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SUMMARY

We have used two approaches to study the molecular basis of cold-induced pollen sterility in rice. Firstly, we studied the effect of cold on sugar metabolism in rice anthers, with the intention to identify genes that are affected by cold. Secondly, we used microarray gene expression profiling to identify rice genes that are affected by cold treatment, and to compare the cold response between a cold-tolerant and a cold-sensitive variety. The work on sugar metabolism has shown that cold treatment of rice anthers leads to an absence of starch accumulation and non-viability of pollen. Starch is an essential source of energy for pollen development and pollen fertility. At the same time, we found that sucrose – the building block of starch – is accumulating in cold-stressed anthers at the cold-sensitive young microspore stage. This indicates that sucrose somehow fails to be converted to starch in the pollen grains, and that the supply mechanism of sugar to the tapetum and developing pollen grains is disturbed by cold. The tapetum, the cell layer in the anther that feeds the pollen grains, and the pollen cells are physically isolated from the rest of the anther at the young microspore stage. Supply of sugars from the rest of the anther to the tapetum and pollen grains occurs via a specialised mechanism involving two enzymes: cell wall invertase and monosaccharide transporters. Biochemical analysis indicated that the activity of anther cell wall invertase was significantly repressed by cold, suggesting that the first step in the sugar transport chain is functioning at reduced capacity. We cloned the gene that encodes this enzyme, *OSINV4*, and found that the expression of this gene is repressed by cold. We subsequently identified two monosaccharide transporter genes: *OSMST8* was repressed by cold, while *OSMST7* was induced by cold. *OSINV4* and *OSMST8* function in the same pathway that supplies sucrose to the tapetum and pollen, while *OSMST7* functions in a different pathway that may lead to starch accumulation in the anther wall. Studying the cold-tolerant Chinese cultivar *R31* revealed that this cultivar did not accumulate sucrose, contained starch-filled fertile pollen grains, and did not repress *OSINV4* and *OSMST8* expression following cold treatment. Thus, there is a strong correlation between these phenotypes and the cold tolerance phenotype, suggesting that we have now some expression markers for cold-tolerance. We have also found that these genes are regulated by the plant hormone ABA; ABA perfectly mimics the effect of cold and it serves as a signal to switch of gene expression, including *OSINV4* and *OSMST8*. ABA-accumulation does not occur to the same extent in *R31* than in *Doongara*, and we have identified an anther ABA biosynthetic gene that is induced by cold (*OSNCED3*). These findings have improved our understanding of the molecular basis of cold-induced pollen sterility significantly, and we are now in the stage of identifying a marker gene that can be used to follow the cold-tolerance trait in a breeding population. We have also made good progress using the microarray approach. By comparing the cold response of *Doongara* and two tolerant cultivars (*R31* and *R32*) we identified a non-redundant set of 329 genes that are expressed differently between the different cultivars. The genes were sequenced and their chromosome location was determined. This gave us more information about other cellular processes that are affected by cold and how these processes are affected differently in tolerant and sensitive cultivars. We are now in the stage of spotting these genes on a smaller diagnostic microarray, and this array will be used to screen doubled haploid lines of a Doongara/R31 cross (prepared by Dr. X. Zhao, Sydney Univ.). This will enable us to identify suitable marker genes for cold tolerance in rice.

1. Background

Cold-induced pollen sterility is the most important yield-limiting factor in the Australian rice industry. On average 10% of the yield is lost annually, but unpredictable cold snaps can cause yield losses of 20-40% on average every 3-4 years. Very little is known about the physiology and molecular basis of the problem, making it very difficult for breeders to carry out a targeted breeding approach for cold-tolerant Australian rice cultivars. Our work uses the latest molecular biology techniques to investigate the molecular basis of the problem. We have carried out a detailed analysis of cold-induced changes in sugar metabolism in rice anthers, and this led to the identification of important sugar metabolic genes that are switched off by cold conditions. We also use DNA chip technology to carry out gene expression profiling of cold-stressed rice anthers, in order to screen differences in response between cold-tolerant and cold-sensitive cultivars. The combination of the available rice genome sequence and the information obtained by our molecular biology approaches will enable us to identify molecular markers for marker-assisted breeding.

2. Objectives

- To study the effect of cold on sugar metabolism in rice anthers.
- To study the involvement of plant hormones (ABA and GA) in triggering cold-induced pollen sterility.
- To use microarray technology to compare the cold response between cold-sensitive (*Doongara*) and cold-tolerant (*R31* and *R32*) rice varieties.
- To identify molecular markers for breeding cold-tolerant rice.

3. Introductory technical information

Anther physiology and molecular biology, and pollen biology in general, remain poorly understood areas of research. Anthers and pollen are small in size, complicated in structure, and pollen grains go through a fast series of developmental stages. Studies on rice anthers in particular are complicated by their small size and the difficulty to access and determine the exact developmental stages of the pollen. All progress in this project is based on the continuous availability of relatively large amounts of dissected rice anthers. Anther harvesting is difficult and time-consuming and simulating the induction of cold-induced sterility under phytotron conditions needs perfect control of the pollen developmental stage. All standard molecular biology techniques needed to be fine-tuned to work with the small quantities of rice anther material. We have been successful to do most expression studies with nanogram amounts of RNA and establishment of RNA amplification techniques, allowing 1000-fold amplification of RNA amounts, allowed us to do microarray screenings from minute quantities of anthers harvested at the exact pollen developmental stage.

4. The Methodology

- Growing rice plants for harvesting anthers at defined stages of anther development (establishment of auricle distance scale).

- Cold treatment of rice plants in temperature-controlled growth chambers at different developmental stages to induce pollen sterility.

- Microscopic analysis of the effect of cold on rice anthers and pollen development.

- Extraction of anther RNA for gene expression studies.

- RNA expression analysis using northern blot hybridisation and RT-PCR.

- Sucrose measurement and invertase enzyme assays in cold-tolerant rice lines.

- ABA injection experiments in panicles for sterility measurements and gene expression studies.

- Preparation of anther cDNA libraries and PCR amplification of 18,000 cDNA insert; these amplified clones were spotted on the cDNA microarray.

- Construction of rice anther cDNA microarrays for gene expression profiling studies.

- Amplification of anther RNA for probe preparation and microarray screening.

- Preparation of anther RNA probes for screening of microarrays.

- Hybridisation of existing microarrays with anther probes, cold vs. control plants.

- Laser scanning and quantification of the microarray data; statistical analysis of the data and establishment of databases.

- Identification of interesting genes. Growing up bacterial clones for plasmid preparation and DNA sequencing.

- DNA isolation and recombinant DNA work for isolating interesting rice anther genes.

- Preparation of recombinant DNA constructs and rice transformation; this is done to do expression studies (promoter-GUS or promoter-GFP fusion constructs).

5. Detailed results

- An auricle distance (AD) scale was established for *Doongara* and microscopic analysis was used to determine the anther developmental stages associated with these AD's. We established the cold treatments to induce pollen sterility in the phytotron (3 days at 12^0C).

- Microscopic analysis after iodine staining indicated that cold-treated pollen grains do not accumulate starch at anthesis. Viability staining showed the starch-less pollen grains are also not viable. These results indicate that cold treatment leads to premature abortion of pollen development.

- Non-reducing sugars, and sucrose in particular, were shown to accumulate in cold-stressed rice anthers to levels that are more than double those in normal anthers. This indicates that sucrose supply is not the limiting factor for starch biosynthesis, but that cold somehow prevents conversion of sucrose to starch.

- Cell wall bound invertase activity is dramatically down-regulated by cold in rice anthers. Cell wall invertase is a sucrose transport enzyme involved in the supply of sucrose from the cell wall space in the anther wall to the tapetum and young microspores – the tapetum and microspores are symplastically isolated from the anther wall cell layers. This suggests that cold blocks the very first step in the pathway leading to starch biosynthesis: the supply of sucrose to the tapetum and young microspores.

- In an attempt to clone the invertase gene that encodes the cell wall invertase enzyme that is affected by cold, we cloned 4 rice invertase genes: two vacuolar invertases (*OSINV2* and *OSINV3*) and two cell wall invertase genes (*OSINV1* and *OSINV4*). We made promoter-GUS and promoter-GFP chimeric gene constructs for all these genes and transformed these constructs to rice, in order to study the expression pattern of these genes. We also studied the expression pattern of these genes at the RNA level using northern blot hybridisation and RT-PCR.

- We found that the cell wall invertase gene *OSINV4* is anther-specific, and its expression is down-regulated by cold. In collaboration with our rice CRC colleagues at Sydney University we found that *OSINV4* is specifically expressed in the tapetum at the cold-sensitive young microspore stage and in the pollen grains from the early binucleate stage until pollen maturity. The expression properties we observed for *OSINV4* correlate very well with our biochemical studies in anthers, suggesting that the repression by cold of this gene is responsible for the decline in cell wall invertase activity and accumulation of sucrose we observed.

- Monosaccharide transporters are responsible for the next step in the transport of sucrose into the tapetum and pollen. Two rice anther monosaccharide transporter genes were cloned. We have shown that the expression of one of these genes (*OSMST8*) matches the expression pattern of *OSINV4*: the gene is repressed following cold treatment. The other gene (*OSMST7*) is induced by

cold treatment. These results confirm that cold causes extreme changes in the fate of sucrose in the anther. Instead of mobilizing sucrose to the tapetum and developing pollen grains, cold causes sucrose to accumulate in the anther wall; later this sucrose is converted to starch that accumulates in the anther wall.

- The work carried by rice CRC colleague Dr. Xiaochun Zhao (Sydney University, Cobbity) provided us with Chinese rice varieties that show good cold tolerance. This provided us with the opportunity to study the effect of cold on the expression of our genes in the cold-tolerant cultivar *R31*. Expression of *OSINV4*, *OSMST7* and *OSMST8* appeared to be expressed at much higher (2-2.5-fold) levels in this variety compared to *Doongara*, and expression after cold treatment remains even higher than in *Doongara* under normal conditions. We also found that most pollen grains accumulated starch and that there is no sucrose accumulation in cold-stressed anthers.

- Injection of ABA in rice panicles at the young microspore stage result in the induction of pollen sterility; this was visualised using starch staining. We also found that ABA injection causes repression of *OSINV4* and *OSMST8* expression, and induction of *OSMST7*. These genes were changing their expression pattern in the same way following cold treatment, indicating that ABA mimics the effect of cold treatment. Our results suggest that ABA is functioning as a signal used by cold to induce pollen sterility.

- We have shown that cold treatment results in increased ABA levels in rice anthers. This increase occurs already after 5 hours of cold treatment and is faster than the increase observed in leaves – which occurs after 24 hours. This could indicate that anther ABA is made in the anther rather than being transported from the leaves.

- ABA levels in the cold tolerant cultivar *R31* are lower than in *Doongara* and the increase in ABA levels following cold treatment are also much lower in the cold-tolerant cultivar.

- We have initiated investigating the role of gibberellic acid (GA) in determining cold tolerance in rice, in order to investigate the correlation between semi-dwarfism and cold-sensitivity. The cold-sensitive semi-dwarf variety *Doongara* contains a deletion of the GA20-oxidase gene *SD1*, which results in lower GA levels throughout the plant and smaller plant stature. ABA and GA often act antagonistically in plant development; therefore plants with lower GA content may be more susceptible to increased ABA levels caused by cold treatment. *R31* is not a semi-dwarf variety and contains the normal *SD1* gene. We have cloned the normal gene from *R31* and made a construct to introduce this gene in *Doongara*. This will allow us to study in the same genetic background whether cold-tolerance can be improved by complementing the *SD1* mutation, and, in doing so, preventing ABA accumulation to high levels. In addition, we will be able to study whether there is any effect on grain quality.

- We have identified two ABA biosynthetic genes that are expressed in rice anthers. One of these genes (*OSNCED3*) is induced by cold and shows much higher expression in *Doongara* than in the cold-tolerant cultivar *R31*. This result proves that in response to cold treatment, the anther induces its own ABA biosynthesis, and this response is stronger in *Doongara* than in *R31*. We are currently investigating how this difference in *OSNCED* gene regulation occurs. If there is a difference in the promoter or coding region of the *OSNCED3* gene of *Doongara* and *R31*, then we may be able to design DNA markers to track this difference in a breeding population.

- We made 5 anther cDNA libraries from different pollen developmental stages, control and cold-treated. The plasmid clones were excised from the phage libraries and random colonies were picked and frozen as glycerol stocks at –80^{o}C.

- We prepared two microarrays: a general anther microarray containing 18,000 cDNA clones, and a tetrad stage array containing 11,000 clones.

- The 18K array was screened with young microspore stage anther RNA, control versus cold-treated. We Identified 550 rice genes that are significantly affected by cold at the young microspore stage. Sequence analysis was carried out and a database has been established.

- The 18K array was also screened with young microspore RNA from drought-stressed anthers, and anthers from plants that were injected with ABA. These results indicate that there is a significant overlap between the cold, drought and ABA response. This result confirms that mechanistically these three stimuli have an overlapping signaling mechanism, and that ABA may be the common signal for the induction of pollen sterility under both cold and drought conditions.

- An RNA amplification method was established that enables us to use small amounts of anther RNA for the screening of microarrays. We were able to amplify RNA quantities 1000-fold, allowing us to use minute quantities of anthers harvested at more precise developmental stages and hence to reduce the time invested in anther harvesting.

- In collaboration with Dr. Zhao (Univ. Sydney), we analysed the effect of cold on anther gene expression at different stages of pollen development: pre-mothercell, mothercell, tetrad, early uni-nucleate and mid uni-nucleate stage (made possible by the RNA amplification method). The results show that there are dramatic changes in the cold response throughout pollen development, especially at the cold-sensitive stage (tetrad to early uni-nucleate stage).

- We have used extensive microarray expression profiling to compare the cold response between *Doongara* and the two cold-tolerant varieties *R31* and *R32* at three different stages of pollen development, centred on the cold-sensitive young microspore stage (pollen mother cell, tetrad and early uni-nucleate stage). This work revealed extensive differences in gene expression between *Doongara* and the tolerant cultivars, but also between the two tolerant cultivars. A non-

redundant set of 329 genes was identified after DNA sequencing, and the chromosome position of these genes was determined.

- We are in the stage of spotting these 329 clones on a smaller diagnostic microarray in order to identify genes that have potential as molecular markers for breeding. We will use this array to screen a set of doubled haploid lines (Dr. X. Zhao, Sydney Univ.) from a Doongara/R31 cross. This will allow us to identify linkage between the genes we identified, and the cold-tolerance phenotype in the doubled haploid lines. When we find genes that are strongly linked to the cold-tolerance phenotype, we will identify closely linked micro-satellite markers to these genes (using search engine on the rice genome project database) and these markers could then be used as DNA markers for breeding.

- One publication has been written about the sugar and invertase work and submitted to Plant, Cell and Environment. The reviewers considered the paper as a significant publication in this field of research. A second equally important publication on the ABA data is currently in preparation, and we are finalising the microarray results for publication.

6. Discussion of results

The project has made significant breakthroughs in understanding the molecular basis of cold-induced pollen sterility in rice. The finding that cold represses sugar transport to the tapetum and the pollen grains has led to the identification of genes that (*OSINV4, OSMST7, OSMST8*) that are affected by cold. The availability of a good cold tolerant cultivar like *R31* has been crucial to prove that there is a strong correlation between the regulation of the sugar metabolism genes and cold tolerance; this cultivar will therefore be an important asset to look for cold-tolerance markers in the future. Promoter analysis of the *OSINV4, OSMST7* and *OSMST8* genes predicted that these would respond to the plant hormone ABA, also called the stress hormone. ABA was known for some time to be involved in a variety of stress responses, including cold and drought. The fact that ABA injection induces pollen sterility and ABA also affects the genes we identified in the same way as cold, encouraged us to look further at ABA as a potential signal to trigger the abortion of pollen development in rice. ABA levels go up in anthers in response to cold treatment, and again the cold-tolerant line *R31* supported the correlation between ABA and pollen sterility: ABA levels in *R31* were lower to start with, and the increase caused by cold was much weaker than in *Doongara*. We also identified two ABA biosynthetic genes in anthers; one of these genes, *OSNCED3*, is induced by cold and is expressed at much lower levels in *R31* compared to *Doongara* – this again supports our hypothesis. One important experiment to carry out in the near future is to study whether the DNA sequence of *OSNCED3* gene in *R31* is different compared to *Doongara* and that this sequence difference causes the gene to be expressed differently in a tolerant and sensitive variety. If this is the case, this will offer potential to develop molecular markers based on the sequence difference of *OSNCED3* in tolerant vs. sensitive cultivars. ABA in the tapetum is high under normal conditions; the hormone is known to play a role in the regulation of the normal apoptosis (controlled cell death) event that degenerates the tapetum from the vacuolated stage of pollen development

onwards. Increasing the level of ABA by imposing cold conditions at the critical young microspore stage, when tapetum is functioning at maximal capacity, may lead to accelerated or premature cell death. This will cause the tapetum to degenerate quicker, before its essential role of feeding the young microspores is finished, leading to abortion of pollen development. The fact that non-dwarf rice varieties are generally more cold-tolerant suggests that the hormone gibberellic acid (GA) also plays a role - and we have started to investigate this. ABA and GA often work antagonistically, ABA preventing growth and inducing a defence response against stress, and GA promoting growth and development. The fact that GA counteracts the damaging effect of ABA under cold conditions may explain why semi-dwarf rice like *Doongara*, which is mutated in one of the ABA biosynthetic genes, is more cold sensitive. GA is made in the tapetum and drives development of the entire flower; high pollen number, and large anthers are most probably controlled by GA and have been correlated with cold-tolerance. We have therefore started to investigate GA function, as a potential avenue to find markers for cold-tolerance.

The microarray work has also started to produce good results. We have identified a set of 329 genes that are affected differently by cold in *Doongara*, *R31* and *R32*. We are making a new diagnostic array using these genes, and with this array we will screen different doubled haploid lines obtained from a cross between *Doongara* and *R31*. This will identify which genes are strongly linked to cold tolerance. Because we know the rice genome sequence, we can then start identifying closely linked DNA markers (eg., micro-satellites) that can be used as molecular markers for breeding.

7. Implications and recommendations

The sugar/hormone work and the mapping of the cold-tolerant loci using microarrays will offer potential for the identification of molecular markers that will greatly improve speed and reliability of breeding cold tolerant rice cultivars. This will lead to improved rice yields and a more reliable harvest under adverse environmental conditions. Availability of cold tolerant Australian rice will also lead to a significant reduction in water use in the field, or the ability to grow more rice with the same amount of water. The fact that the mechanism of cold-tolerance may be related to drought-tolerance may lead to cross-protection against heat and drought conditions in the cold-tolerant cultivars. The availability of DNA markers may provide opportunities to apply for IP and plant variety rights.

8. A description of the Project Intellectual Property

The characterisation of the molecular basis of cold-induced pollen sterility has led to the identification of many genes that are regulated differently between a cold-tolerant and a cold-sensitive rice cultivar. These genes, or the biochemical processes they are involved in, could be used as diagnostic markers for cold-tolerance/sensitivity. We are now in the stage of developing these gene expression markers further into DNA markers that will be much easier to use. DNA molecular markers need a simple PCR assay and will not need flowering plants or cold treatments; instead, selection of interesting breeding varieties can be done anytime at the seedling stage. Such markers may provide an opportunity to apply for IP and Australian rice breeders will adopt this technology to create new cold-tolerant rice lines and have Plant Variety Rights.

9. Recommendations

We have made significant progress in identifying the molecular basis of cold-induced pollen sterility in rice and this has put us in a strong position to start exploiting this information for the development of molecular markers for marker-assisted breeding. The availability of the rice genome sequence will greatly facilitate this task, but some further research efforts have to invested in:

9.1 The identification of polymorphism at the DNA level in the ABA signalling mechanism that controls abortion of pollen development in a cold-sensitive cultivar. This will require sequencing the *OSNCED3* gene of *Doongara* and *R31*, and studying further what is different about the expression of this gene between the two cultivars. We will also look at the two other *OSNCED* ABA biosynthetic genes that rice has; we know that another gene (*OSNCED1*) is expressed in anthers, and preliminary results show that this gene is not induced by cold – this needs further confirmation. The presence of different *OSNCED* genes may offer the opportunity for transgenic approaches to make cold-tolerant rice, for instance by knocking out expression of the cold-induced *OSNCED3* gene. We will study the "proof-of-concept" potential of this approach.

9.2 We will further investigate the role of GA, as this may also offer opportunities for marker development.

9.3 We will start screening the doubled haploid lines (Dr. X. Zhao) and look for linkage of the 329 microarray genes with cold-tolerance. This will lead to the identification of genes that are closely linked to cold tolerance. Once we know these genes, we will study the effect of manipulating the expression of these genes on cold-tolerance to confirm their role and compare this gene between tolerant and sensitive varieties. We will also look for DNA markers using the tools from the Rice Genome Project website and we will test these markers using the double haploid lines and segregating populations of crosses between *R31* and Australian cold-sensitive cultivars.

10. References.

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