male reproductive process is the formation of viable mature pollen which is the key determinant for pollination and establishment of grain set. This important agronomic trait has been used in selecting for drought tolerance in rice (Venuprasad et al., 2007). Many studies have attempted to identify the molecular mechanism of pollen abortion in cereals under abiotic stresses in general and drought in particular (Barnabas et al., 2008). It is quite well known from the literature that the reduction of viable pollen will potentially result in a decline of grain set under adverse conditions. This was also the case in the present work. The results presented in this study clearly demonstrate that osmotic stress equal to or more intense than -0.5 MPa successfully reduced grain set which attributed to a decrease in the number of viable pollen (Chapter 3).

Moreover, the causal relationship between grain set and pollen viability appears to rely on the development of pollen at early stages, as a strong correlation between grain set and viable young microspore has been demonstrated (Chapter 3). It is likely that the reduction of young microspore viability will later determine the number of sterile mature pollen leading to poor or failure of pollination. Hence, the reduction of viable young microspores was demonstrated to indirectly affect the formation of grain set (Chapter 3). Therefore the young microspore viability could be used as an indicator of male fertility at an early stage. Apart from this, young microspore viability could be used to measure the immediate effects of drought on fertility (Chapter 3).

6.3. Molecular mechanism of microspore abortion under drought

Previous work illustrated that carbohydrate metabolism during male reproduction of wheat and rice anthers is disturbed by chilling and drought stress (Dorion et al., 1996; Sheoran & Saini, 1996; Koonjul et al., 2005; Oliver et al., 2005).
Failure to supply hexoses, important substrates of respiratory pathways, has been assumed to cause pollen abortion in cold stressed rice. Chilling stress in rice has resulted in a blockage of sugar supply from the connective tissues to the tapetum and anther locules caused by transcriptional down-regulation of OsINV4 and OsMST8 (Oliver, 2004; Alfred, 2006). However, the results in this study demonstrated that the supply of both sucrose and hexoses from the anther walls to the locules might not be restricted after water deficit stress (Chapter 4). Up-regulation of OsSUT5 and OsMST7 for all three stages of anther development supports this finding (Chapter 4). Clearly the developing microspores were not starved of sugar, a crucial substrate for initial steps in downstream respiratory pathways. Moreover, stable expression of OsFKI and OsHXK3 at the most sensitive period e.g. the PMC and tetrad stage (Chapter 3), confirmed that initial steps of sugar phosphorylation in the glycolytic pathway were not affected by water deficit stress (Chapter 4). Still, one could argue that although OsCIN4 was not affected at the most sensitive stages, selective transcriptional down-regulation of other invertase genes, as occured in drought-stressed wheat (Koonjul et al., 2005), might affect hydrolysis of sucrose within the anther locules leading to a shortage of hexose supply for young microspores. However, results from sugar assays indicated that water deficit caused an increase not only of non-reducing sugar (mainly sucrose), but also of reducing sugar (mainly glucose and fructose) (Chapter 4). As mentioned in Chapter 4, the down-regulation of invertase genes and their translated protein as reported from other research (Dorion et al., 1996; Sheoran & Saini, 1996; Koonjul et al., 2005) might be potentially regulated by increased levels of sugars (Koch, 1996; Roitsch, 1999; Ho et al., 2001; Ramanjulu & Bartels, 2002; Rolland et al., 2006; Huang et al., 2007). At this point, one might infer that immediate reduction of male fertility after drought could potentially result from perturbation of one or several crucial pathways providing energy
for developing microspores. Of those, respiratory pathways such as glycolysis and TCA cycle are particularly interesting. Intriguingly, results from the Chapter 5 strongly suggested that water deficit has induced the production of hydrogen peroxide, a major form of ROS, above the redox balance, which in turn caused detrimental effects to cellular DNA resulting in a PCD in the anthers. In addition, hydrogen peroxide accumulation effectively influenced ATP synthesis leading to a decrease in the level of ATP in the anthers (Chapter 5). Excessive hydrogen peroxide accumulation after drought could be the consequence of insufficient activity of an antioxidant system, which has been illustrated by qRT-PCR expression analysis of major antioxidant genes (Chapter 5). A down-regulation of those genes would increase the chance for oxidative damage, in contrast stable or up-regulated expression of them showed lesser oxidant damage (Chapter 5). All of these findings suggest that oxidative stress might be a direct cause of abortion of young microspores under water deficit, which has been illustrated in CMS crops from other studies (Li et al., 2004; Jiang et al., 2007; Wan et al., 2007).

6.4. Causal relationship between oxidative stress and sugar accumulation

Many studies have attempted to assess the relationship between oxidative stress and increased levels of carbohydrate under abiotic stress, however direct consequential effects of ROS on sugar levels or vice versa have not been confirmed. Sugars have been shown to be associated with both ROS producing and scavenging pathways via NADPH metabolism (Couee et al., 2006; Ying, 2008). Soluble sugars can take part in ROS producing pathways such as increased respiration or activation of NADPH oxidases (Foreman et al., 2003; Ying, 2008). On the other hand, they can enhance NADPH producing metabolism, e.g. oxidative pentose-phosphate pathway, which contributes to the ROS scavenging system (Couee et al., 2006).
Some examples on causal effects of sugars on the production and scavenging of ROS have been demonstrated in yeast and animals. Although sugars, especially glucose, are the most favourable nutrition for yeast growth, excessive amounts of sugar supply in the absence of additional nutrients has been reported to induce cell death in yeast in conjunction with rising levels of ROS (Granot et al., 2003). Likewise, high glucose concentrations have been reported to activate NADPH oxidase, which catalyses the production of superoxide (O$_2^-$) (Babior, 1999), one of the reasons for glucotoxicity in animal cells (Bonnefont-Rousselot, 2002). Similarly, Susztak et al. (2006) illustrated that high concentrations of extracellular glucose increased ROS production by activation of both plasma membrane NADPH oxidase and mitochondrial ROS generation, which later triggers apoptosis at the onset of diabetic nephropathy.

In contrast, the presence of glucose has been demonstrated to protect hamster ovary cells against oxidative damage caused by exogenous hydrogen peroxide. The role of glucose in this protection was attributed to activation of the pentose phosphate pathway and hence enhanced activity of antioxidant enzymes such as catalase and glutathione (Averillbates & Przybytkowski, 1994). Likewise, increased levels of sugars have been reported to provide protection to cells from oxidant damage in plants. High levels of sugars have been demonstrated to maintain cellular NADPH concentrations, an important precursor for the production of antioxidant substrates such as ascorbic acid and glutathione in an ascorbate-glutathione cycle (Figure 6.1) (Noctor & Foyer, 1998). Consistent with this hypothesis, elevated levels of NADPH in transgenic rice resulting from over expression of a reductase gene YK1, has been thought to enhance the antioxidant pool and hence reduce cell death caused by exogenous hydrogen peroxide (Hayashi et al., 2005; Takahashi et al., 2006).
Figure 6.1: Involvement of NADPH in ascorbate – glutathione cycle. Adapted from Noctor & Foyer (1998). Where: GSH glutathione; GSSG oxidized glutathione; AA ascorbate; DHA dehydroascorbate; MHDA monohydroascorbate; GR glutathione reductase; DHAR dehydroascorbate reductase; MDHAR monodehydroascorbate reductase; APX ascorbate peroxidase; SOD superoxide dismutase.
Moreover, high levels of glucose could directly increase the production of ascorbic acid, an important substrate of the anti-oxidant system (Figure 6.2) (Wheeler et al., 1998). In support of this hypothesis, Smirnoff & Pallanca (1996) postulated that feeding excised leaves of barley (*Hordeum vulgare*) seedlings either glucose or sucrose in the dark partially recovered the ascorbate pool. Similarly, Nishikawa *et al.* (2005) reported that application of exogenous sucrose can compensate for the loss of ascorbate content in excised broccoli (*Brassica oleracea* L. var. *italica*) florets.

**Figure 6.2:** The role of glucose as building block of ascorbic acid. Adapted from Wheeler *et al.* (1998). Enzymes of reactions from 1-9: 1, hexose phosphate isomerase; 2, phosphomannose isomerase; 3, phosphomannose mutase; 4, GDP-D-mannose pyrophosphorylase; 5, GDP-D-mannose-3,5-epimerase; 8, L-galactose dehydrogenase; 9, L-galactono-1,4-lactone dehydrogenase.
In the present study, higher levels of sugars (both total and reducing) at all three stages (Chapter 4) seemed to be associated with some measure of protection to the anthers against oxidative stress (Chapters 3 & 5). Induced expression of OsSUT5 and OsMST7 might have maintained the high level of sugar in the tapetum and the locules (Chapter 4), which alleviated oxidant damage caused by excessive ROS generation (Chapter 5). As a result, the higher the levels of sugars in the tapetal layer, the less cellular damage was observed (Chapters 4 & 5). Consequently, less damage to the anther and young microspores was recorded (Chapters 3 & 5). In addition, this assumption could be further inferred by reviewing abnormalities observed in chilling and drought stressed anthers from other research. It has been well demonstrated that stressed anthers from rice and wheat showed abnormal phenomena called tapetal swelling, which is assumed to be related to tapetal dysfunction (Lalonde et al., 1997a; Li, 2005; Mamun et al., 2006). Water stress in the present study might have induced excessive ROS production in the tapetum where many mitochondria were located (Chapter 5). High concentrations of sugar in the tapetum and the locules could be a natural response to oxidative stress and therefore increased turgor pressure which in turn causes tapetal swelling or hypertrophy, a common symptom associated with pollen sterility often observed in chilling stressed anthers (Nishiyama, 1984; Li, 2005; Mamun et al., 2006). Similar abnormalities have also been postulated for wheat anthers under water stress (Lalonde et al., 1997a). In the present study, sugar flux to the tapetum and anther locules was increased after water deficit, which might help alleviate detrimental effects caused by excessive ROS generation. Nevertheless, as mentioned at the start of this section, sugars are not simply accumulated to enhance antioxidant defence; instead they appear to play a dual role in response to water deficit stress. Therefore, this causal relationship between sugars and ROS needs to be further characterised.
6.5. Sensitive stages of anther development to water deficit

It has been well documented in the literature that one of the most sensitive stages of anther development to drought stress is around anther meiosis. This rule applies for most cereal crops in general and rice in particular (Saini & Westgate, 2000; Yang et al., 2007) and results presented in this current work concur (Chapter 3). Nevertheless, there has been no proper interpretation of this phenomenon to date. As discussed in the aforementioned sections, there is accumulating evidence of oxidative stress in the anther after a short water deficit period, which detrimentally affects microspore development (Chapter 5). As mitochondrial respiration is the main source of ROS production (Moller, 2001), it is suggested that the increased number of mitochondria located in the tapetal cells are the main reason for the increased susceptibility of anthers to water deficit around anther meiosis to tetrad breakup. Fewer mitochondria are present at other stages such as microspore and vacuolated microspore and this may contribute to milder oxidative effects observed (Chapters 3 & 5). Nevertheless, this assumption needs to be further validated with proper quantitative methods for mitochondrial activity.

6.6. Involvement of signalling molecules to pollen sterility under drought

As mentioned in Chapter 1, chilling and drought stress have been known to result in abnormal starch accumulation in the connective tissue of rice and wheat anthers (Lalonde et al., 1997a; Mamun et al., 2006). The cause of this abnormal starch accumulation remains unknown although it is found to be associated with pollen sterility (Lalonde et al., 1997a; Li, 2005). As discussed in Chapter 5, down regulation of 14-3-3 gene at the tetrad stage took place in conjunction with a serious decline in plant fertility (Chapter 3). It appears that expression of 14-3-3 gene is not only relevant to ATP synthesis and apoptosis, but also to other events such as starch synthesis. Down-
regulation of 14-3-3 protein has been shown to result in a two to four fold increase in starch accumulation in Arabidopsis leaf (Sehnke et al., 2001). It is possible that water stress in the present study might also induce starch accumulation in the anther and thus this would take place in parallel with decreased expression of 14-3-3 gene as discussed in Chapter 5. This finding suggests the role of 14-3-3 transcripts as a signalling molecule under drought conditions. Abnormal behavior of this molecule would signal a disturbance in metabolic activity in anthers leading to pollen sterility in the present study.

In addition, recent reports have demonstrated the role of 14-3-3 proteins as intermediates of abscisic acid (ABA) signal transduction (Schoonheim et al., 2007; Wasilewska et al., 2008). The accumulation of ABA as a water deficit stress response has been well documented in the literature (Davies & Zhang, 1991; Shinozaki & Yamaguchi-Shinozaki, 2007) although the molecular mechanism of this phenomenon is not clear. Nevertheless, the involvement of ABA on reproductive tissue has been long considered as part of a signalling system under chilling and drought stress (Saini & Westgate, 2000; Oliver et al., 2007). This phytohormone has been considered as an endogenous sporocide that can induce spikelet sterility under water stress in some reports (Saini & Westgate, 2000). Indeed, high levels of endogenous ABA have been found to be associated with pollen and spikelet sterility in wheat under drought (Morgan, 1980; Saini & Aspinall, 1982; Westgate et al., 1996) and in rice under chilling (Oliver et al., 2007). Decreased levels of endogenous ABA have been hypothesised to be associated with cold tolerance in rice (Oliver et al., 2007). Accumulation of ABA under drought stress has been regarded as a key inducer of ROS production and induction of ABA is an upstream event of ROS generation (Guan et al., 2000; Jiang &
Zhang, 2002b; Hu et al., 2005; 2006), which in turn causes direct detrimental effects on tissues.

However, there is also some evidence that an increase in endogenous ABA does not affect fertility in wheat under drought (Dembinska et al., 1992). Water deficit-induced ABA accumulation has been shown to enhance the antioxidant system and hence provide a protection against ROS production in maize seedlings (Jiang & Zhang, 2002a). A very recent study on water stressed rice during meiosis illustrated that the levels of endogenous ABA are not different between sensitive and drought resistant cultivars (Yang et al., 2007). In contrast, the inhibition of endogenous ABA synthesis has been shown to cause spikelet sterility for both drought stressed and control plants in rice suggesting a protective role of elevated ABA under drought condition (Yang et al., 2007). Therefore, it poses an interesting question on the underlying molecular mechanism of ABA accumulation in plant tissues under drought. It is likely that the actual impact of ABA on plant tissues strongly depends on its concentration. Low concentrations of ABA have been reported to enhance antioxidant defence against oxidative damage, whereas high concentrations result in excessive accumulation of ROS and lead to oxidative damage (Jiang & Zhang, 2001). More work needs to be done to unveil the actual impacts of ABA on plants during drought.

This information suggests that signalling molecules such as 14-3-3 proteins and ABA appear to act upstream of ROS production and antioxidant defence in plants. Further work on these molecules might therefore give an interesting picture on how they influence plant fertility under water shortage condition.

6.7. Concluding remarks

This thesis has contributed to the understanding of the cellular and molecular basis of pollen sterility in rice under water shortage conditions. New information
regarding the possible molecular mechanisms of pollen sterility under drought conditions has proposed and discussed. Oxidative stress appears to be a plausible reason for the decline of pollen viability and grain set in the present work. Indeed, transgenic rice engineered with MnSOD, a ROS scavenging enzyme, does show better tolerance to drought induced by PEG in comparison to the wild type (Wang et al., 2005). Nevertheless, a more detailed investigation of mitochondrial respiration in rice anthers is required to further examine this problem. Moreover, this thesis has suggested new ideas regarding the role of rising sugar levels to cope with oxidative stress in anthers. Sugar accumulation might have provided protection against oxidant damage by strengthening the antioxidant system. However, the interplay between sugar and oxidative stress is not straightforward and needs to be further characterised. In-depth investigations on the interaction between sugar signaling and oxidative stress responses may help indentify the role of sugars in protecting anthers under water deficit.

Although many studies on drought and chilling stresses in rice anthers have been performed, the causal mechanism of male sterility, to some extent, is still poorly understood. Findings presented in this thesis may contribute to understandings of drought stress responses in rice. Furthermore, the mechanisms underlying water deficit induced pollen sterility in this study can be not only applied for rice plants but also extended to other cereals crops. Findings from this thesis may assist in developing better breeding strategies for improving drought tolerance in rice.
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### Components of SOC medium

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Bacto® Trypton</td>
<td>2.0g</td>
</tr>
<tr>
<td>Bacto® yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>1M NaCl</td>
<td>1ml</td>
</tr>
<tr>
<td>1M KCl</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>2M Mg$^{2+}$ stock, filter-sterilized</td>
<td>1ml</td>
</tr>
<tr>
<td>2M glucose, filter-sterilized</td>
<td>1ml</td>
</tr>
<tr>
<td>Sterile ddH$_2$O</td>
<td>make up to 100 ml</td>
</tr>
</tbody>
</table>

### Components of ampicillin LB plate

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto® Trypton</td>
<td>5 g</td>
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<tr>
<td>Bacto® yeast extract</td>
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<tr>
<td>NaCl</td>
<td>2.5 g</td>
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<tr>
<td>Agar</td>
<td>7.5 g</td>
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<tr>
<td>100 mg/ml stock ampicillin</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Sterile ddH$_2$O</td>
<td>make up to 500 ml</td>
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</table>