Identification of drought responsive microRNAs and functional analysis of a sample microRNA for drought tolerance in barley (*Hordeum vulgare* L.)

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A thesis submitted to the University of Adelaide

in fulfilment of the requirements for the degree of Doctor of Philosophy

Faculty of Sciences

School of Agriculture, Food and Wine

The University of Adelaide

February 2016

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Abstract

Declining water resources coupled with the dwindling size of agricultural land necessitate the development of drought tolerant crop varieties. However, genetic control of drought tolerance is complex because of the involvement of a large number of genes. Studies in plant biotechnology for improved plant stress tolerance mostly concentrate on implementing and manipulating downstream gene, involved in the physiological responses. Recently, the upstream gene regulatory network involving small, non-coding RNAs such as microRNAs (miRNAs) and their target genes has been discovered. However, the drought regulatory relationship between miRNAs and their targets in barley, one of the major cereal crops, is still largely unknown. In this PhD project, potential drought responsive miRNAs and their targets were identified and validated in barley genotypes, and the drought tolerance of transgenic barley over-expressing miR827, a previously reported miRNA that conferred drought tolerance in *Arabidopsis*, was examined.

To identify and validate drought responsive miRNAs and their targets, we conducted expression analysis of several drought responsive miRNAs under drought treatment in four barley genotypes that were reported to perform well in the drought prone areas of Australia. Differences in expression of four miRNAs; *Ath*-miR169b, *Osa*-miR1432, *Hv*-miRx5 and *Hv*-miR166b/c were observed between drought-treated and well-watered barley samples, and this expression varied among the experimental genotypes. Generally, miRNA-mediated cleavage of the target mRNAs was observed at the ideal cleavage site, however we also found miRNA-mediated cleavage not limited to the canonical position. We detected the spatial accumulation of potential drought responsive miRNAs and targets that mostly localized in the mesophyll tissues, and inverse correlation of expression between mature miRNAs and their target mRNAs.

These results suggested a contribution of miRNAs to the molecular control of target gene expression, genotype-specific regulation under drought treatment, and the promise of miRNA: mRNA as biomarkers for genotypic selection in barley.

To examine the drought tolerance of transgenic barley expressing miR827, we monitored the performance under drought of miR827 over-expressing transgenic barley plants under the control of the constitutive promoter *CaMV-35S* and drought-inducible promoter *Zm-Rab17*. We observed that the ectopic overexpression of *Ath*-miR827 resulted in unwanted side-effects, that is reduced shoot area, delayed anthesis and reduced whole plant water use efficiency (WUE_{wp}) and weight of seeds per plant in the transgenic compared with the wild type counterpart or null plants. In contrast, the drought inducible expression of *Hv*-miR827 caused several promising phenotypes; that is, the transgenic barley plants did not show reduced shoot area or delayed anthesis compared to the wild type, while the WUE_{wp} and leaf relative water content (RWC) were improved. Further, we observed the advantages of drought inducible *Hv*-miR827 over-expression in the recovery of transgenic plants after drought stress. These findings suggest that the over-expression of *Hv*-miR827 is promising for improving plants' performance under drought and that miRNA-mediated drought tolerance or intolerance also depends on the nature of promoter.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Acknowledgements

While my name may be alone on the front cover of this thesis, I am forever indebted to Professor Peter Langridge and Dr. Penny Tricker, my academic supervisors, for their excellent guidance, great thought-provoking impulses and encouragement.

Peter, you are the brightest person I have ever seen. You are intensely knowledgeable, wise and patient, confident and humble. Your brilliant advice and cooperation have been priceless for me. Your availability and huge experience were the key motivations throughout my PhD. I appreciate all your contributions of time, ideas, and encouragement to make my PhD experience productive and enjoyable.

Penny, I found you extremely organized. Your wise counsel, immense knowledge, strategic thinking and persistent personality are something those have been improving me ever since I have got chance to work with you. Your effort on setting up recurrent meetings was one of the strengths of my PhD project that have put me right on the track. You have foreseen that I will have these many publications and made me believe that. Once I started to believe that, my brain had no choice but to make it reality. Without the unique set of people like you two, I have no doubt that I would not have lasted long. It has been an honour to be supervised by you!

I must express my heartiest gratitude to my independent advisor Dr. Julie Hayes for her availability, valuable input and support in to my project. I am also thankful to Dr. Bu-jun Shi, who provided his time and bright input for initiating this project.

It was also a real privilege to be mentored by Dr. Fahimeh Shahinnia at some stage of my PhD. Fahimeh, your motivational advice has brought me positivity. I learnt from you that time I enjoy wasting is not wasted time. Meeting you was really necessary for my journey.

I express my sincere gratitude to The University of Adelaide for ASI scholarship and to the Australian Centre for Plant Functional Genomics (ACPFG) for research

facilities and supplementary top-up scholarship. I am also thankful to the Grains Research and Development Corporation (GRDC) for my travel awards to present my work at the ‘Mini-symposium on Cereal Genomics to address Grand Challenges’ in Grasmere, Cumbria, England.

I must thank Professor Robbie Waugh of University of Dundee at James Hutton Institute (JHI). I visited Robbie and his group in 2015 at JHI where my visit was made to feel very welcome. Robbie cooperated me for the use of RNA sequencing dataset from the JHI that ultimately led to one of the important findings to this thesis.

I would like to extend my thanks to all the excellent people at the ACPFG specially, Dr. Ryan Whitford, Dr. Nick Collins, Dr. Ursula Langridge, Dr. Takashi Okada, Dr. Nataliya Kovalchuk, Margaret Pallotta, Larissa Chirkova, Raghuveeran Anbalagan, Vahid Eichi, Yuan Li, Hui Zhou, Priyanka Kalambettu, Dr. Gwenda Mayo from Waite Research Institute & School of Agriculture, Food and Wine, and Adelaide Microscopy, University of Adelaide Waite Facility, also Asmini Athman from Plant Research Centre for their cooperation during my PhD.

I would also like to thank Huihui, Dilrukshi, Saba, Judith, Taj, Yanfei, Shefat and Ali for being such good friends along my journey at the University of Adelaide.

I am grateful to the Bangladesh Rice Research Institute (BRRI), the Ministry of Agriculture for providing me study leave to pursue this PhD program.

I especially thank my wonderful parents for their encouragement and mental support. I am particularly grateful to my inspirational partner Jamil, a fun-guy, for his everlasting support and care. Jamil, you are the one in my life who has made me more than I am!

Last but not least, I would have never been able to accomplish this task without the inspiration of the Almighty.

List of Publications

1. Ferdous J., Hussain, S.S. & Shi, B.J. (2015a) Role of microRNAs in plant drought tolerance. Plant Biotechnology Journal 13, 293–305.

2. Ferdous J., Li Y., Reid N., Langridge P., Shi B.J. & Tricker P.J. (2015b) Identification of reference genes for quantitative expression analysis of microRNAs and mRNAs in barley under various stress conditions. PLoS ONE 10(3), e0118503.

3. Ferdous J., Sanchez-Ferrero J.C., Langridge P., Milne L., Chowdhury J. & Tricker P.J. (2015) Differential expression of microRNAs and potential targets under drought stress in barley (Accepted by Plant, Cell & Environment, doi: 10.1111/pce.12764).

List of Awards

Best Poster Award (2014)

Awarded for the best poster presentation at the Research Day, School of Agriculture, Food and Wine, the University of Adelaide.

Travel Award (2015)

Awarded by the Grains Research and Development Corporation, Australia to attend the "Mini-symposium on Cereal Genomics to address Grand Challenges" in Grasmere, Cumbria, England.

CHAPTER 1

General introduction

Structure of this thesis

The thesis is presented as a series of four papers. Two manuscripts have been published, one manuscript is in press and these are included in the published/accepted format. The remaining chapter is drafted as a manuscript for publication. In Chapter 1 (this chapter), a general introduction sets out the context of the thesis and indicates the research objectives. Chapter 2 is a published review and provides the broad background for the work presented in the thesis in order to rationalize the research objectives. Chapters 3, 4 and 5 are the three experimental papers. Each experimental chapter includes: Abstract, Introduction, Materials and Methods, Results, Discussion and References. Each chapter is prefaced by a statement of authorship of the paper that describes each author's contribution, and a link page that serves to connect the chapter to the broader hypotheses addressed by this thesis. In Chapter 6, the general discussion of this thesis is presented which covers the broader significance of outcomes generated in this PhD project. Future research directions are also suggested. This thesis is in agreement with the specification of thesis of the Adelaide Graduate Centre Higher Degree by Research, University of Adelaide, South Australia. This "thesis by publication" format might show some unavoidable repetition, especially in the Materials and Methods sections, but this has been kept to a minimum. Figure 1 demonstrates the schematic structure of this thesis.

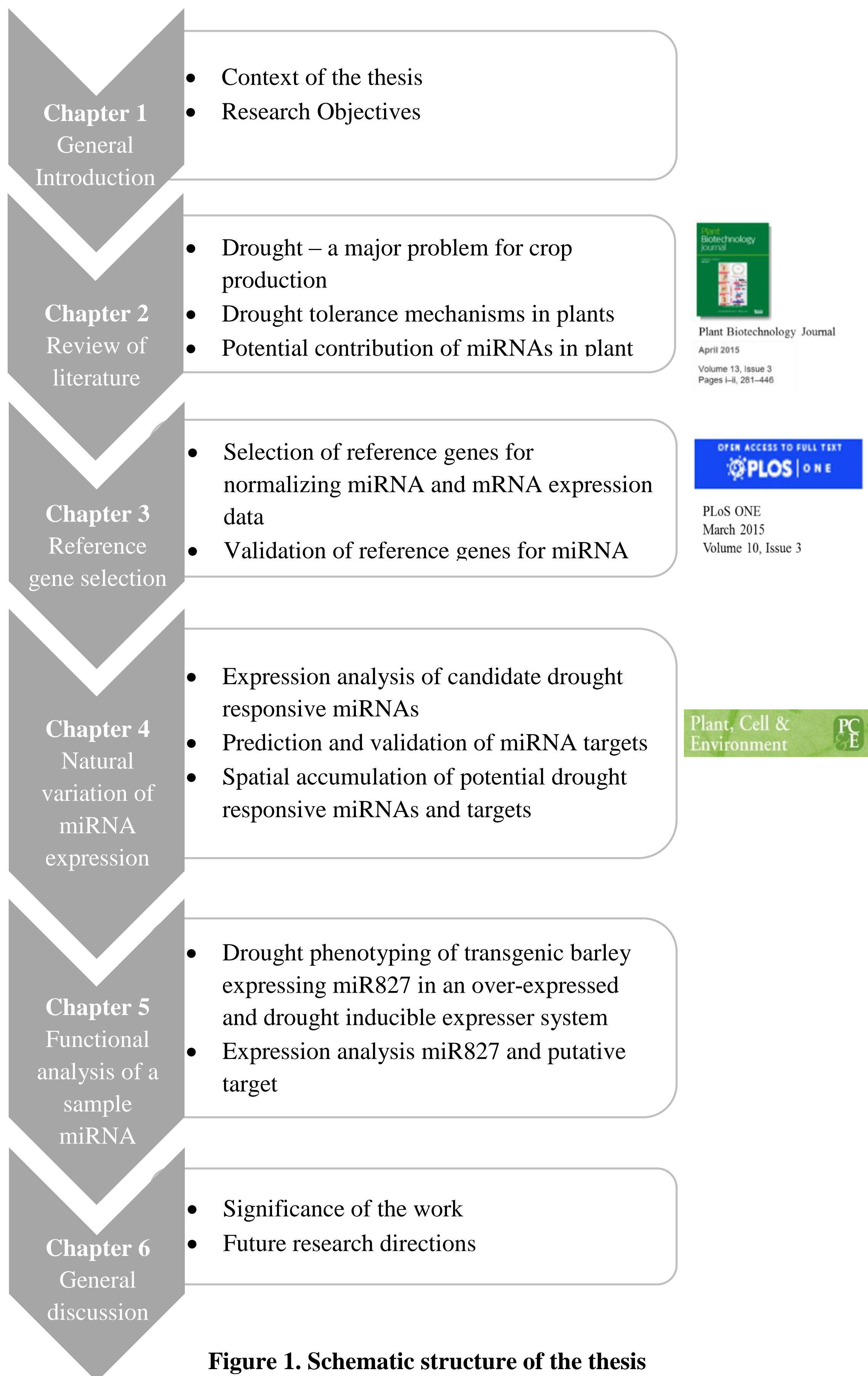

Figure 1. Schematic structure of the thesis

Context of the thesis

A significant area of the world is affected by drought, one of the major factors that limits crop production (Ceccarelli *et al*. 2007). The incidence and severity of drought have increased, putting growing pressure on irrigated crop production in arid and semi-arid areas (Boyer and Westgate 2004). The capacity to increase agricultural land is closely related to available water resources. Currently, agriculture demands over 70% of the world's available water, but the water available for agriculture is expected to decline to 40% by 2050 as the overall demand is projected to increase by 55% (Blum, 2015). Moribund water resources and the dwindling size of agricultural land necessitate the development of drought tolerant crop varieties. However, varietal development for enhancing drought tolerance is hindered by the complexity and low heritability of drought tolerance traits which involve a large number of genes (Shinozaki and Yamaguchi-Shinozaki, 2007). Today, the ultimate vision of crop improvement combines traditional plant breeding coupled with promising biotechnological innovations.

In recent years, RNA interference (RNAi) has become an advanced technology in crop genetic improvement for regulation of specific genes. Naturally occurring RNAi is an important mechanism in plants and animals that uses small RNAs (approx. 20–30 nucleotides) to recognize and manipulate complementary nucleic acids in order to regulate gene expression (Obbard *et al*., 2009; Sinha, 2010; Tanguy and Miska, 2013). One of the central molecules for naturally occurring RNA interference is microRNA (miRNA). miRNAs are non-protein coding small RNA molecules, approximately 18-21 nucleotides (nt), single-stranded and highly conserved within eukaryotes (Reinhart *et al*. 2002; Jones-Rhoades *et al*., 2006). Single stranded miRNAs bind to Argonaute proteins (AGO) to form the RNA induced silencing complex (RISC). miRNAs then guide the

RISC to bind to the target mRNA in a sequence specific mode. Binding of miRNAs to their sequence specific targets leads to mRNA transcript cleavage, where AGO performs the slicer activity (Lee *et al.*, 1993; Fagard *et al.*, 2000; Baumberger and Baulcombe, 2005; Naqvi *et al.*, 2012). Under stressful growing conditions, plants perceive stress signals and respond to reprogramme gene expression. Thus, plants maintain the re-establishment of cellular homeostasis by the alteration of gene expression under adverse conditions. This process is partly determined by miRNAs, which play an important regulatory role for gene expression (Zhu, 2002; Sunkar *et al.*, 2012).

Barley (*Hordeum vulgare*) is an economically important crop all over the temperate world (Newton *et al.*, 2011). miRNAs have been identified in many plant species, including barley, and sequence based profiling and prediction of miRNAs and target genes have become an exhilarating research topic in molecular biology in recent years (Sunkar *et al.*, 2008; Zhang *et al.*, 2009; Song *et al.*, 2010; Barrera-Figueroa *et al.*, 2011; Schreiber *et al.*, 2011; Lv *et al.*, 2012). Computational prediction of miRNAs in some *Triticeae* species has revealed numerous conserved miRNA precursors and mature miRNAs (Dryanova *et al.*, 2008, Schreiber, *et al.*, 2011). However, very few studies have demonstrated varying expression of mature miRNAs in barley genotypes to evaluate the promise of miRNAs as gene regulatory molecules for plant drought tolerance (Kantar *et al.*, 2010, Hackenberg *et al.*, 2015). Identification and validation of drought responsive barley miRNAs and their target genes, and functional analysis of miRNAs for phenotyping their performance under drought remain a big challenge. Studying the involvement of drought associated miRNAs would help our understanding of their potential contribution to drought tolerance in barley.

Research objectives

The objectives of this PhD project were to:

1. Analyse natural variation in miRNA expression among barley genotypes under drought stress to assess the scope for selecting drought tolerant genotypes based on miRNA expression profiles.
2. Examine the potential value of miRNA over-expression as a tool to enhance drought tolerance using a sample miRNA in transgenic plants and phenotyping performance under drought.

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CHAPTER 2

Role of microRNAs in plant drought tolerance

Statement of Authorship

Title of Paper	**Role of microRNAs in plant drought tolerance**
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Ferdous J., Hussain S. S., Shi B. J. (2015). Role of microRNAs in plant drought tolerance. Plant Biotechnology Journal 13, 293–305. 10.1111/pbi.12318. This is a review article summarizes the current understanding of the regulatory mechanisms of plant microRNAs (miRNAs), involvement of plant miRNAs in drought stress responses and adaptive mechanisms under drought. Potential strategies and directions for future miRNA research are discussed which is closely related to the subject matter of this thesis.

Principal Author

Name of Principal Author (Candidate)	Jannatul Ferdous		
Contribution to the Paper	Collected, analysed and interpreted literature, wrote manuscript.		
Overall percentage (%)	60%		
Certification:	The literature research I conducted during the period of my candidature, and this article has not been accepted for any other University award. I am the primary author of this paper. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	09/02/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Syed S. Hussain		
Contribution to the Paper	Wrote, evaluated and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	14/01/2016

Name of Co-Author	Bu-Jun Shi		
Contribution to the Paper	Wrote, evaluated and edited the manuscript. Acted as the corresponding author. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	10/12/2015

Link to chapter 2

This chapter sets the scene for the challenges in cereal drought research, potential involvement of miRNAs in plant drought tolerance and provides an overview of the literature associated with the current understanding of gene regulatory mechanisms of plant miRNAs and the contribution of miRNAs in drought stress responses in different plant species. In this chapter, we also discuss the involvement of miRNAs in plants' drought-adaptive mechanisms. Possible directions for future miRNA research regarding the utilization of miRNAs as a tool for the improvement of drought tolerant cereal crops are also discussed. This chapter (Chapter 2) has been published as follows: Ferdous J., Hussain Syed S. & Shi B.J. (2015) Role of microRNAs in plant drought tolerance. Plant Biotechnology Journal 13, 293–305.

Plant Biotechnology Journal

Plant Biotechnology Journal (2015) **13**, pp. 293–305 doi: 10.1111/pbi.12318

Role of microRNAs in plant drought tolerance

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Received 14 July 2013;
revised 27 November 2014;
accepted 29 November 2014.
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Keywords: microRNA, drought stress, functional mechanism, genetic modification.

Summary

Drought is a normal and recurring climate feature in most parts of the world and plays a major role in limiting crop productivity. However, plants have their own defence systems to cope with adverse climatic conditions. One of these defence mechanisms is the reprogramming of gene expression by microRNAs (miRNAs). miRNAs are small noncoding RNAs of approximately 22 nucleotides length, which have emerged as important regulators of genes at post-transcriptional levels in a range of organisms. Some miRNAs are functionally conserved across plant species and are regulated by drought stress. These properties suggest that miRNA-based genetic modifications have the potential to enhance drought tolerance in cereal crops. This review summarizes the current understanding of the regulatory mechanisms of plant miRNAs, involvement of plant miRNAs in drought stress responses in barley (*Hordeum vulgare* L.), wheat (*Triticum* spp.) and other plant species, and the involvement of miRNAs in plant-adaptive mechanisms under drought stress. Potential strategies and directions for future miRNA research and the utilization of miRNAs in the improvement of cereal crops for drought tolerance are also discussed.

Introduction

Drought is one of the most common environmental stresses affecting growth, development and yield of plants (Ceccarelli and Grando, 1997). Understanding plant tolerance to drought is important for the improvement of crop productivity (Lawlor, 2013). During evolution, plants have developed different defence strategies against drought. One of them is to escape the drought by timing the most sensitive stages of development (e.g. reproductive stage) to occur when the stress is less severe. Another strategy is drought avoidance, involving maintenance of high tissue water potential. The third strategy combines enhanced water acquisition using a deep root system with minimization of water loss by restraining transpiration. Mechanisms of drought tolerance include maintenance of turgor through osmotic adjustment, increased cell elasticity and decreased cell size as well as desiccation tolerance via protoplasmic tolerance. In molecular terms, many genes have been implicated in drought tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007). However, transgenic plants overexpressing some drought-responsive genes did not exhibit significant improvements or had no improvement at all for drought tolerance (Bartels and Sunkar, 2005). This may reflect the fact that the plant drought stress responses, tolerance mechanisms and genetic control of tolerance are complex.

Expression of microRNAs (miRNAs) has been found to be altered in plants during drought stress. This finding helps shed light on drought response mechanisms which can potentially be targeted in development of new drought tolerant crops (Chen *et al.*, 2012; Kantar *et al.*, 2010; Niu *et al.*, 2006; Zhao *et al.*, 2007). The focus of this review is to provide an update on microRNAs and their involvement in responses to stresses, particularly in cereal crop species against drought. Firstly, we outline the knowledge on biogenesis and functions of plant miRNAs. Another section addresses the behaviour and roles of miRNAs under drought stress in barley and wheat. Then, the work regarding the involvement of miRNAs in potential drought-adaptive mechanisms of plants is discussed. Finally, we discuss the scope for utilizing miRNAs for improving drought tolerance of crop plants, especially barley and wheat.

MiRNAs: discovery, biogenesis and mechanisms

miRNA were first discovered in the nematode *Caenorhabditis elegans* in 1993 at which time they were considered as small temporal RNAs (stRNAs; Lee *et al.*, 1993). In 2001, miRNAs were formally named and recognized as a distinct class of RNAs with regulatory functions (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Plant miRNAs were identified 10 years after animal miRNAs (Reinhart *et al.*, 2002). Now, 7385 mature miRNAs and 6150 precursor miRNAs (pre-miRNAs) have been identified in 72 plant species (miRBase, 20 June 2013; Griffiths-Jones *et al.*, 2008). miRNAs are single-stranded noncoding RNAs sized usually between 20 and 24 nucleotides (nt) that serve as gene regulators in a wide range of organisms (Lee *et al.*, 1993; Reinhart *et al.*, 2002; Shabalina and Koonin, 2008). They affect many biological processes including development of organs such as roots, stems, leaves and flower parts (Bartel, 2004; Bian *et al.*, 2012; Chen, 2004; Chen *et al.*, 2011; Kim *et al.*, 2005; Liu and Chen, 2009; Maizel and Jouannet, 2012; Ronemus and Martienssen, 2005; Vaucheret *et al.*, 2004; Wang *et al.*, 2005, 2008). A growing body of evidence suggests that miRNAs play key roles in plant responses to biotic and abiotic stresses. miRNAs mediate the responses by modulating the amount of themselves, the amount of mRNA targets or the activity/mode of action of miRNA–protein complexes. In turn, these changes modify the

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timing, location and amount of proteins expressed from other genes upon exposure to the stress. Much of the gene regulation by miRNAs in response to plant biotic and abiotic stresses occurs at the post-transcriptional level (Ding *et al.*, 2013; Feng *et al.*, 2013; Floris *et al.*, 2009; Gupta *et al.*, 2012; Jian *et al.*, 2010; Liu *et al.*, 2008, 2012; Ozhuner *et al.*, 2013; Sunkar *et al.*, 2006; Wang *et al.*, 2014; Xie *et al.*, 2012; Yang *et al.*, 2012; Zhang *et al.*, 2009, 2011). In this section, we briefly describe how miRNAs are generated and functional in organisms.

miRNAs are transcribed from genes, but the transcripts are not translated into proteins. The primary transcript (pri-miRNAs) base pairs with itself to create a structure containing stem-loop and double-stranded RNA sections, and which is then processed by an RNase III enzyme [called Dicer-like1 (DCL1) in plants and Drosha in animals] into stem-loop structures of approximately 70 nt (pre-miR NAs). The release of a miRNA/miRNA* duplex from a pre-miRNA is carried out in the nucleus by DCL1 in both plants and animals. 'miRNA' refers to the strand that will become the miRNA, and 'miRNA*' refers to the strand that is complementary to the miRNA. Each strand is methylated to avoid degradation before being transported to the cytoplasm where the miRNA strand of the miRNA/miRNA* duplex is incorporated into a protein complex known as RISC (RNA-induced silencing complex), which is a multiprotein complex that incorporates one strand of miRNA or a small interfering RNA (siRNA). For a detailed description of miRNA biogenesis, readers are referred to the studies by Bao *et al.* (2004), Baumberger and Baulcombe (2005), Khraiwesh *et al.* (2010), Lee *et al.* (2004), Park *et al.* (2005), Voinnet (2009), and Wu *et al.* (2010).

Plant miRNAs are derived by processing of their RNA precursors. Such precursors are occasionally transcribed from an intron or exon of a protein coding region, but most precursors are transcribed from the intergenic regions of genomes (Chen, 2004; Jones-Rhoades *et al.*, 2006; Kim, 2005; Reinhart *et al.*, 2002). miRNA biogenesis involves multiple steps to form mature miRNAs from miRNA genes (Chen, 2009; Jones-Rhoades *et al.*, 2006; Kim, 2005; Park *et al.*, 2005; Voinnet, 2009). Mirtrons, a type of miRNAs, originating from the introns can bypass the microprocessor complex (a multisubunit complex comprising the RNAse III enzyme) and directly enter as pre-miRNA into the miRNA maturation pathway (Zhu *et al.*, 2008). A few miRNAs can be generated independently of the splicing pathway, but details of their maturation are obscure (Johanson *et al.*, 2013). Both strands of a miRNA duplex can be incorporated into the Argonaute (AGO)-containing RISC complex used for silencing the target (Okamura *et al.*, 2008). From this fact, defining which strand is miRNA and which strand is miRNA* is difficult. Perhaps for this reason, currently, the miRNA and miRNA* terms are widely replaced by the '3p' and '5p' suffixes according to their positions in the precursor miRNAs (pre-miRNAs). Surprisingly, loop-derived miRNAs were recently identified and shown to be functional (Hackenberg *et al.*, 2013; Okamura *et al.*, 2013; Winter *et al.*, 2013). However, how these loop-derived miRNAs are generated has not yet been elucidated. Once a miRNA is incorporated into the RISC, it would guide AGO by base pairing with mRNA to cleave the target (Baumberger and Baulcombe, 2005; Jones-Rhoades *et al.*, 2006) or inhibit translation of the target (Arteaga-Vazquez *et al.*, 2006; Aukerman and Sakai, 2003; Brodersen and Voinnet, 2009; Brodersen *et al.*, 2008; Chen, 2004; Gandikota *et al.*, 2007; Gu and Kay, 2010; Vazquez *et al.*, 2010). While miRNA mediated post-transcriptional gene regulation is common, in human cells miRNAs also regulate genes at the transcriptional level (Kim *et al.*, 2008; Place *et al.*, 2008). The mechanism of action is unclear, but it is likely to be via miRNA-directed DNA methylation, which occurs at cytosine in all sequence contexts (Axtell, 2013; Chellappan *et al.*, 2010; Khraiwesh *et al.*, 2010; Vazquez *et al.*, 2008; Wu *et al.*, 2009, 2010; Zhu *et al.*, 2008). This way of regulation is very similar to siRNA-directed DNA methylation (Chan *et al.*, 2005; Matzke *et al.*, 2009). siRNA is a class of double-stranded small RNAs of 21–24 base pairs in length, which plays important roles in the RNA interference (RNAi) pathway. In contrast to siRNAs, miRNA-directed DNA methylation is affected by multiple factors such as the Dicer member (Khraiwesh *et al.*, 2010), miRNA size (Wu *et al.*, 2010), AGO member (Axtell, 2013; Chellappan *et al.*, 2010; Khraiwesh *et al.*, 2010; Vazquez *et al.*, 2008; Wu *et al.*, 2010) and stability of the duplex miRNA (Khraiwesh *et al.*, 2010). In plants, miRNAs mainly function at the post-transcriptional gene silencing (PTGS) level and guide the AGO protein to cleave the target mRNA between positions 10 and 11 (relative to the 5′ end of the miRNA). Although the exact way of translational inhibition is still obscure, it is assumed that, during translation, miRNAs do not result in mRNA cleavage, but arrest translation by blocking read-through of the ribosome (Wang *et al.*, 2008). A recent study showed that miRNA-directed translation inhibition occurs at the endoplasmic reticulum (ER) and requires ALTERED MERISTEM PROGRAM1 (AMP1) (Li *et al.*, 2013). Given that homologues of AMP1 are present in animal genomes, it is possible that the connection between the ER and translation inhibition by miRNAs is conserved across plants and animals (Li *et al.*, 2013). Taken together, it is suggested that miRNAs may regulate the expression of their target genes via a combination of the aforementioned mechanisms (Eulalio *et al.*, 2008). Large amounts of data have indicated that miRNA regulatory activity has effects on growth and development as well as on responses to environmental stresses (Berger *et al.*, 2009; Khraiwesh *et al.*, 2012; Llave, 2004; Meng *et al.*, 2009; Reyes and Chua, 2007; Rodriguez *et al.*, 2010; Schommer *et al.*, 2008; Sunkar and Zhu, 2004). As the biogenesis and functional mechanism of miRNAs have already been reviewed extensively, we will not discuss those aspects further.

miRNA responses to drought stress

Drought stress has been revealed to alter expression of many genes/metabolites, including dehydrins, vacuolar acid invertase, glutathione S-transferase (GST), abscisic acid (ABA)-inducible genes [LEA (late embryo abundant), RAB (responsive to abscisic acid), COR (cold regulated), Rubisco (5-bisphosphate carboxylase-oxygenase)], helicase, proline and carbohydrates (Nezhadahmadi *et al.*, 2013; references therein). miRNAs as gene regulators are expected to participate in the regulation of these drought-responsive genes. Studies have shown that the expression of miRNAs is themselves altered in response to drought stress. Drought-responsive miRNAs have been reported in many plant species such as *Arabidopsis* (Sunkar and Zhu, 2004), rice (Zhou *et al.*, 2010), cowpea (Barrera-Figueroa *et al.*, 2011), tobacco (Frazier *et al.*, 2011), soya bean (Kulcheski *et al.*, 2011), *Phaseolus vulgaris* (Arenas-Huertero *et al.*, 2009) and so on and have been summarized in Table 1. In *Arabidopsis*, miR156, miR159, miR167, miR168, miR171, miR172, miR319, miR393, miR394a, miR395c, miR395e, miR396 and miR397 are up-regulated, while miR161, miR168a, miR168b, miR169, miR171a and miR319c are down-regulated, under drought stress (Liu *et al.*,

Table 1 Drought-responsive miRNAs in different plant species

miRNA	Target name and functions*	Species[†]	Source
miR156	SBP family of transcription factors—promote phase transitions, flowering time	*Ath*↑, *Tdi*↑, *Hvu*↑, Rice↓ *Peu*↑, *Ppe* (slightly)↑, *Pto*↓	Eldem *et al.* (2012), Kantar *et al.* (2011), Liu *et al.* (2008), Ren *et al.* (2012), Wu and Poethig (2006) and Zhou *et al.* (2010)
miR157	SBP family of transcription factors	*Ppe*↑↓	Eldem *et al.* (2012)
miR159	MYB and TCP transcription factors—ABA response, Nacl stress response, floral asymmetry and leaf development	*Ath*↑ Rice↓ *Ppe*↓	Arenas-Huertero *et al.* (2009), Eldem *et al.* (2012), Jones-Rhoades and Bartel (2004), Liu *et al.* (2008), Reyes and Chua (2007) and Zhou *et al.* (2010)
miR160	ARF 10, ARF 16 and ARF 17—seed germination and postgermination stages	*Ppe*↑, *Pto*↑, *Ptc*↓	Eldem *et al.* (2012), Jones-Rhoades and Bartel (2004), Liu *et al.* (2007), Ren *et al.*, (2012) and Shuai *et al.* (2013),
miR162	DCL1—miRNA biogenesis	*Pto*↑	Ren *et al.* (2012) and Xie *et al.* (2003)
miR164	NAC domain TF—lateral root development	*Mtr*↓, *Ptc*↓, *Bdi*↓	Shuai *et al.* (2013) and Wang *et al.* (2011)
miR165	HD-ZIPIII transcription factor—axillary meristem initiation, leaf and vascular development	*Ppe*↓	Eldem *et al.* (2012)
miR166	HD-ZIPIII transcription factor—axillary meristem initiation, leaf and vascular development	*Tdi*↓, *Gma*↑	Kantar *et al.* (2011), Li *et al.* (2011a,b), Sun (2012) and Williams *et al.* (2005)
miR167	ARF6 and ARF8—gynoecium and stamen development	*Ath*↑, *Ppe*↓, *Pto*↑	Eldem *et al.* (2012), Liu *et al.* (2008), Ren *et al.* (2012) and Wu and Poethig (2006)
miR168	ARGONAUTE1, MAPK—miRNA biogenesis and mRNA degradation, plant development	*Ath*↑ Rice↓ *Z. mays*↓	Liu *et al.* (2008), Wei *et al.* (2009) and Zhou *et al.* (2010)
miR169	NF-YA transcription factor subunit A-3, NF-YA transcription factor subunit A-10, SlMRP1—Plant development and Flowering timing, response to different abiotic stresses	*Ath*↓, Tomato↑, Rice↑, *Mtr*↓, *Ppe*↓, *Gma*↑, *Pto*↓, *Peu*↑	Eldem *et al.* (2012), Li *et al.* (2008), Li *et al.* (2011a,b), Qin *et al.* (2011), Ren *et al.* (2012), Trindade *et al.* (2010), Wang *et al.* (2011), Zhang *et al.* (2011), Zhao *et al.* (2007) and Zhou *et al.* (2010)
miR170	SCL transcription factor—radial patterning in roots, floral development and shoot branching	*Ath*↓, Rice↓	Sun (2012) and Zhou *et al.* (2010)
miR171	GRAS transcription factors—response to abiotic stresses and floral development	*Ath*↑, *Tdi*↓, Rice↑↓, *Mtr*↓, *Ppe*↑, *Pto*↓,	Eldem *et al.* (2012), Kantar *et al.* (2011), Llave *et al.* (2002), Liu *et al.* (2008), Ren *et al.* (2012), Wang *et al.* (2011) and Zhou *et al.* (2010)
miR172	cDNA floral homeotic protein APETALA2, bZIP transcription factor family protein—flowering time, floral organ identity, cold stress response	*Ath*↑,Rice↓, *Pto*↑	Jones-Rhoades and Bartel (2004), Ren *et al.* (2012) and Zhou *et al.* (2010)
miR319	TCPcell differentiation, leaf development and biosynthesis of jasmonic acid	*Ath*↑, Rice↑↓, *Pto*↑	Efroni *et al.* (2008), Ren *et al.* (2012), Sarvepalli and Nath (2011), Schommer *et al.* (2008), Sunkar and Zhu (2004) and Zhou *et al.* (2010)
miR390	ARF—auxin-mediated transcriptional activation/suppression	*Pto*↓	Allen *et al.* (2005) and Ren *et al.* (2012)
miR393	TIR1 and AFB2 and AFB3—susceptibility to virulent bacteria	*Ath*↑ *Ppe*↓	Liu *et al.* (2008), Navarro *et al.* (2006) and Eldem *et al.* (2012)
miR394	Dehydration-responsive protein and F-box proteins—abiotic stress-response pathway	*Pto*↑, Ptc↓, *Gma*↑	Li *et al.* (2011a,b), Ren *et al.*(2012) and Shuai *et al.* (2013)
miR395	Sulphate transporter—response to sulphate deprivation	Rice↑, *Ppe*↓, *Pto*↓	Eldem *et al.* (2012), Liang *et al.* (2010), Ren *et al.* (2012) and Zhou *et al.* (2010)
miR396	GRL transcription factors; ceramidasegenes —leaf and cotyledon development	*Ath*↑ Rice↓ *Mtr*↓ *Ppe*↓	Eldem *et al.* (2012), Kantar *et al.* (2011), Liu *et al.* (2008), Liu and Yu (2009), Sun (2012), Wang *et al.* (2011) and Zhou *et al.* (2010)
miR397	Laccases—lignin biosynthesis, ion absorption and stress response	*Ath*↑, Rice↓, *Ppe*↓, *Pto*↓	Abdel-Ghany and Pilon (2008), Ding and Zhu (2009), Eldem *et al.* (2012), Ren *et al.*(2012), Sunkar and Zhu (2004) and Zhou *et al.* (2010)
miR398	Copper superoxide dismutases; cytochrome C oxidase subunit V—Copper	*Mtr*↑, *Tdi*↑, *Mtr*↓, *Ppe*↓	

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Table 1 Continued

miRNA	Target name and functions*	Species†	Source
	homoeostasis, oxidative stress; enzyme involved in respiration		Eldem *et al.* (2012), Jones-Rhoades and Bartel (2004), Kantar *et al.* (2011), Sunkar *et al.* (2006), Trindade *et al.* (2010) and Wang *et al.* (2011)
miR399	Phosphate transporter–role in response to phosphate starvation	*Mtr*↑, *Pto*↑↓	Bari *et al.* (2006), Jones-Rhoades and Bartel (2004), Ren *et al.* (2012) and Wang *et al.* (2011)
miR403	AGO2—miRNA functioning	*Pto*↑	Allen *et al.* (2005) and Ren *et al.* (2012)
miR408	Chemocyanin precursor, cDNA phosphatidylinositol 3 and 4—kinase family protein, Peptide chain release factor—pollen tube growth	Rice↓, *Ath*↑, *Mtr*↑, *Ppe*↓, *Pto*↓, *Ptc*↓	Eldem *et al.* (2012), Liu *et al.* (2008), Ren *et al.* (2012), Shuai *et al.* (2013), Trindade *et al.* (2010) and Zhou *et al.* (2010)
miR474	Kinesin, a pentatricopeptide repeat (PPR) family protein-Motor functions; organelle biogenesis	Rice↑ *Tdi*↑	Kantar *et al.* (2011), Lu *et al.* (2005) and Zhou *et al.* (2010)
miR528	POD—Elimination of ROS	*Z. mays*↓	Wei *et al.* (2009)
miR827	NAD (P)-binding and SPX (SYG1/Pho81/XPR) proteins—activate in signal transduction pathways	*Z. mays*↑	M. Aukerman and W. Park (unpubl. data) and Zhang *et al.* (2009)
miR1432	Poly (ADP-ribose) polymerase; calcium-binding EF hand domains—activate in signal transduction pathways	*Tdi*↑	Kantar *et al.* (2011) and Zhang *et al.* (2009)
miR1444‡	Polyphenol oxydase—Probable role for improving plant water stress	*Ptc*↓	Khraiwesh *et al.* (2012), Shuai *et al.*, (2013) and Thipyapong *et al.* (2004)
miR2118	TIR-NBS-LRR domain protein—response to salinity, drought, cold and ABA stress	*Mtr*↑	Jagadeeswaran *et al.* (2009) and Wang *et al.* (2011)

*AFB, Auxin F-box protein; AGO2, Family member of ARGONAUT protein; AP2, APETALA2; ARF, auxin response factors; bHLH, basic helix–loop–helix; bZIP, Basic leucine zipper domain; CBF, CCAAT-binding factor; DCL1, Dicer Like1; GRAS, GAI, RGA, SCR; GRL, growth-regulating factor; GRML, Gibberellin response modulator-like protein; HD-ZIP, class III homeodomain leucine zipper; L-RTMK, Leucine-rich repeat transmembraneprotein kinase; MAPK, Mitogen-activated protein kinase; NAC domain TF, (NAM, ATAF1/2 and CUC2) domain proteins; NB-ARC domain protein, NB, ARC1 and ARC2 (functional ATPase domain—Probable regulation for activating the resistance proteins); NBS-LRR domain protein, Nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins; NF-YA, Nuclear factor Y subunit A; PDC, pyruvate decarboxylase isozyme1; PPRs, pentatricopeptide repeat (PPR) proteins; POX/POD, Peroxidase; SBP, Squamosa promoter-binding protein; SCL, scarecrow-like; SlMRP1, Multidrug resistance-associated protein gene; SNF7, Vacuolar-sorting protein; TCP-TEOSINTE BRANCHED/CYCLOIDEA/PCF) transcription factor genes; TF, transcription factor; TIR1, transport inhibitor response1.

†↑, up-regulation by drought; ↓, down-regulation drought; *Ath*, *Arabidopsis*; *Bdi*, *Brachypodium distachyon*; *Gma*, *Glycine max*; *Hvu*, *Hordeum vulgare*; *Mtr*, *Medicago truncatula*; *Peu*, *Populus euphratica*; *Ptc*, *Populus trichocarpa*; *Pto*, *Populus tomentosa*; *Ppe*, *Prunus persica*; *Tdi*, *Triticum dicoccoides*; *Z. mays*, *Zea mays*.

‡This miRNA was only found in *Populus trichocarpa*. All the rest miRNAs listed in Table 1 are present in more than three plant species and hence are considered as conserved miRNAs.

2008; Sunkar and Zhu, 2004). The up-regulated miRNAs were also shown to be involved in different developmental stages (Alonso-Peral *et al.*, 2012; Curaba *et al.*, 2013; Vaucheret *et al.*, 2006; Wu and Poethig, 2006; Wu *et al.*, 2006; Xie *et al.*, 2014; Zhu and Helliwell, 2011), suggesting that the regulation of drought tolerance and development by miRNAs is tightly linked, which probably undergoes via the same mechanism. It is very common that the expression level or drought responsiveness of a miRNA is species dependent (Arenas-Huertero *et al.*, 2009; Barrera-Figueroa *et al.*, 2012; Frazier *et al.*, 2011; Kantar *et al.*, 2011; Kulcheski *et al.*, 2011; Liu *et al.*, 2008; Lu *et al.*, 2008; Trindade *et al.*, 2010; Zhao *et al.*, 2007; Zhou *et al.*, 2010). For example, drought up-regulates miR156 in *Arabidopsis*, *Prunus persica*, barley, *Panicum virgatum* and *Triticum dicoccoides* (Eldem *et al.*, 2012; Kantar *et al.*, 2010, 2011; Sun *et al.*, 2012b; Sunkar and Zhu, 2004), but down-regulates it in rice and maize (Wei *et al.*, 2009; Zhou *et al.*, 2010). Similarly, drought stress down-regulates miR169 in *Arabidopsis*, *P. persica*, *P. virgatum* and *Medicago truncatula* (Li *et al.*, 2008), but up-regulates it in rice, *Glycine max*, *Populus euphratica* and tomato (Li *et al.*, 2011a,b; Qin *et al.*, 2011; Zhang *et al.*, 2011; Zhou *et al.*, 2010). miR1510 is up-regulated in *Glycine max* but down-regulated in *M. truncatula* and miR396 is down-regulated in *M. truncatula* and *Vigna unguiculata*, but up-regulated in *G. max* (Mantri *et al.*, 2013). In some plant species, members of the same families were found to be differently expressed under drought stress, for example, drought stress down- and up-regulates respective members of the miR319 family in rice (Zhou *et al.*, 2010). In fact, even the same miRNA in the same plant species can show different responses to drought depending on the exact conditions. For instance, in one study expression level of miR398a/b in *M. truncatula* was increased under drought stress (Trindade *et al.*, 2010), while in another study, expression level of the same miRNA in the same plant species decreased under drought stress (Wang *et al.*, 2011). Such differences may reflect different degrees of drought stress (Wang *et al.*, 2011) and high sensitivity of some miRNAs to subtle differences in growing conditions. Indeed, with different externally applied concentrations

of polyethyleneglycol (PEG), a chemical that simulates drought conditions, the same miRNAs such as miR167, miR172, miR393, miR395, miR396, miR398 and miR399 in tobacco plants showed different degrees of up- or down-regulation (Frazier *et al.*, 2011). It is possible that differential expression of the same miRNA in the same plant species under drought conditions is the result of different spatial–temporal manner. It is likely that under drought conditions regulators of miRNA genes change their expression, which in turn leads to the change in expression of miRNAs and ultimately that of miRNAs' targets (Reyes and Chua, 2007; Trindade *et al.*, 2010). It is worth mentioning here that although miRNAs are conserved across different plant species, their targets may not be (Lu *et al.*, 2005). Therefore, the targets of miRNAs need to be identified in individual plant species. Target validation can also help provide functional evidence of the conserved and specific miRNAs in plant species.

miRNAs are also differentially expressed between different tissues or developmental stages under drought stress (Reinhart *et al.*, 2002). This has been the case for miR169, which in rice is induced more prominently in the roots than in the shoots. Members of the miR169 family are encoded by many loci within some plant species. However, in *Arabidopsis*, only miR169a and miR169c are substantially down-regulated by drought stress (Li *et al.*, 2008). Compared to other miR169 loci, the miR169a locus produces 90% of the total miR169 population, suggesting that miR169a would play a major role in response to drought stress. If this is true, then miR169 is likely to be regulated by drought stress at the transcriptional level at their loci. A previous study showed that transgenic overexpression of drought down-regulated miR169a and miR169c in *Arabidopsis* increased drought sensitivity of the plants (Li *et al.*, 2008). However, two members of the miR169 family in rice, miR169g and miR169n/o and one member in tomato, miR169c, were up-regulated by drought stress (Zhang *et al.*, 2011; Zhao *et al.*, 2009). In addition, overexpression of miR169c in tomato reduced stomatal conductance and water loss compared to nontransgenic tomato and hence enhanced drought tolerance (Zhang *et al.*, 2011). These differences in outcomes of overexpressing miR169c in different plant species have been suggested to be caused by different timing, duration and intensity of the stress that was applied in the different studies (Covarrubias and Reyes, 2010). It is likely that the level of miRNA169 could vary during the course of the stress treatment. This has been the case for miR398 in *Arabidopsis* (Jia *et al.*, 2009). The reduced accumulation of miRNAs under drought could be because of interference with their biogenesis pathway (Covarrubias and Reyes, 2010). The contribution of miR169 to drought tolerance or intolerance could depend on its promoter because two dehydration-responsive elements (DREs) were identified in the promoter of MIR169g (Zhao *et al.*, 2007). Further studies showed that miR169 targets nuclear factor Y (NF-Y) transcription factor (TF), known as a heme-activated protein (HAP) or CCAAT-binding factor (CBF), by reducing the NF-Y mRNA level (Li *et al.*, 2008). Furthermore, transgenic overexpression of NFYA5, a subunit of the NF-Y TF, has been shown to increase drought tolerance (Li *et al.*, 2008). These pieces of evidence indicate that the contribution of miR169 to drought tolerance is via the NF-Y TF and that the down-regulation of miR169 contributes to the high level of NFYA5 observed under drought stress.

Transgenic overexpression of osa-miR319 in creeping bentgrass and of miR394 in soya bean also increased drought tolerance (Ni *et al.*, 2012; Zhou *et al.*, 2013). Both miRNAs are up-regulated in most plant species under drought stress (Ni *et al.*, 2012; Zhou *et al.*, 2010). However, under other stress conditions such as salinity, cadmium toxicity or low iron and sulphate, the regulation of miR394 shows differences between plant species (Huang *et al.*, 2010; Kong and Yang, 2010). One of the miR394's targets has been identified to encode an F-box protein (At1g27340) involved in the regulation of leaf curling-related morphology in *Arabidopsis* (Song *et al.*, 2012). miR393 is another key miRNA for the regulation of the F-box genes in many plant species including *Arabidopsis*, rice, *M. truncatula*, *Pinguicula vulgaris* and sugarcane (Ferreira *et al.*, 2012), whose expression is altered by drought stress. Like miR394, miR393 is up-regulated by drought stress in most plant species and is responsive to other abiotic stresses such as salinity, low temperature and aluminium toxicity (Arenas-Huertero *et al.*, 2009; Liu *et al.*, 2008; Sunkar and Zhu, 2004; Trindade *et al.*, 2010; Zhao *et al.*, 2007). Transgenic overexpression of miR393 in rice increased salinity tolerance, suggesting the native gene may regulate salinity tolerance (Gao *et al.*, 2011). Transgenic overexpression of miR393 in rice resulted in hyposensitivity to synthetic auxin analogue treatments (Xia *et al.*, 2012), suggesting that native miR393 may regulate auxin signalling and would thus reduce plant growth under drought stress. Under drought, endogenous concentrations of auxin, gibberellin and cytokinin usually decrease, whereas ABA and ethylene increase (Nilsen and Orcutte, 1996). In line with this hypothesis, miR393 was found to target transport inhibitor response 1 (TIR1), known as an auxin receptor and positive regulator of auxin signalling that acts via degradation of Aux/IAA proteins (Dharmasiri and Estelle, 2002; Windels and Vazquez, 2011). However, how miR393 regulates its targets remains unclear. Table 2 summarizes studies in which transgenic alteration of miRNA expression was tested for effects on drought tolerance.

A number of legume-specific miRNAs were identified in *Phaseolus vulgaris* plants treated by drought and ABA, and targets of these miRNAs were annotated to be involved in diverse cellular processes unique to legumes (Arenas-Huertero *et al.*, 2009). Using deep sequencing technology, Kulcheski *et al.* (2011) identified 256 miRNAs from genotypes of soya bean that were susceptible or resistant to drought or rust. Of these miRNAs, 71 belonged to conserved miRNA soya bean families, while 15 miRNAs belonging to six families were conserved in other plant species. Twenty-nine miRNAs belonging to 24 novel families were reported for the first time in soya bean. The authors also reported 121 alternative isoforms (miRNA variants) derived from 22 conserved miRNA families and four novel miRNA families. An interesting point is that among 11 miRNAs analysed, all were expressed differently from each other during drought stress. However, the majority were up-regulated in a susceptible genotype but down-regulated in a tolerant genotype under drought. This distinct miRNA behaviour across the two genotypes may reflect regulation of the genes associated with drought stress tolerance or intolerance. Similarly, Barrera-Figueroa *et al.* (2011) used deep sequencing of sRNA libraries from two cowpea genotypes (drought tolerant and susceptible) to identify 157 miRNAs which belonged to 89 families. Forty-four drought-responsive miRNAs belonging to 28 families were identified by comparing expression levels in stressed versus control plants. Of them, 30 miRNAs were up-regulated while 14 miRNAs were down-regulated. These drought-responsive miRNAs included miRNA families which were already known to be drought-

Table 2 miRNA transgenics for drought tolerance

Overexpressed miRNA	Species	Transgenic plants exhibited	Possible mechanism	References
miR164	*Arabidopsis*	Leaf longevity	Ethylene signalling molecule, EIN2	Kim *et al.* (2009)
miR169c	Tomato	Reduced stomatal conductance and transpiration rate	Unknown	Zhang *et al.* (2011)
miR169a	*Arabidopsis*	Increased leaf water loss and greater sensitivity to drought stress	Unknown	Li *et al.* (2008)
Osa-miR319	Creeping bentgrass	Increased leaf wax content and water retention capacity	Unknown	Zhou *et al.* (2013)
miR393	Rice	Increased tillering, early flowering and reduced tolerance to salt and drought	Hyposensitivity to auxin	Xia *et al.* (2012)
Gma-miR394a	*Arabidopsis*	Recovery from drought stress	Possible involvement of F-box proteins in abiotic stress-response pathway	Ni *et al.* (2012)

responsive in other plant species, indicating that these miRNA families may be involved in conserved drought-response pathways. In addition, predicted target genes of 32 miRNAs were shown to have diverse predicted physiological functions. Most of these predicted targets were TFs.

Drought up- or down-regulated miRNAs are both potentially relevant for engineering plant drought tolerance, as miRNA targets probably include genes that contribute both positively or negatively to tolerance. The up-regulation of miRNAs means that their targets are down-regulated under the same conditions and vice versa. Enhancing the accumulation of target(s) contributing to drought tolerance could be achieved either by overexpressing target genes, or by silencing the corresponding miRNA (Sunkar *et al.*, 2007). For example, down-regulated miR168 and miR528 under drought stress resulted in accumulation of their targets, mitogen-activated protein kinase (MAPK) and peroxidase (POD) (Wei *et al.*, 2009). In this experiment, ABA levels significantly increased in maize tissues, which in turn enhanced the formation of reactive oxygen species (ROS), which further up-regulated MAPK for inducing the expression of antioxidant genes and antioxidant enzymes. Both ABA and ROS are important signalling molecules that regulate many developmental processes and stress-adaptive processes in plants (Cutler *et al.*, 2010). Antioxidant enzymes also limit ROS levels to help achieve drought stress. Likewise, an increased level of POD also results in the elimination of ROS and alleviation of drought injury (Wei *et al.*, 2009). Therefore, the down-regulation of miR168 and miR528 under drought stress is expected to increase drought tolerance. A study conducted by Shuai *et al.* (2013) showed that the down-regulation of miR160 and miR164 in drought-stressed *P. trichocarpa* also allows increased expression of their targets, ARF and NAC domain TFs. Overexpression of these TFs in rice has been shown to enhance drought stress tolerance in the field under severe drought stress conditions at the reproductive stage (Hu *et al.*, 2006) as well as at the seed germination and postgermination stages (Liu *et al.*, 2007). Drought down-regulated miRNAs in *P. trichocarpa* also included miR408, miR1444 and miR394, which target dehydration-responsive proteins such as early responsive dehydration-related protein (ERD) and polyphenol oxidase (PPO) (Shuai *et al.*, 2013). Increased expression levels of these targets help lessen drought injury in transgenic plants (Shuai *et al.*, 2013). In the same study, two novel miRNAs Ptc-miRn6 and Ptc-miRn16 were also confirmed to be down-regulated in *P. trichocarpa*, but the function of the targets of these two miRNAs are unknown (Shuai *et al.*, 2013). Therefore, alteration in miRNA profiles seems to play crucial roles in attenuating plant growth and development under stresses. In a nutshell, these findings highlight the importance of detailed characterization of stress-responsive miRNAs in plants.

Drought-responsive miRNAs in wheat and barley

Wheat and barley are two of the most important cereals in the world and are crops that are seriously affected by drought. Furthermore, both *Triticeae* species contain large and repetitive genomes, which are, respectively, much larger than that of rice or *Arabidopsis*. Therefore, in this section, we particularly summarize the recent knowledge on drought-responsive miRNAs in these two crops, which were as yet given little attention before.

In miRBase (Release 20: June 2013), 69 miRNAs from barley and 43 miRNAs from wheat were described. Additional barley and wheat miRNAs were described in published papers (Colaiacovo *et al.*, 2010; Curaba *et al.*, 2012; Dryanova *et al.*, 2008; Hackenberg *et al.*, 2012a,b, 2014; Han *et al.*, 2014; Jin *et al.*, 2008; Kantar *et al.*, 2010, 2011; Li *et al.*, 2013; Lucas and Budak, 2012; Lv *et al.*, 2012; Meng *et al.*, 2013; Schreiber *et al.*, 2011; Sun *et al.*, 2014; Wang *et al.*, 2014; Wei *et al.*, 2009; Xin *et al.*, 2010; Yao *et al.*, 2007, 2010). Barley miRNAs were initially predicted from available barley EST sequences by Dryanova *et al.* (2008). These included 28 conserved miRNAs belonging to 15 miRNA families. A more sophisticated computational prediction approach was then used to extend this to 156 miRNAs belonging to 50 miRNA families (Colaiacovo *et al.*, 2010). In 2011, 100 barley miRNAs were experimentally identified by deep sequencing small RNAs of barley cultivar Golden Promise (Schreiber *et al.*, 2011). Of these miRNAs, 56 were shown to be expressed as orthologs in other species, while 44 miRNAs were known to be expressed only in barley. Soon after, deep sequencing of small RNAs from a different barley cultivar, clipper, identified 259 miRNAs, of which133 were novel (Lv *et al.*, 2012). Using psRNA target, a plant small RNA target analysis server (http://plantgrn.noble.org/psRNATarget/), 267 targets of barley miRNAs were predicted (Lv *et al.*, 2012). These targets were predicted to be

involved in many developmental processes such as seed germination, vegetative and reproductive phase changes, flowering initiation and seed production (Lv *et al.*, 2012). However, the validation of these miRNA targets is likely to be complex as each miRNA may control many genes and each gene can be controlled by many miRNAs (Yang and Qu, 2013).

Wheat miRNAs were first computationally predicted in 2005 (Zhang *et al.*, 2005), at which time only 16 miRNAs belonging to nine conserved miRNA families were identified from wheat EST databases. In 2007, 58 miRNAs belonging to 43 miRNA families were discovered by cloning and sequencing of wheat small RNAs (Yao *et al.*, 2007). So far, 270 known miRNAs have been reported in wheat (Dryanova *et al.*, 2008; Jin *et al.*, 2008; Kantar *et al.*, 2010, 2011; Lucas and Budak, 2012; Pandey *et al.*, 2014; Wei *et al.*, 2009; Xin *et al.*, 2010). The identified barley and wheat miRNAs provide a platform for further analysis of expression profiles of miRNAs and characterization of drought-responsive miRNAs in barley and wheat.

Of the miRNAs identified in barley and wheat, relatively few are drought responsive. Of 28 miRNAs in barley studied by Kantar *et al.* (2010), only four (hvu-miR156a, hvu-miR166, hvu-miR171 and hvu-miR408) were found to be differentially expressed under dehydration stress conditions. All four dehydration-regulated miRNAs were found to be induced by drought in barley leaves (Kantar *et al.*, 2010). By contrast, in barley roots, hvu-miR166 expression was suppressed by drought and the expression of the other three miRNAs was unchanged by it (Kantar *et al.*, 2010). As expected, the targets were found to be inversely expressed relative to the respective miRNAs in these tissues, with the exception of miR408's target, whose expression could not be detected in leaf (Kantar *et al.*, 2010). Later, a further three conserved miRNAs (miR156d miR396d and miR399b) and three novel miRNAs (miR-n026a*, miR-n029 and miR-n035) were found to be up-regulated under drought in barley leaves (Lv *et al.*, 2012). The three novel miRNAs were also shown to be up-regulated by salinity in barley leaves (Lv *et al.*, 2012). Very recently, 31 barley miRNAs were detected in barley cv. Golden Promise treated by drought, of which 13 were significantly down-regulated, while one miRNA (hvu-miR5049b) was significantly up-regulated, under the drought conditions (Hackenberg *et al.*, 2014). Hvu-miR399 was not expressed under drought (Hackenberg *et al.*, 2014), indicating that the expression of this miRNA may be drought dependent. Of 74 conserved miRNAs detected in Golden Promise, 20 belonging to ten miRNA families were significantly drought down-regulated, while one miRNA (gma-miR6300) was significantly up-regulated (Hackenberg *et al.*, 2014). However, some drought-regulated miRNAs were inconsistently expressed across different barley tissues. Moderately expressed hvu-miR166a was drought up-regulated in barley leaves but down-regulated in roots. Hvu-miR168-5p was only drought up-regulated in leaves while in root tissues its expression level was unchanged. Osa-miR393a and hvu-miRX35 were expressed in leaf but not in root (Hackenberg *et al.*, 2014). All the drought-regulated miRNAs detected in Golden Promise showed expression patterns that were similar to those reported for the corresponding miRNAs in other barley cultivars, under the same drought conditions, as judged by Northern hybridization or quantitative real-time reverse transcription PCR (qRT-PCR) (Hackenberg *et al.*, 2014). The regulation of these miRNAs by drought may be partly associated with drought-related TFs such as DREB TFs (Hackenberg *et al.*, 2012a; Morran *et al.*, 2011).

Drought-regulated miRNAs were identified by the micro-array approach from a wild wheat, *T. turgidum* ssp. *dicoccoides* (Kantar *et al.* (2011). At 4 and 8 h postdrought treatment, 438 miRNAs were identified in leaf and root tissues while in control plants only 205 miRNAs were detected (Kantar *et al.*, 2011). A comparison showed that 13 miRNAs (miR1867, miR896, miR398, miR528, miR474, miR1450, miR396, miR1881, miR894, miR156, miR1432, miR166 and miR171) were differentially expressed between the drought and water conditions (Kantar *et al.*, 2011). However, none of these miRNAs have their targets experimentally validated (Kantar *et al.*, 2011). miR1450 was drought up-regulated in *T. dicoccoides* (Kantar *et al.*, 2011), but drought down-regulated in *P. trichocarpa* (Lu *et al.*, 2008). miR1450 was also down-regulated in *P. trichocarpa* by saline conditions (Lu *et al.*, 2008). The results for miR1450 suggest that this miRNA may be controlled by different regulatory networks in different plant species. Transgenic rice overexpressing miR159 from wheat was found to be more sensitive to heat stress, indicating that miR159 might participate in a heat stress-related signalling pathway and influence heat stress tolerance (Wang *et al.*, 2012). Intriguingly, the transgenic rice also delayed heading and increased male sterility (Wang *et al.*, 2012). Targets of miR159 were identified as *MYB33* and *MYB101* which are important players in responses to ABA accumulation under drought stress (Reyes and Chua, 2007). Taken together, these data indicate that drought-responsive miRNAs can be used as a tool in the genetic modification for future improvement of cereal crops tolerant to drought.

Mechanisms of drought stress responses of miRNAs

Abscisic acid-responsive elements in miRNA genes

Suppression of lateral root growth by drought stress has been widely accepted as an adaptive response, because it allows redirection of resources towards production of deeper roots, enabling more efficient extraction of water from deep in the soil. Epoxycarotenoid cleavage-derived ABA has been shown to serve as a specific stress signal in plants (Nambara and Marion-Poll, 2005). Under drought stress, ABA is formed in the dehydrating roots, which inhibits lateral root growth (Xiong *et al.*, 2006). miRNA393 was found to be strongly up-regulated by ABA (Sunkar and Zhu, 2004). An ABA hypersensitive mutant of *A. thaliana (fry 1)* was shown to reduce lateral root growth at an elevated miR393 level (Chen *et al.*, 2012). Hence, miR393 was proposed to be a regulator of root adaptation under drought stress. Known targets of miR393, two auxin receptors (TIR1 and AFB2), undergo post-transcriptional silencing through miR393-guided cleavage—a process that is required for drought inhibited induced lateral root growth (Chen *et al.*, 2012). Promoters of most ABA-responsive genes have a conserved cis element as well as ABA-response elements (ABREs), which have been considered to play a role in stress-responsive expression (Mundy *et al.*, 1990; Xu *et al.*, 1996). In *Arabidopsis*, miR167 is up-regulated under drought stress and has ABREs in the promoter of the corresponding gene (Liu *et al.*, 2008). miR167 targets two auxin response factors (ARFs) that play a role in root architecture (Wu *et al.*, 2006). In this regard, miRNA393 and miR167 seem like good candidate miRNAs for use in studying drought-adaptive mechanisms.

Abscisic acid is also known to play an important role in seed dormancy. It is responsible for the instigation and maintenance of dormancy (Rodriguez-Gacio *et al.*, 2009). Reyes and Chua (2007)

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showed that in *Arabidopsis* miR159 levels increased with the addition of exogenous ABA or under drought treatment during seed germination (Reyes and Chua, 2007). miRNA159 mediates cleavage of MYB101 and MYB33 transcripts that function as positive regulators of ABA responses in the plants (Reyes and Chua, 2007). In support, overexpression miR159 suppresses MYB33 and MYB101 transcript levels in the transgenic plants and renders the plants hyposensitive to ABA (Reyes and Chua, 2007). Consistent with this, transgenic plants overexpressing cleavage-resistant forms of MYB33 and MYB101 are also hypersensitive to ABA (Reyes and Chua, 2007). By facilitating seed dormancy under stress (when endogenous ABA is high), the target mRNA of miR159 might therefore have a crucial role in ensuring avoidance to drought. Recent studies showed that drought and ABA up-regulated miRNAs also include miR169, miR319, miR397, miR2118, miR393 and miR167 (Khraiwesh *et al.*, 2012). However, it is unknown whether the promoters of these miRNA genes contain ABRE cis elements that are important for abiotic stress responses (Mundy *et al.*, 1990; Xu *et al.*, 1996). miR168 and miR396 contain the ABRE cis elements in their promoter regions and up-regulated by drought stress (Liu *et al.*, 2008). ABRE elements present in the promoter regions of miRNA genes could influence drought tolerance mechanisms.

Ethylene signalling and regulation of miRNAs

miRNAs could influence leaf senescence (Lim *et al.*, 2007). Leaf senescence is regarded as a drought avoidance mechanism, as it can reduce canopy size and transpiration, and allow remobilization of water and nutrients to organs more crucial for survival and reproduction of the plant (Griffiths *et al.*, 2014). In the study by Kim *et al.* (2009), miR164 was proposed to be a regulator of leaf senescence in *Arabidopsis*, based on the fact that *EIN2* (ETHYLENE INSENSITIVE 2), an ethylene signalling protein in *Arabidopsis*, down-regulates miR164 in older leaves. This results in increasing levels of its targets *NAC1*, *ORE1* and At5g61430. Accordingly, miR164 overexpression and/or lack of its target *ORE1* activity resulted in enhanced leaf longevity. The study further indicated that Ath-miR164 negatively regulates cell death and senescence in younger leaves through down-regulation of *ORE1* (Kim *et al.*, 2009). Drought stress triggers ethylene production in higher plants, which in turn enhances leaf senescence (Apelbaum and Yang, 1981; McKeon *et al.*, 1982; McMichael *et al.*, 1972) indicating a further link of the aforementioned pathway to drought adaptation.

In drought-resistant wild emmer wheat, miR166 was shown to be down-regulated by drought stress (Kantar *et al.*, 2011). Expression of miR166 is also regulated by two members of the GRAS family of TFs, SHORT-ROOT (SHR) and SCARECROW (SCR). SHR and SCR are both sensitive to ABA (Cui *et al.*, 2012). miR166 activated by SHR and SCR in turn down-regulates the HD-Zip TFs (Carlsbecker *et al.*, 2010; Miyashima *et al.*, 2011; Williams *et al.*, 2005). By contrast, the HD-Zip TF-encoded gene *Hahb-4* was up-regulated under drought stress and ABA treatments (Dezar *et al.*, 2005). An ethylene-responsive element was found in the promoter region of *Hahb-4* (Manavella *et al.*, 2006). Correspondingly, in *Arabidopsis*, *Hahb-4* was found to be up-regulated during ethylene-mediated leaf senescence and transgenic overexpression of this gene enhanced drought tolerance (Manavella *et al.*, 2006). However, it is unclear how miR166 regulates HD-Zip TFs and what genes are regulated by HD-Zip TFs.

Other drought-inducible promoter elements in MIR genes

According to the unpublished data of M. Aukerman and W. Park, up-regulated miR827 is considered to be necessary for drought tolerance in maize. miR399 and miR2111 have also been reported to be up-regulated in *M. truncatula* under drought stress (Wang *et al.*, 2011). These three miRNAs are reported to be up-regulated by phosphate starvation in *Arabidopsis* (Bari *et al.*, 2006; Hackenberg *et al.*, 2012b; Hsieh *et al.*, 2009; Pant *et al.*, 2008). Previously, a member of the MYB TF super family was found to be involved in phosphate starvation signalling (Rubio *et al.*, 2001). On the other hand, MYB TF-binding sites are reported to be drought-inducible promoter elements in *Arabidopsis* (Liu *et al.*, 2008). It is possible that the MYB TF promoter-binding sites facilitate both drought and phosphate starvation-induced expression of MIR genes. In comparison of *Arabidopsis* and rice, little is known about cis-regulatory elements in the promoters of miRNA genes in barley and wheat.

Strategies for functional analysis of miRNAs and their targets in plants

miRNAs are negative regulators of genes. Their short sequence length makes it relatively easy for them to base pair with other sequences, potentially allowing regulation of multiple genes. This, combined with the existence of gene–gene interaction networks, makes the biological implications of miRNA action difficult to ascertain. Generally, two transgenic strategies can be adopted to determine the functions of miRNAs. One is to use gain of function to increase miRNA expression and the other is loss of function to reduce or abolish miRNA expression. Gain of function can be achieved by overexpressing the miRNA, using a constitutive promoter such as the 35S or polyubiquitin promoters, or an inducible promoter that is activated only under certain conditions. Loss of function can be accomplished by overexpressing antisense miRNAs. Antisense miRNAs inactivate miRNA activities by base pairing with miRNAs and have been widely used in the functional analysis of animal miRNAs (Thomson *et al.*, 2011). Artificial miRNAs (amiRNAs), generated by replacing the miRNA duplex regions in native miRNA precursors, can be used to achieve either gain or loss of function (Ossowski *et al.*, 2008). Compared to miRNAs and antisense miRNAs, amiRNA sequences can be optimized for high efficiency because they are generated from the same locus in their precursors (Warthmann *et al.*, 2008). A website is currently available for the automated design of amiRNAs (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd).

If miRNA's targets are known, then miRNA functions can be analysed by modulating the expression of the targets. The knockdown or abolishment of the transcription of miRNA's targets can be achieved using amiRNAs, which can specifically silence single or multiple genes of interest (Alvarez *et al.*, 2006; Duan *et al.*, 2008; Khraiwesh *et al.*, 2008; Molnar *et al.*, 2009; Ossowski *et al.*, 2008; Schwab *et al.*, 2006; Warthmann *et al.*, 2008). Unlike antisense, the amiRNA sequence does not have to be perfectly complementary to the target sequence (Schwab *et al.*, 2006; Warthmann *et al.*, 2008). Therefore, specific nucleotides within the amiRNAs can be optimized to particular gene(s), which do not affect the pre-miRNA processing and the biogenesis of mature miRNAs (Niu *et al.*, 2006; Vaucheret *et al.*, 2004; Warthmann *et al.*, 2008; Zeng *et al.*, 2002) and result in more

accurate gene silencing (Duan *et al.*, 2008; Park *et al.*, 2009; Tang, 2010). miRNA mimics are another way to analyse functions of both miRNAs and their targets in both plants and animals (Franco-Zorrilla *et al.*, 2007; Thomson *et al.*, 2011). miRNA mimics can be designed to target gene promoters, and these have been shown to work in human cells (Place *et al.*, 2008). Different from endogenous miRNAs, miRNA mimics act in a gene-specific manner. Either miRNA mimics, antisense miRNAs or amiRNAs can be used in transient assays for quickly examining the expression relationship between miRNAs and their targets (Johansen and Carrington, 2001).

Conclusion and future directions

Changing climate, variable weather patterns and other environmental stresses are a matter of concern for agricultural crop production. Drought is a stress limiting crop production and yield across the world. Drought tolerance is a complex trait involving a number of gene regulatory networks that miRNAs participate in. However, the mechanisms of miRNAs involvement in stress tolerance and their target regulatory networks are not well understood. This is partly due to the possibility of each endogenous miRNA regulating multiple genes and each gene being regulated by multiple miRNAs. Therefore, although many miRNAs have been identified from a variety of plants, some of which are shown to be drought regulated, the targets of these miRNA are still largely unknown. Hence, the major challenge ahead will be to discover the miRNA targets and how miRNAs function on the targets. This information will allow the identification of miRNAs/targets that influence drought tolerance. The other challenge will be to characterize the cis-regulatory elements in the miRNAs genes, to determine the corresponding TFs and to describe how the miRNAs are regulated by drought. These data would offer new insights for understanding the action of miRNAs and their potential to be used to engineer enhanced drought stress tolerance.

Acknowledgement

We would like to express our sincere thanks to Dr. Nick Collins for his excellent English-editing help.

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CHAPTER 3

Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions

Statement of Authorship

Title of Paper	Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Ferdous J., Li Y., Reid N., Langridge P., Shi B.J., Tricker P.J. (2015) Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions. PLoSONE 10(3), e0118503. doi:10.1371/journal.pone.0118503. This is an experimental article about reference gene selection for accurate and reliable gene expression analysis of microRNA and mRNA targets using quantitative real-time reverse transcription PCR (qPCR). This article is closely related to the subject matter of this thesis.

Principal Author

Name of Principal Author (Candidate)	Jannatul Ferdous		
Contribution to the Paper	Conceived and designed the experiments, performed data analysis, critical interpretation and wrote manuscript.		
Overall percentage (%)	70%		
Certification:	I conducted this experiment during the period of my candidature, and this article has not been accepted for any other University award. I am the primary author of this paper. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Yuan Li		
Contribution to the Paper	Performed the qRT-PCR and data normalization. I hereby certify that the statement of the contribution is accurate.		
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Name of Co-Author	Nicolas Reid		
Contribution to the Paper	Provided data analysis tools and helped in data analysis. I hereby certify that the statement of the contribution is accurate.		
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Name of Co-Author	Peter Langridge		
Contribution to the Paper	Supervised the experiment. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/16

Name of Co-Author	Bu-Jun Shi		
Contribution to the Paper	Conceived the experiment, supervised designing the experiments. Edited the manuscript and acted as the corresponding author. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	10/12/2015

Name of Co-Author	Penny J. Tricker		
Contribution to the Paper	Conceived the experiment, supervised designing the experiments, data analysis and interpretation. Wrote and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/16

Link to chapter 3

Experimental validation of miRNAs is difficult because of their short length (18–21 nt) and lack of common sequence features. Furthermore, insufficient information is available on the selection of suitable reference genes for miRNA- quantitative real-time reverse transcription PCR (qRT-PCR) in barley. Therefore, selecting the reference genes for miRNA-qRT-PCR data normalization was a big challenge for the expression analysis of miRNAs and their target genes for the drought experiments. Here, we determined reference genes for accurate and reliable gene expression analysis for quantifying candidate mature miRNAs and their targets using qRT-PCR. We studied the expression stability of four commonly used housekeeping genes; Actin (*ACT*), alpha-Tubulin (*α-TUB*), Glycolytic glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ADP-ribosylation factor 1-like protein (*ADP*), four snoRNAs; (U18, U61, snoR14 and snoR23) and two microRNAs (miR168, miR159) as candidate reference genes in six barley genotypes under five experimental stresses, drought, fungal infection, boron toxicity, nutrient deficiency and salinity. This chapter (Chapter 3) has been published as follows: Ferdous J., Li Y., Reid N., Langridge P., Shi B.J. & Tricker P.J. (2015) Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions. PLoS ONE 10(3), e0118503. The findings of this work suggested that *ADP*, snoR14 and snoR23 were the best reference genes across diverse samples for miRNA and target mRNA expression analysis in the same samples. The combination of *ADP*, snoR14 and snoR23 showed increased sensitivity of detection of differential expression of miRNA and mRNA for other stress treatments also. Therefore, snoR14, snoR23 and *ADP* were used for qRT-PCR data normalization of all the miRNA and mRNA quantification for the remaining studies reported in this thesis.

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CORRECTION

Correction: Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions

The *PLOS ONE* Staff

The Abstract of the published article is incorrect. Please view the correct Abstract here:

For accurate and reliable gene expression analysis using quantitative real-time reverse transcription PCR (qPCR), the selection of appropriate reference genes as an internal control for normalization is crucial. We hypothesized that non-coding, small nucleolar RNAs (snoRNAs) would be stably expressed in different barley varieties and under different experimental treatments, in different tissues and at different developmental stages of plant growth and therefore might prove to be suitable reference genes for expression analysis of both microRNAs (miRNAs) and mRNAs. In this study, we examined the expression stability of ten candidate reference genes in six barley genotypes under five experimental stresses, drought, fungal infection, boron toxicity, nutrient deficiency and salinity. We compared four commonly used housekeeping genes; Actin (*ACT*), alpha-Tubulin (*α-TUB*), Glycolytic glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ADP-ribosylation factor 1-like protein (*ADP*), four snoRNAs; (U18, U61, snoR14 and snoR23) and two microRNAs (miR168, miR159) as candidate reference genes. We found that *ADP*, snoR14 and snoR23 were ranked as the best of these candidates across diverse samples. Additionally, we found that miR168 was a suitable reference gene for expression analysis in barley. Finally, we validated the performance of our stable and unstable candidate reference genes for both mRNA and miRNA qPCR data normalization under different stress conditions and demonstrated the superiority of the stable candidates. Our data demonstrate the suitability of barley snoRNAs and miRNAs as potential reference genes for miRNA and mRNA qPCR data normalization under different stress treatments.

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Citation: The *PLOS ONE* Staff (2015) Correction: Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions. PLoS ONE 10(5): e0126167. doi:10.1371/journal.pone.0126167

Published: May 6, 2015

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Reference

1. Ferdous J, Li Y, Reid N, Langridge P, Shi B-J, Tricker PJ. (2015) Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions. PLoS ONE 10(3): e0118503. doi: 10.1371/journal.pone.0118503 PMID: 25793505

RESEARCH ARTICLE

Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions

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Citation: Ferdous J, Li Y, Reid N, Langridge P, Shi B-J, Tricker PJ (2015) Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions. PLoS ONE 10(3): e0118503. doi:10.1371/journal.pone.0118503

Academic Editor: Jürgen Brosius, University of Münster, GERMANY

Received: August 21, 2014

Accepted: January 19, 2015

Published: March 20, 2015

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Funded by the Australian Centre for Plant Functional Genomics.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

For accurate and reliable gene expression analysis using quantitative real-time reverse transcription PCR (qPCR), the selection of appropriate reference genes as an internal control for normalization is crucial. We hypothesized that non-coding, small nucleolar RNAs (snoRNAs) would be stably expressed in different barley varieties and under different experimental treatments, in different tissues and at different developmental stages of plant growth and therefore might prove to be suitable reference genes for expression analysis of both microRNAs (miRNAs) and mRNAs. In this study, we examined the expression stability of ten candidate reference genes in six barley genotypes under five experimental stresses, drought, fungal infection, boron toxicity, nutrient deficiency and salinity. We compared four commonly used housekeeping genes; Actin (*ACT*), alpha-Tubulin (*α-TUB*), Glycolytic glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ADP-ribosylation factor 1-like protein (*ADP*), four snoRNAs; (U18, U61, snoR14 and snoR23) and two microRNAs (miR168, miR159) as candidate reference genes. We found that *ADP*, snoR14 and snoR23 were ranked as the best of these candidates across diverse samples. For accurate and reliable gene expression analysis using quantitative real-time reverse transcription PCR (qPCR), the selection of appropriate reference genes as an internal control for normalization is crucial. We hypothesized that non-coding, small nucleolar RNAs (snoRNAs) would be stably expressed in different barley varieties and under different experimental treatments, in different tissues and at different developmental stages of plant growth and therefore might prove to be suitable reference genes for expression analysis of both microRNAs (miRNAs) and mRNAs. In this study, we examined the expression stability of ten candidate reference genes in six barley genotypes under five experimental stresses, drought, fungal infection, boron toxicity, nutrient deficiency and salinity. We compared four commonly used housekeeping genes; Actin (*ACT*), alpha-Tubulin (*α-TUB*), Glycolytic glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ADP-ribosylation factor 1-like protein (*ADP*), four snoRNAs; (U18, U61, snoR14 and snoR23) and two microRNAs (miR168, miR159) as

candidate reference genes. We found that *ADP*, snoR14 and snoR23 were ranked as the best of these candidates across diverse samples. Additionally, we found that miR168 was a suitable reference gene for expression analysis in barley. Finally, we validated the performance of our stable and unstable candidate reference genes for both mRNA and miRNA qPCR data normalization under different stress conditions and demonstrated the superiority of the stable candidates. Our data demonstrate the suitability of barley snoRNAs and miRNAs as potential reference genes for miRNA and mRNA qPCR data normalization under different stress treatments.

Introduction

The study of gene expression has become increasingly widespread in numerous organisms. Real time quantitative (reverse transcription) polymerase chain reaction (qPCR) is a commonly used technique due to its high sensitivity, accuracy and reproducibility. Among several quantification strategies, a commonly used method is relative quantification where data are normalized to an internal control gene [1]. The internal control gene is called a reference or house-keeping gene (HKG) and, while subjected to the same experimental factors during sampling and cDNA preparation as the gene(s) of interest, is not differentially expressed, providing a constant for relative quantification of differences in expression of the gene(s) of interest. Hence, the selection of appropriate reference genes is important for obtaining valid results and proper interpretation from the analysis [2]. The most frequently used HKGs in plant qPCR analysis are protein-coding genes, such as Actin (*ACT*), alpha-Tubulin (*α-TUB*), Cyclophilin, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ADP-ribosylation factor 1-like protein (*ADP*) and ribosomal RNAs [3–9]. However, several studies have shown that the expression of these genes varies considerably in different cells and tissues under different experimental conditions and, in these comparisons, they become unsuitable for qPCR data normalization [9–16].

Until two decades ago it was believed that the DNA between protein-coding genes was nothing more than junk DNA [17]. This idea was challenged by the discovery of small regulatory RNAs in eukaryotes. MicroRNAs (miRNAs) are a class of small RNAs, approximately 18–24 nucleotides (nt) long, non-coding and single-stranded molecules. miRNAs play pivotal roles in cellular homeostasis by the alteration of gene expression under stress conditions [18, 19]. Recent advances in high-throughput sequencing and bioinformatics analyses have enhanced the discovery of miRNAs in different plant species [20–23]. However, the regulatory function of miRNAs is just beginning to be understood [24–26]. miRNAs negatively regulate their target messenger RNAs (mRNAs) and the regulation is subject to various levels of control [18]. To determine the function of miRNAs, the expression levels of miRNAs and their target mRNA *in vivo* must be precisely compared. To investigate the differential expression of miRNAs, sequencing and computational analyses require the experimental validation of expression profiles. However, miRNA experimental validation is difficult because of their short length (∼ 18–24 nt) and lack of common sequence features (e.g. polyA) [27].

Barley (*Hordeum vulgare*) is one of the most important cereal crops cultivated in the world and, with a large amount of genetic and genomic data available, including its full genome sequence [28], is a model for cereal genomics studies. Although several reports have suggested suitable reference genes for RNA quantification in cereals, all show some variation in expression amongst plant tissues, species and experimental conditions so that there is no known

reference genes suitable for all experiments [29–32]. Additionally, inadequate information is available on suitable reference genes for miRNA-qPCR. Studies of miRNA expression using qPCR often provide limited information on the stability of reference genes, but, where investigated, lower expression stability of some commonly used HKGs has been found in miRNA-qPCR experiments [2, 33, 34]. The use of miRNAs [1, 27, 35–37] and small nucleolar RNAs (snoRNAs) [38, 39] as reference genes has been proposed for better normalization in miRNA-qPCR expression analysis in both plants and animals. However, barley miRNAs and snoRNAs have never been assessed for their expression stability as reference genes under biotic and abiotic stresses.

The present work was designed to identify and evaluate suitable reference genes in barley under drought, salinity, boron toxicity, low nutrient stress and fungal infection. The selected internal controls were used for miRNA and mRNA expression profiling in barley to validate the performance of stable reference genes under drought stress treatment. Candidate reference genes were examined and compared with commonly used HKGs and ranked with three statistical algorithms. We found that the expression of four commonly used HKGs varied with experimental conditions. By contrast, snoRNAs and miRNAs were suitable internal controls for miRNA and mRNA-qPCR expression analysis in barley.

Materials and Methods

Plants and Growth Environments

Drought treatment. *H. vulgare* cvs. 'Fleet' and 'Commander' were grown in a glasshouse at 22–23°C day (d)/16°C night temperatures, with a day length of 12 hours (h). Plants were grown in pots with coco-peat soil. 30 individual plants of each genotype were grown. Drought treatment was applied as follows: plants were initially well-watered, then water withheld until visible wilting. Samples were collected after 21 d, after flag leaf emergence and 10 d after anthesis. The sampled materials corresponded to three developmental stages: tillering, booting and grain filling. Consistently well-watered plants were sampled as control plants. Leaf and root materials were sampled at tillering and booting stages and grains were harvested at the grain filling stage, 18 d post-anthesis.

Boron treatment. *H. vulgare* cv. 'Clipper' plants were grown hydroponically for 21 d in sufficient and excessive concentrations of boron with temperatures and day length as above. Seedlings were grown in a nutrient solution containing 5 mM NH_4NO_3, 5 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 100 μM KH_2PO_4, 50 μM NaFe(III) EDTA, 50 μM $B(OH)_3$, 5 μM $MnCl_2$, 10 μM $ZnSO_4$, 0.5 μM $CuSO_4$ and 0.1 μM Na_2MoO_3. After 7 d, half of the seedlings were transferred to a nutrient solution with an additional 3 mM H_3BO_3. Leaves were harvested after 14 d.

Fungal infection. *H. vulgare* cv. 'Sloop' seeds were sown in pots with coco-peat soil in a growth chamber at 18°C with a 14/10 h day/night light period and 60±10% relative humidity (RH). After three weeks, three individual plants were infected with *Rhynchosporium commune*. After inoculation the seedlings were maintained in darkness for 24 h at 100% RH, then returned to the 14/10 h day/night light period with 80±10% RH until the leaf samples were harvested, 14 d after inoculation. Same age uninfected plant leaves from three individual plants were harvested as the control group.

Salt treatment. *H. vulgare* cv. 'WI4330' seedlings were grown hydroponically as described for the boron treatment. At the appearance of the third leaf (approx. 10 d), half of the seedlings were subjected to the treatment by transferring them to a nutrient solution with an additional 50 mM NaCl. NaCl was added to the nutrient solution twice daily in increments of 50 mM, to

a final concentration of 250 mM. Roots were harvested for RNA extraction when the third leaf was fully expanded approximately after a further 12 d.

Nitrate treatment. *H*. *vulgare* cv. 'Golden Promise' seedlings were grown in a fully-supported hydroponics set-up [40] in a glasshouse with a temperatures ranging between 19–23°C. Sufficient and low nitrate treatments were achieved by supplying 5 mM and 0.5 mM nitrate to the nutrient solution, respectively. The nutrient solution contained (in mM): 2.0 $MgSO_4.7H_2O$, 0.1 KH_2PO_4, 0.5 $Na_2Si_30_7$, 0.05 NaFe(III)EDTA, 0.05 H_3BO_3, 0.005 $MnCl_2.4H_2O$, 0.01 $ZnSO4.7H_2O$, 0.0005 $CuSO4.7H_2O$ and 0.0001 $Na_2MoO_4.2H_2O$ with 2 KNO_3 and 1.5 Ca $(NO_3)_2.4H_2O$ in the sufficient NO_3^- treatment (5 mM NO_3^-) and 0.25 KNO_3 and 0.125 Ca $(NO_3)_2.4H_2O$ in the low NO_3^- treatment (0.5 mM NO_3^-). In order to maintain similar K^+ and Ca^{2+} levels to the sufficient NO_3^- treatment, the low NO_3^- treatment also comprised (in mM): 0.875 K_2SO_4 and 1.375 $CaCl_2.2H_2O$. The treatments were established at the start of the experiment and the nutrient solution was replaced every 10 d to ensure that nutrients were not depleted. Following 21 d of sufficient and low nitrate treatment, the 2nd leaf blade was collected.

All harvested materials were immediately frozen in liquid N_2 and stored at −80°C.

Candidate Reference Gene Selection and Primer Design

Four common HKGs (*ACT*, *α-TUB*, *GAPDH* and *ADP*), four snoRNAs (U18, U61, snoR14 and snoR23) and two miRNAs (miR168 and miR159) were selected. The sequences of *Hvu-Actin* (AY145451.1: http://www.ncbi.nlm.nih.gov/nuccore/AY145451), *Hvu-Tubulin* (U40042.1: http://www.ncbi.nlm.nih.gov/nuccore/U40042.1), *Hvu-GAPDH* (X60343.1: http://www.ncbi.nlm.nih.gov/nuccore/X60343.1) and *Hvu-ADP* (AJ508228.2: http://www.ncbi.nlm.nih.gov/nuccore/AJ508228.2) were downloaded from the NCBI Genbank while the sequences of all barley snoRNAs were downloaded from the plant snoRNA database (http://bioinf.scri.sari.ac.uk/cgi-bin/plant_snorna/home). The sequences of miR168 (accession number MIMAT0018216) and miR159 (accession number MIMAT0018210) were obtained from miRBase (http://www.mirbase.org/index.shtml). The selection of these two miRNAs was based on our previous miRNA expression studies in drought-treated *H*. *vulgare* cv. 'Golden Promise', where these two miRNAs were relatively invariant across drought treated and well-watered samples (data not shown).

Primers for *ACT*, *α-TUB*, *GAPDH*, *ADP* and snoRNAs were designed using AlleleID software (Premier Biosoft International, Palo Alto, CA, USA) considering amplicon sizes in the range of 60–80 bases. To eliminate the possibility of an inhibiting effect of the RNA secondary structure during cDNA synthesis, the primers for snoRNAs were designed in their loop regions [41]. miRNA specific stem-loop RT primers and appropriate forward and reverse primers for individual miRNA were designed following the previously described method [42, 43]. A minimum of three primer pairs were designed and tested for each gene. Primer pairs for 10 genes were selected on the basis of their amplification efficiency and specificity and are listed in Table 1. All primers used in this study were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA).

Specific qPCR amplification for all candidate internal controls was confirmed by a single, distinct melt peak in melt curve analysis (S1 Fig.) and a single band of desired size in 3% agarose gel electrophoresis (S2 Fig.). Further confirmation of these qPCR amplified products was done by sequencing using the respective reverse primers each containing M13 reverse primer sequence and a spacer (S1 Table), and all of these qPCR products showed correct sequences.

Table 1. Ten candidate reference genes and primer sequences used for their qPCR.

Candidate reference genes	Accession number	Annotation	Primer (5′–3′)	Amplicon (bp)	PCR efficiency	Regression coefficient (R^2)
Hvu-U61	-	Small nucleolar RNA	Fw GAGGAAACGAAACCTGTGC	65	93.94	0.999
			Rev ACTTCTTAGAGGGTTGTGTTAC			
Hvu-U18	-	Small nucleolar RNA	Fw GTGATGAAGAAAAGTTGGTC	67	89.25	0.999
			Rev AGAAGTTTATTAAGGATGGTTATC			
Hvu-snoR14	-	Small nucleolar RNA	Fw GATGTTTATGTATGATAGTCTGTC	67	95.55	0.999
			Rev GTCGGGATGTATGCGTGTC			
Hvu-snoR23	-	Small nucleolar RNA	Fw TCGGCAGTGGTGTCATC	64	98.31	0.999
			Rev CTCAGTGGAAAGAGAAGTCG			
Hvu-ADP	AJ508228.2	ADP-ribosylation factor 1-like protein	Fw GCTCTCCAACAACATTGCCAAC	77	100.79	0.999
			Rev GAGACATCCAGCATCATTCATTCC			
Hvu-α-TUB	U40042.1	Tubulin alpha-2 chain	Fw GTCCACCCACTCCCTCCTTG	78	106.49	0.999
			Rev CGGCGGCAGATGTCATAGATG			
Hvu-ACT	AY145451.1	Actin	Fw CCACGAGACGACCTACAAC	80	102.36	0.999
			Rev CACTGAGCACGATGTTTCC			
Hvu-GAPDH	X60343.1	Glycolytic glyceraldehyde-3-phosphate dehydrogenase	Fw GCCAAGACCCAGTAGAGC	78	92.12	0.999
			Rev CACATTTATTCCCATAGACAAAGG			
Hvu-miR168	MIMAT0018216	microRNA	Fw CTCACGTCGCTTGGTGCAGAT	60	107.31	0.997
			Rev GAGCTGGGTCCGAGGT			
			Stem-loop RT primer GTCGTATCCAGAGCTGGGTCCGAGGTATTCGCTCTGGATACGACGTCCCG			
Hvu-miR159	MIMAT0018210	microRNA	Fw CGTGGGTTTGGATTGAAGGGA	61	107.21	0.999
			Rev GTGCAGGGTCCGAGGT			
			Stem-loop RT primer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGAGC			

doi:10.1371/journal.pone.0118503.t001

qPCR Analysis

Total RNA was extracted from each of the samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, except for grain RNA extraction, which included additional 2% (w/v) polyvinylpyrrolidone (PVP-40) (Sigma). From each of the five experiments RNA was isolated from a minimum of three biological replicates from each of the treated and control conditions.

The isolated RNA samples were treated with DNA-free reagents (Ambion, Life Technologies, Grand Island, NY, USA) twice according to the manufacturer's instruction in order to completely remove genomic DNA (gDNA). A polymerase chain reaction (PCR) was carried out to ensure no gDNA contamination in the DNAse-treated RNA samples (S3 Fig.). The concentration and integrity of the DNA-free RNA was determined by Agilent-2100 Bioanalyzer using RNA 6000 NanoChips (Agilent Technologies, Santa Clara, CA, USA). RNA samples with RNA integrity numbers (RIN) ≥ 5 were used for cDNA synthesis. cDNAs were synthesized in a final volume of 40 μl. In each 40 μl reaction, the reaction mixture contained 2 μg RNA, 2 μl (10 mM) dNTP mix and a cocktail of 2 μl (1 μM) appropriate stem loop primer, 2 μl (10 mM) appropriate snoRNA specific reverse primers and 2 μl 50 μM Oligo $(dT)_{20}$ (Life Technologies, Carlsbad, CA, USA) (Table 1). cDNA synthesis was carried out using SuperScript III RT (Life Technologies, Carlsbad, CA, USA). All investigated samples were transcribed by the pulsed RT method recommended for stem-loop primers [43]. In brief, the samples were incubated at 16°C for 30 minutes (min) followed by pulsed RT of 60 cycles at 30°C for 30 seconds (s), 42°C for 30 s and 50°C for 1 s. Finally, the reaction was terminated by incubating the samples at 85°C for 5 min.

To provide a template for the standard curve, four (three technical replicates and one no template control) 20-μL PCR reaction mixtures were combined and purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The purified product was then quantified using the QUBIT fluorometer and the Qubit dsDNA HS assay kit (Life Technologies) according to the manufacturer's instructions. An aliquot of this solution was diluted to produce a stock solution containing10^9 copies of the PCR product per μL. A dilution series covering six orders of magnitude was prepared from the 10^9 stock solution to produce solutions covering 10^7 to 10^1 copies per μL to derive a standard curve (S4 Fig.) for each assay. Reactions were prepared in triplicates and a no template control.

qPCR assays were assembled using the fluid-handling robotic platform CAS-1200 (QIAgility, Qiagen, Valencia, CA, USA). The cDNA samples were diluted 20 times in sterile milli Q water and the diluted cDNA solution was used in the qPCR reaction. Each assay contained: 2 μl of cDNA solution (or water as a no template control), 5 μl of KAPA Sybr Fast qPCR Universal Readymix (Geneworks, Adelaide, Australia), 1.2 μl of each of the forward and reverse primers at 4 μM and 0.6 μl water to make up the total reaction volume of 10 μl. Amplifications were performed in an RG 6000 Rotor-Gene Real-Time Thermal Cycler (Qiagen, Valencia, CA, USA) with 2 min at 95°C followed by 50 cycles of 1 s at 95°C, 1 s at 60°C, 25 s at 72°C, and fluorescent acquisition at 72°C, followed by melt curve analysis of temperature increasing from 60°C to 95°C with fluorescence readings acquired at 0.5°C increments (S1 Fig.).

Gene Expression Stability Ranking of Candidate Reference Genes

The stability of candidate reference genes' expression was analysed using three software packages, geNorm (version 3.5) ([3] http://www.biogazelle.com/genormplus/website), Normfinder (version 0.953) ([44] http://www.mdl.dk/publicationsnormfinder.htm webcite) and BestKeeper (version 1) ([4] http://genequantification.com/bestkeeper.html). Raw expression values of candidate reference genes obtained from the qPCR under five experimental treatments were

analysed by geNorm and NormFinder according to the instruction manual. geNorm was used to make pairwise comparisons and exclude values stepwise until the most stable pair of genes remained [3]. NormFinder was used to examine the expression stability of each candidate independently [44]. Both software packages were used to calculate the average expression stability (M) of candidate reference genes, with the highest M value indicating the least stable candidate reference genes and the lowest M value the most stable [3] and to rank their performance accordingly. BestKeeper analysis was used to calculate the geometric mean of the Cycle threshold (Ct) values of the candidate reference genes [4] and estimate gene expression stability based on the standard deviation of the Ct value, coefficient of correlation (r) and percentage covariance, to rank the candidates from the most to least stably expressed.

Determination of the Optimal Number of Reference Genes

To determine the optimal number of reference genes, the pairwise variation, V, for all datasets was estimated using geNorm (as before). V was calculated between two sequential normalization factors NFn and NFn+1 to determine the optimal number of reference genes [3]. If V was below 0.15, the addition of a third gene did not result in a noticeable improvement for the normalization. In particular, if Vn/n+1 was less than 0.15, using n+1 reference genes did not significantly improve the normalization [3].

Validation of Candidate Reference Genes' Utility

To validate the reliability of putatively more stable candidate reference genes, the relative expression levels of an mRNA (*Superoxide dismutase*) and an miRNA (miR5048) (S2 Table) were also quantified for all the samples described above by qPCR (as before). Melt curves and standard curves in qPCR for S*uperoxide dismutase* (AK363344.1) and miR5048 (MIMAT0020544) are given in S5 Fig. and S6 Fig., respectively. Expression of miR5048 was compared with previously described deep sequencing data [45].

Results

Gene Expression Stability and Ranking of Candidate Reference Genes by the geNorm analysis

Average expression stabilities (M) for candidate reference genes using pairwise comparison are shown in Fig. 1. snoR14 and *ADP* had the lowest M value (0.399), that is the most stable expression amongst all 10 candidate reference genes across all tissue samples. In contrast, miR159 had the highest M value (2.056) that is the least stable expression across all samples (Fig. 1A). Under drought stress, the most stable reference genes were *ADP* and snoR14, and the least stable was again miR159 (Fig. 1B). Under boron treatment the most stable reference genes were snoR23 and *ADP*, and the least stable reference gene was *GAPDH* (Fig. 1C). Under both fungal infection (Fig. 1D) and nitrate treatment (Fig. 1E) snoR14 and snoR23 were the most stable candidates. *α-TUB* was the least stable gene under both fungal infection (Fig. 1D) and nitrate treatment (Fig. 1E). Under salt treatment, U61 and snoR14 were the most stable candidates while *ACT* was the least stable candidate (Fig. 1F).

Gene Expression Stability and Ranking of Candidate Reference Genes by the Norm Finder analysis

The most stable reference gene identified by NormFinder across all samples was snoR14. *ADP* and snoR23 were ranked second and third, respectively. snoR14, *ADP* and snoR23 also performed well under boron, fungal and nitrate stresses (Table 2). miR159 was the least stable

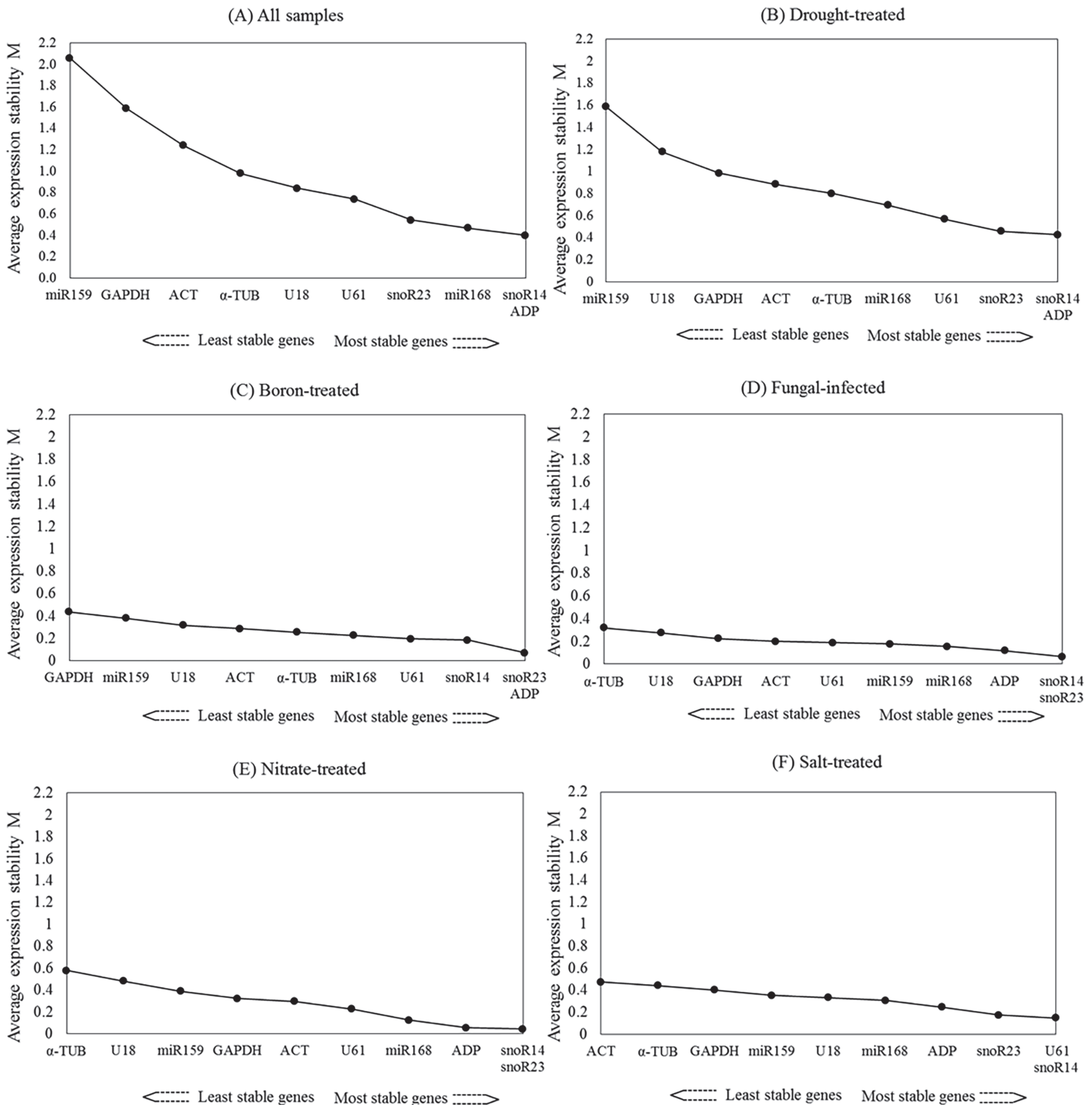

Fig 1. geNorm analysis of average expression stability values and ranking of ten candidate reference genes based on pairwise comparison. Genes on the x-axis in order of increasing stability (y-axis M value) for (A) all samples, (B) drought-treated samples, (C) boron-treated samples, (D) fungal-infected samples, (E) salt-treated samples and (F) nitrate-treated samples. Under individual treatment condition, reference genes' expression stability was obtained comparing to the untreated (control) condition.

doi:10.1371/journal.pone.0118503.g001

(M >1) when used as a universal reference gene across all experimental samples. Though the M value was < 1, *α-TUB* was the most unstable candidate in fungal and nitrate treatment conditions (Table 2). *ACT* and *GAPDH* were the least stable candidates in salinity and boron

Table 2. Expression stability ranking of candidate reference genes as calculated by NormFinder and BestKeeper.

	Rank	Experimental conditions					
		All samples	Drought	Boron	Fungus	Salt	Nitrate
NormFinder (Stability value, M)	1	snoR14 (0.380)	U61 (0.154)	snoR23 (0.044)	*ADP* (0.041)	snoR14 (0.119)	snoR23 (0.04)
	2	*ADP* (0.554)	snoR14 (0.184)	snoR14 (0.061)	snoR14 (0.078)	U61 (0.141)	snoR14 (0.045)
	3	snoR23 (0.641)	miR168 (0.223)	*ADP* (0.063)	snoR23 (0.081)	snoR23 (0.151)	*ADP* (0.066)
	4	miR168 (0.708)	*ADP* (0.309)	U61 (0.102)	miR168 (0.104)	miR168 (0.200)	miR168 (0.238)
	5	U61 (0.765)	snoR23 (0.481)	miR168 (0.185)	U61 (0.106)	*ADP* (0.233)	U61 (0.305)
	6	U18 (0.801)	*ACT* (0.567)	*α-TUB* (0.242)	miR159 (0.112)	miR159 (0.259)	*GAPDH* (0.323)
	7	*ACT* (0.883)	*α-TUB* (0.587)	U18 (0.254)	*ACT* (0.129)	U18 (0.269)	*ACT* (0.325)
	8	α-TUB (0.976)	*GAPDH* (0.899)	*ACT* (0.291)	*GAPDH* (0.245)	*GAPDH* (0.294)	miR159 (0.353)
	9	*GAPDH* (1.88)	U18 (1.244)	miR159 (0.346)	U18 (0.260)	*α-TUB* (0.311)	U18 (0.455)
	10	miR159 (2.205)	miR159 (2.165)	*GAPDH* (0.435)	*α-TUB* (0.326)	*ACT* (0.380)	*α-TUB* (0.657)
BestKeeper (SD)	1	*ADP* (0.573)	snoR23 (0.39)	*ADP* (0.27)	snoR14 (0.075)	miR168 (0.155)	miR168 (0.518)
	2	snoR14 (0.772)	*ADP* (0.45)	snoR14 (0.28)	snoR23 (0.111)	U61 (0.2)	snoR14 (0.813)
	3	snoR23 (0.816)	snoR14 (0.61)	snoR23 (0.313)	*ADP* (0.177)	snoR23 (0.222)	snoR23 (0.871)
	4	miR168 (0.972)	miR168 (0.7)	U61 (0.328)	U61 (0.184)	snoR14 (0.23)	*ADP* (0.935)
	5	*GAPDH* (1.074)	U61 (0.82)	miR168 (0.333)	miR168 (0.185)	*ADP* (0.259)	*GAPDH* (1.35)
	6	*ACT* (1.094)	*GAPDH* (0.89)	*GAPDH* (0.4)	*GAPDH* (0.193)	U18 (0.273)	U61 (1.48)
	7	U61 (1.377)	*ACT* (0.93)	U18 (0.46)	miR159 (0.222)	*GAPDH* (0.298)	*α-TUB* (1.64)
	8	*α-TUB* (1.557)	*α-TUB* (1)	*α-TUB* (0.51)	*ACT* (0.307)	*α-TUB* (0.31)	*ACT* (1.745)
	9	U18 (1.913)	U18 (1.41)	*ACT* (0.56)	U18 (0.439)	miR159 (0.36)	U18 (1.75)
	10	miR159 (3.046)	miR159 (2.73)	miR159 (0.752)	*α-TUB* (0.563)	*ACT* (0.449)	miR159 (1.8)

doi:10.1371/journal.pone.0118503.t002

treatments respectively. These results were broadly consistent with the pairwise analysis using the geNorm algorithm (Fig. 1).

Gene Expression Stability and Ranking of Candidate Reference Genes by Bestkeeper analysis

Expression of the 10 candidate reference genes was measured and Ct values are shown in Fig. 2. Average Ct values for the 10 candidates among all experimental samples varied between

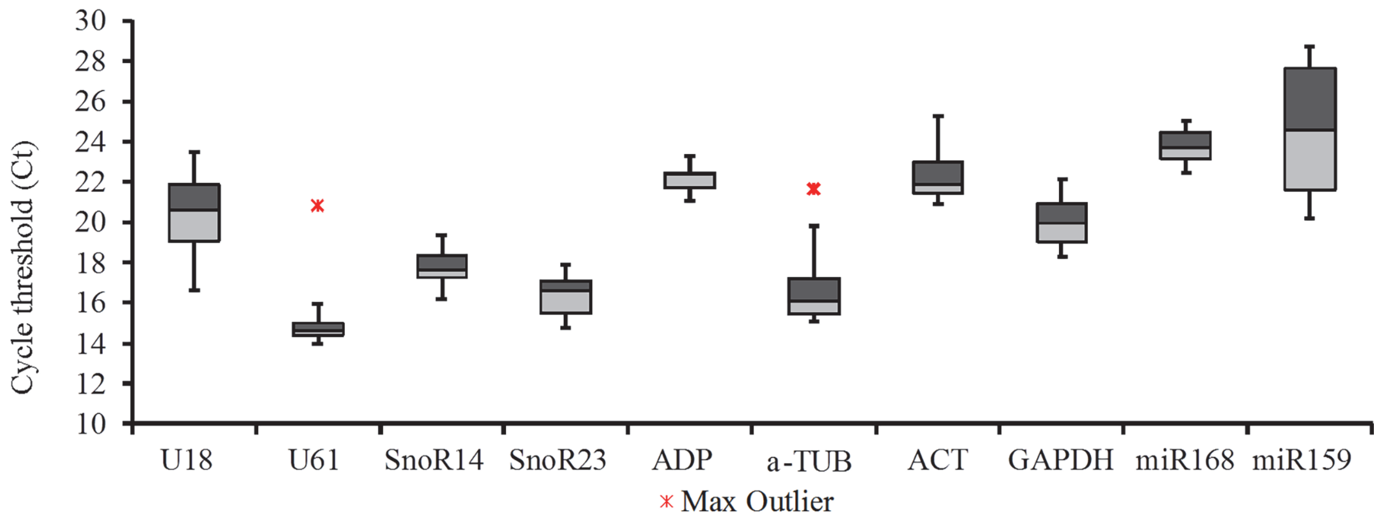

Fig 2. Gene expression of ten candidate internal controls in all samples. Data points are cycle threshold (Ct) values for each candidate reference gene in all samples. Boxes indicate the 25th (light shading) and 75th (dark shading) percentiles, the line indicates the median and whiskers depict the maximum and minimum values.

doi:10.1371/journal.pone.0118503.g002

14.98 and 24.35 (Fig. 2); in which higher and lower abundance of mRNA is represented by lower and higher Ct values, respectively. It should be noted that the Ct values obtained for the most stable genes ($\sim$ 20) make them suitable for the normalization of a wide range of experimental targets, but they would be less suitable for certain targets with very high or low expression.

The Bestkeeper analysis suggested that *ADP* (0.573) was the most stable reference gene followed by snoR14 (0.772) and snoR23 (0.816), while miR159 (3.046) was the least stable across all samples (Table 2). SnoR14, snoR23 and *ADP* also performed well in drought, boron and fungal treatment experiments (Table 2). Under salt and nitrate treatments miR168 was the most stable reference gene. miR159 was the least stable candidate under drought, boron and nitrate treatments, while *α-TUB* and *ACT* were the least stable reference genes under fungal and salt treatments, respectively.

Consensus ranking performance of each candidate reference gene in all samples ($n = 44$) was evaluated by the three algorithms (Fig. 3). For each candidate, the consensus ranking was calculated as geometric mean of three rankings suggested by three algorithms; geNorm, Norm Finder and Bestkeeper.

The Optimal Number of Reference Genes

The optimal number of reference genes under individual treatment was determined by geNorm analysis (Fig. 4). Under boron, fungal, salt and nitrate treatments, the $V_{2/3}$ values were less than 0.15 (Fig. 4). This analysis suggested that, apart from the two most stable reference genes found in the respective treatment conditions, the addition of a third, reference genes did not improve the normalization so that the use of the two most stable reference genes would be sufficient for normalization. Under drought treatment, $V_{2/3}$-$V_{7/8}$ was ≤ 0.15, while $V_{8/9}$ and $V_{9/10}$ was > 0.15 (Fig. 4). In such a situation, the use of between two and seven reference genes resulted in reliable normalization. It is worth noting that the V value is only a guideline for the determination of an optimal number of reference genes and thus should not be considered as an exact cut-off point.

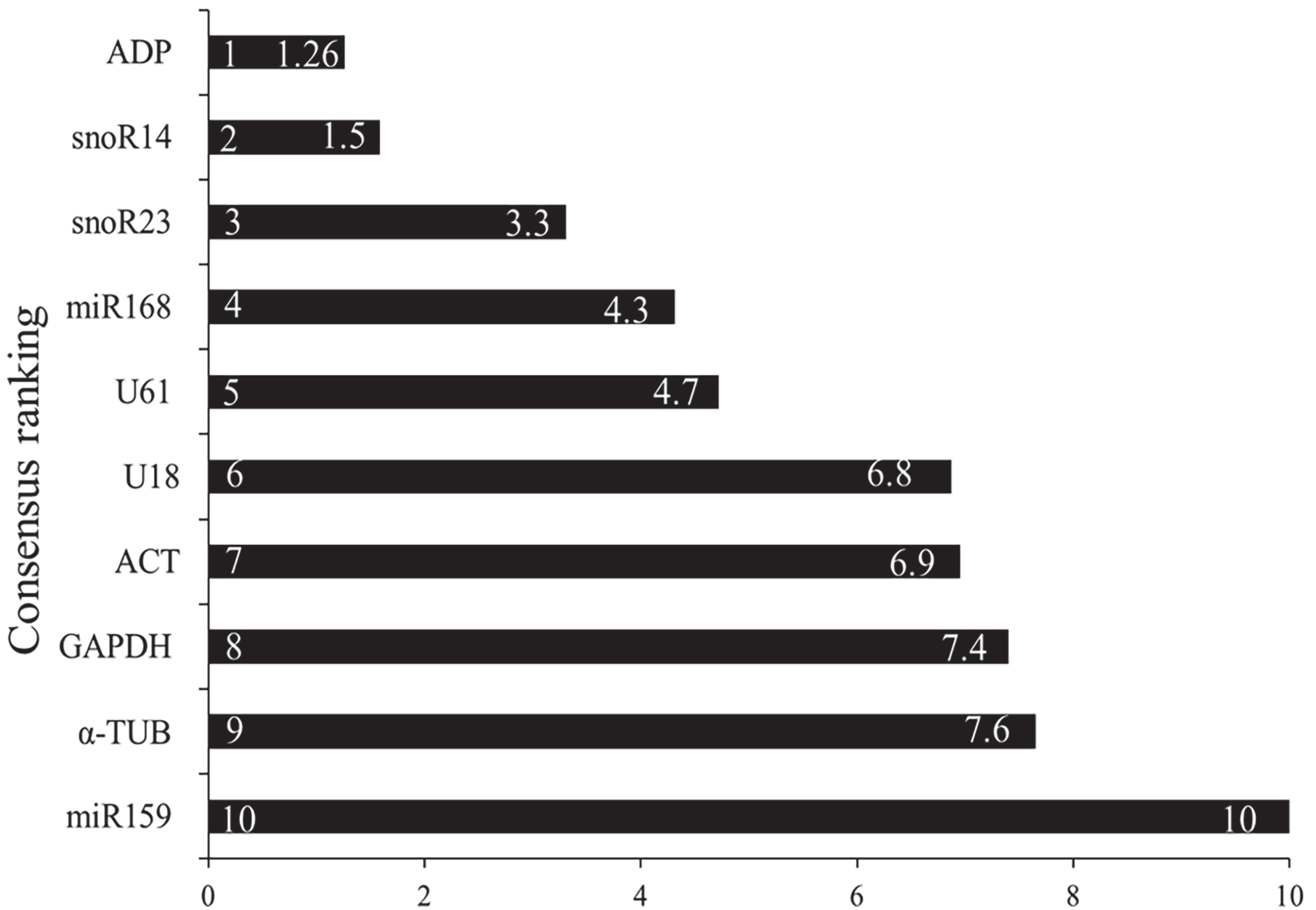

Fig 3. Consensus ranking of ten reference genes. Each candidate gene was assessed in the composite of all samples (n = 44). For each candidate, the consensus ranking was calculated as geometric mean of all rankings suggested by three algorithms; geNorm, Norm Finder and Bestkeeper.

doi:10.1371/journal.pone.0118503.g003

Validation of Stable Reference Genes for mRNA and miRNA Expression Under Various Stress Conditions

To validate the stability of reference genes, we used two groups for normalization. Group 1 denotes the three putatively stable reference genes, *ADP*, snoR14 and snoR23, suggested by the consensus ranking, and group 2 denotes the three commonly used HKGs, *ACT*, *α-TUB* and *GAPDH*. For each experimental condition, the expression of *SUPEROXIDE DISMUTASE* was normalized to group 1 and group 2 in the treated and control samples (Fig. 5). The formation of reactive oxygen species (ROS) in plants is triggered by various environmental stresses, such as drought, nutrient deficiency, nutrient toxicity, salinity and pathogen attack [46, 47]. *SUPEROXIDE DISMUTASE* is one of the first line of defence antioxidant enzymes. Though its expression is not broadly reported for each particular stress condition, it is well-known to be up-regulated under drought stress in plant species including barley [48–55]. As expected, normalized expression of *SUPEROXIDE DISMUTASE* to group 1 and group 2 resulted in lower expression under well-watered (control) than under drought-treated samples (Fig. 5A). However, the expression difference between the treated and control samples was greater when normalized with group 1 candidates (Fig. 5A). In the boron treatment experiment, both normalizer

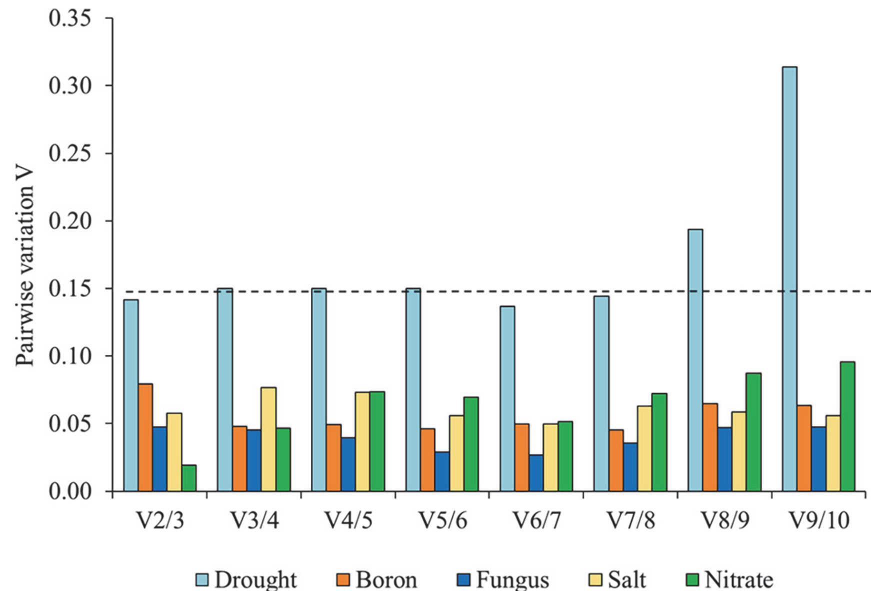

Fig 4. Determination of the optimal number of reference genes for normalization of qPCR data in each experimental condition. If the V (variation) value is below 0.15, the addition of a further reference gene does not result in any improvement of normalization.

doi:10.1371/journal.pone.0118503.g004

groups showed similar results where *SUPEROXIDE DISMUTASE* was significantly up-regulated in the treated samples (Fig. 5B). In the fungal infected samples the expression level of *SUPEROXIDE DISMUTASE* was reduced compared to the control samples when normalized to group 1. However, when normalized to group 2 the expression level of *SUPEROXIDE DISMUTASE* was not significantly different (Fig. 5C). In the nitrate and salt treatment samples, group 1 normalization resulted in significantly higher *SUPEROXIDE DISMUTASE* expression than in the control samples (Fig. 5D & E). However, in both of these experimental conditions, group 2 normalization did not show any significant difference of *SUPEROXIDE DISMUTASE* expression between the treated and control samples (Fig. 5D & E).

We also quantified the relative expression of a barley miRNA, miR5048, normalizing with the same groups of candidates. The normalization against group 1 reference genes resulted in down-regulation of miR5048 under drought treatment compared with the control conditions (Fig. 5F) which was consistent with our pre-existing deep sequencing data (see inset in Fig. 5F) [45]. In contrast, normalizing the expression level of miR5048 to group 2 candidates resulted in comparatively higher expression of miR5048 with no significant difference between the treated and control samples. This result was inconsistent with the normalized expression using group 1 candidate reference genes as well as inconsistent with our pre-existing deep sequencing result (Fig. 5F and inset). These data indicated an increased sensitivity of detection of differential expression using the putatively more stable group 1 candidates.

We also took advantage of the available boron treated, fungal infected, nitrate treated and salt treated samples to compared miR5048 expression in these conditions normalizing the expression level to the two normalizer groups to check for the groups' comparative sensitivity.

Under the boron treatment, miR5048 expression normalized to group 1 showed that miR5048 was down-regulated in the treated samples compared to the control samples. However, normalization to group 2 did not show any difference between the treated and control samples (Fig. 5G). Under the fungal infection, miR5048 expression was not abundant and there was no significant difference in expression with treatment detected using either normalizing

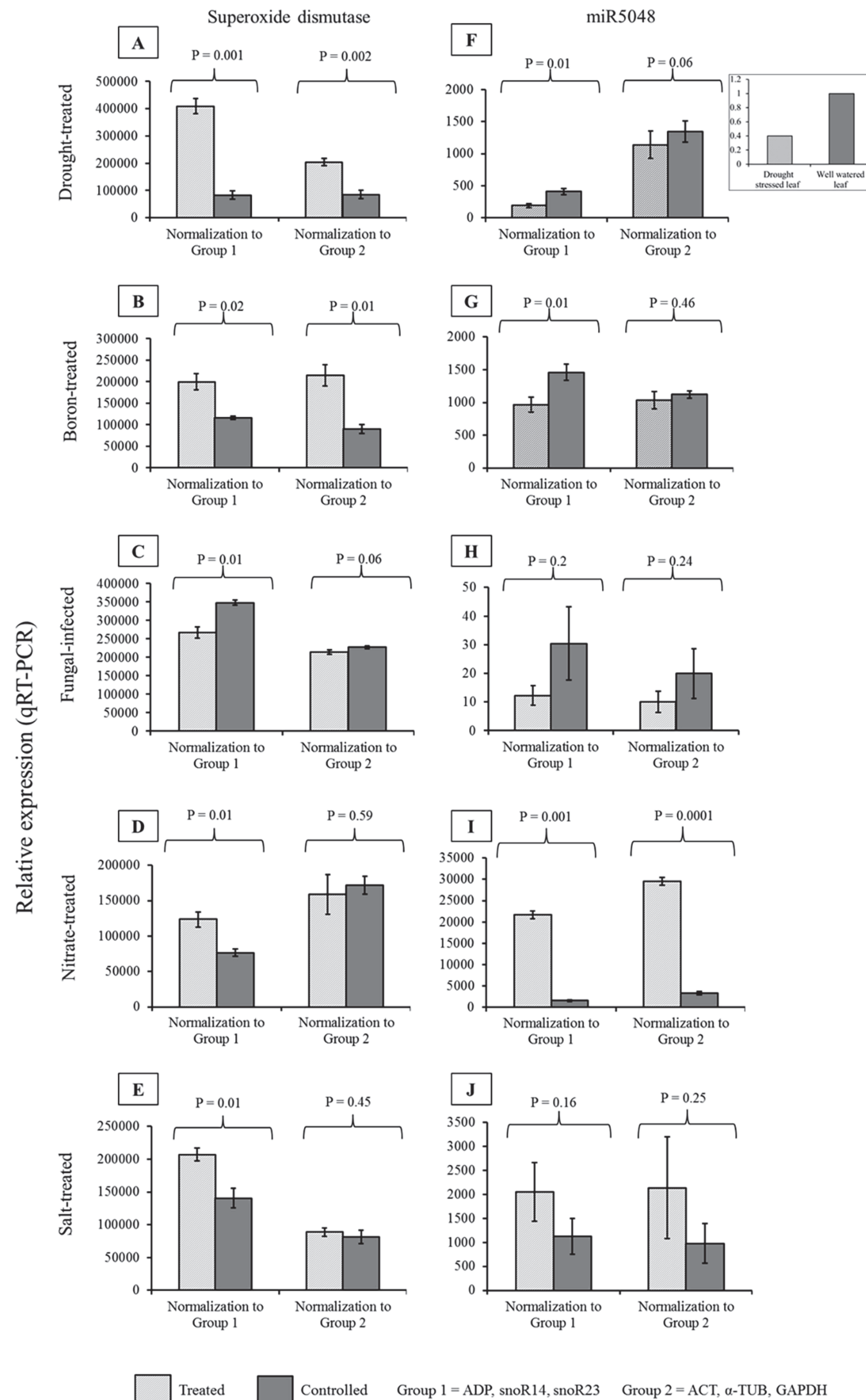

Fig 5. Validation of putatively stable reference genes. Comparison of relative expression of (A, B, C, D, E) *SUPEROXIDE DISMUTASE* (AK363344.1) and (F, G, H, I, J) miR5048 (MIMAT0020544) in drought-treated, boron treated, fungal infected, nitrate treated and salt treated samples and their respective controls by qPCR when normalized to group1 (a combined group of three stable reference genes; snoR14, *ADP*, snoR23) and group 2 (a combined group commonly used housekeeping genes; *ACT*, *α-TUB*, *GAPDH*). The error bars indicate the standard deviation of the mean. Statistical analysis by t-test. (Inset in 5F) The relative expression of miR5048 from deep sequencing of drought-stressed and well-watered barley leaves. Reads per million (RPM) of 1 was the highest number of counts.

doi:10.1371/journal.pone.0118503.g005

group of reference genes (Fig. 5H). Under the nitrate treatment, upon normalization to both normalizer groups, miR5048 was significantly up-regulated compared to the control samples (Fig. 5I). The salt treatment did not result in a significant up-regulation of miR5048 expression with either normalizing group (Fig. 5J).

Discussion

Normalization is an important requirement for the study of gene expression by qPCR. Random selection of reference genes, which may be influenced by experimental treatments, could cause the misinterpretation of results [1, 11]. An appropriate reference gene should have an invariant level of expression regardless of experimental conditions; however, such genes may be hard to find as plant gene expression is affected by environmental conditions [56–59]. It is clear from recent studies that internal reference genes for qPCR assays should be specifically selected for the experimental treatment of interest in both plants and animals [32, 33, 60, 61].

In our study, we evaluated the expression stabilities of ten candidate reference genes, *ACT*, *α-TUB*, *GAPDH*, *ADP*, snoR14, snoR23, U61, U18, miR159 and miR168, in samples from barley plants grown in five stressed conditions. There is no universally accepted method for reference gene selection and stability analysis. We hypothesized that small, non-coding RNAs could be more suitable reference genes for qPCR normalization of miRNAs when compared with commonly used protein-coding reference genes using three popular algorithms in different computer programs such as geNorm, NormFinder and BestKeeper. Using these different analytical algorithms the selected snoRNAs (snoR14 and snoR23) consistently ranked amongst the best and most suitable reference genes, whether individually or in combination, with only *ADP* amongst the more commonly used protein-coding reference genes ranked more highly than other snoRNAs in a composite of all samples by two of the three algorithms.

It is necessary to validate reference genes under each set of experimental conditions [62]. geNorm, NormFinder and BestKeeper analyses were performed separately for each experimental treatment and indicated that two of the candidate snoRNA reference genes, snoR14 and snoR23, were steadily stable in an experiment specific manner. snoR14, in particular and in combination with at least one other reference gene, had potential as a universal reference gene in all the studied sample conditions. Amongst protein-coding genes, *ADP* was indicated as a good potential internal control under drought treatment. This result is consistent with a previous study in barley [9]. Under salt stress, the snoRNA U61 showed stable expression and consistently ranked in the top two potential reference genes by three algorithms. U61 also ranked as the fourth most stable reference gene under boron treatment, miRNAs were also proposed as reference genes for qPCR data normalization of both miRNA and protein-coding genes in an experiment in soybean [1]. The miRNAs, miR168 and miR159, behaved very differently from each other as reference genes in the experiments reported here, underlining the importance of empirical selection and knowledge of the individual gene's differential expression with treatment. miR168 was selected as a candidate because it was reportedly not significantly affected by drought in *Arabidopsis* [63] and rice [64]. Genome-wide deep sequencing of peach also indicated that there was no change in the expression of miR168 in drought-stressed leaf and root tissues [65]. Our results confirmed the comparatively consistent stability of miR168 as a reference gene under each of the experimental conditions, where it ranked in the top five using the three algorithms.

geNorm and BestKeeper specify a gene expression stability threshold value above which a candidate reference gene should be considered as an unreliable internal control. The threshold value is M = 1.5 in geNorm, a value exceeded by miR159 and *GAPDH* in a composite of all

samples. The reliability threshold of SD = 1 in BestKeeper was also a value exceeded by miR159 and *GAPDH*, as well as U61, U18, and *α-TUB* in a composite of all samples.

Though the selection of miR159 was based on our previous miRNA expression studies in drought-treated barley 'Golden Promise' (data not shown), miR159 was ranked as the least stable candidate in drought-treated samples by three algorithms. An explanation of this inconsistency under drought treatment may reside in the use of different varieties, developmental stages and the method of quantification which may lead to changes in miRNA expression as well as impact on gene regulation. Additionally, miR159 was ranked as the least stable candidate under boron and nitrate treatments by BestKeeper. miR159 expression also varied in roots under drought stress in peach [65] and showed variable patterns in rice between young and old leaves [66]. Hence we do not consider miR159 a suitable reference gene.

Conventionally used HKGs, those involved in basic cellular mechanisms, have been used extensively for quantification of transcript expression. However, it has also been reported that their expression levels are not completely independent of the exogenous conditions [9, 13–16]. In our study, we found that some of the commonly used housekeeping genes such as *ACT*, *α-TUB* and *GAPDH* were not the most suitable HKGs under specific experimental conditions as in most cases they were ranked lower than the other candidates (Table 2). Additionally, in BestKeeper analysis, the reliability threshold SD = 1 was exceeded by *ACT*, *GAPDH* and *α-TUB* when used as HKGs for nitrate-treated samples demonstrating the importance of selecting reference genes specific to the experimental treatments. We found that the non-coding RNAs generally outperformed *ACT*, *GAPDH* and *α-TUB*. These results suggest that the common HKGs for qPCR data normalization should be used carefully, demanding a thorough evaluation for every experimental set of samples before use. Additionally, comparison of different algorithms for reference genes selection may facilitate a reliable evaluation of stably expressed genes as well as precise data normalization.

In our analysis of the stability of candidate reference genes, we validated their performance by normalizing the relative expression of an mRNA and an miRNA in barley under five experimental conditions. For evaluating mRNA expression, we selected *SUPEROXIDE DISMUTASE* whose enzymatic product rapidly scavenges reactive oxygen species in plants under various environmental stresses [46, 47, 67–70]. Increased expression of *SUPEROXIDE DISMUTASE* has been identified under drought stress in many plant species including cotton [48], pea [49], *Coffea* [50], common bean [51], rice [52], *Populus* [53] and wheat [54, 55]. To validate the performance of stable candidate internal controls for expression data normalization, we used the combination of *ADP*, snoR14 and snoR23, the top three stable reference genes suggested by the consensus ranking. In comparison to this normalization to these putatively stable candidates, we also considered the combination of relatively unstable candidates *ACT*, *α-TUB*, *GAPDH* suggested by the consensus ranking. With both sets of normalizers, *SUPEROXIDE DISMUTASE* expression increased in the drought treatment but the difference of expression between drought and well-watered samples was greater when the stable group of normalizers was used. This increased sensitivity of detection of differential expression was also evident in the fungal infection, low and high nitrate and salt stress experimental samples using the putatively stable group of reference genes. This demonstrated that the choice of appropriate and stable reference genes does increase the sensitivity of experimental assays in qPCR and validated our comparative ranking of the candidates.

To evaluate the putatively stable candidates for normalizing miRNA expression, we selected miR5048 for comparison with our pre-existing deep sequencing data for expression of this miRNA in the barley variety 'Golden Promise' under drought and well-watered conditions. Deep sequencing is well-recognised to reflect RNA presence and quantity from a genome at a given stage [71] and should correlate with the expression level identified through qPCR [72].

However, it should be noted that different methods of library construction including choice of adapters and even barcodes could generate significant bias during RNA sequencing profiling of miRNAs [73, 74]. Using the same set of putatively more stable reference genes for miR5048 expression data normalization, miR5048 was down-regulated under drought treatment consistent with the deep sequencing result. However, normalization with the putatively unstable, commonly used *ACT*, *α-TUB* and *GAPDH* failed to detect statistically significant down-regulation of miR5048 under drought stress, conflicting with our deep sequencing result. Thus, the validation of candidate reference genes for normalizing miRNA relative expression profiles increased our confidence in the analysis of stability of *ADP*, snoR14 and snoR23 in drought-stressed barley and highlighted how the use of inappropriate reference genes might lead to erroneous results.

Apart from the drought treatment, as there is lack of reference for miR5048 expression, we could not compare our validation results under individual experimental conditions. Nonetheless, for miR5048 as well as for *SUPEROXIDE DISMUTASE*, there was an increase in the sensitivity of detection of differential expression for boron, fungal and salt treatments when our higher-ranked stable candidates were used, once again validating their superiority.

Conclusion

We evaluated ten candidate reference genes in different tissues, genotypes and experimental treatments for their ability to normalize miRNA qPCR data. The expression stability of these candidate genes was evaluated across a set of 44 samples using the computer programs geNorm, NormFinder and BestKeeper. We found that two putative snoRNAs, snoR14 and snoR23, outperformed the generally used HKGs in barley. To our knowledge, this work is the first stability evaluation of a set of commonly used and novel putative reference genes for qPCR in barley under different experimental conditions. This study suggests our set of evaluated candidates *ADP*, snoR14 and snoR23 as potential reference genes in barley. Additionally, this work proposes a list of potential candidate reference genes under five, different experimental conditions. We recommend to use a combination of *ADP*, snoR14 and snoR23 as potential reference genes for miRNA and mRNA qPCR data normalization under the experimental conditions mentioned in this study. As the arbitrary selection of internal controls, or the use pre-identified reference genes, may yield inaccurate results, reference gene selection needs to be optimized for individual assays counting a number of candidate reference genes for evaluation. We also recommend the use of multiple reference genes for more reliable and valid normalization of gene expression in barley.

Supporting Information

S1 Fig. Melt curves of the ten candidate internal controls.
(TIF)

S2 Fig. Agarose gel (3%) electrophoresis showing amplification of the qPCR product of expected size for each candidate internal controls tested in this study.
(TIF)

S3 Fig. A polymerase chain reaction (PCR) using the mixture of RNA samples as no template controls for each primer sets.
(TIF)

S4 Fig. Standard curves of the ten candidate internal controls.
(TIF)

S5 Fig. Melt curve and standard curve of *SUPEROXIDE DISMUTASE*.
(TIF)

S6 Fig. Melt curve and standard curve of miR5048.
(TIF)

S1 Table. Primers used for sequencing individual candidate reference genes.
(DOCX)

S2 Table. Primers used for qPCR of barley *Superoxide dismutase* and miR5048.
(DOCX)

Acknowledgments

The authors thank and acknowledge Julie Hayes, Jessica Hilary Bovill, Rhiannon Schilling and Jamil Chowdhury for providing boron, salt, nitrate and fungal stress experiment RNA samples, respectively. The authors would specially like to thank Lorenzo Giusti for early discussion and his helpful advice on qPCR experiments.

Author Contributions

Conceived and designed the experiments: JF PT BS. Performed the experiments: JF YL. Analyzed the data: JF NR YL. Wrote and edited the paper: JF PT BS PL.

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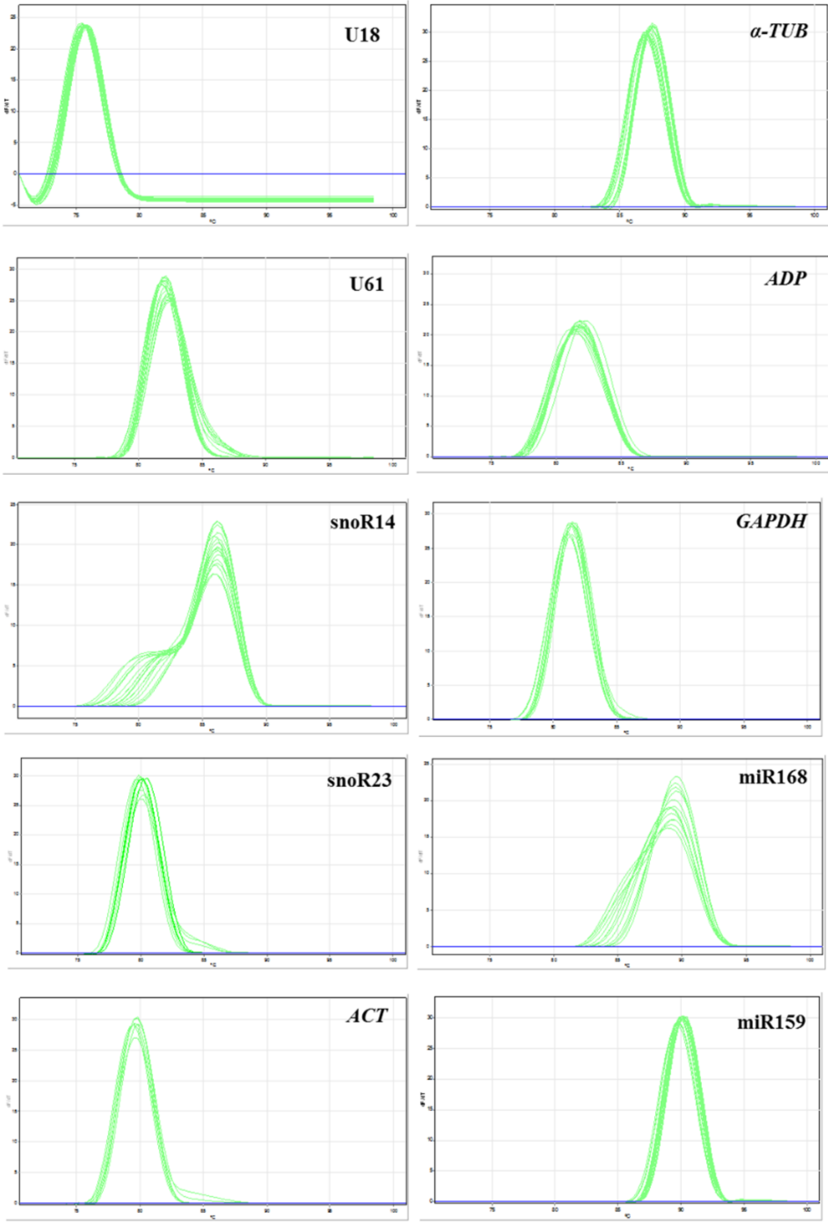
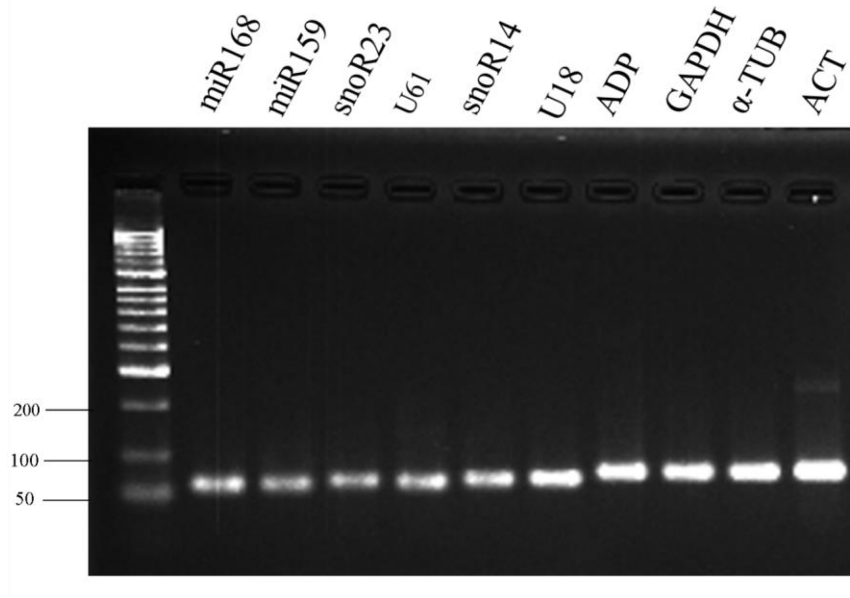
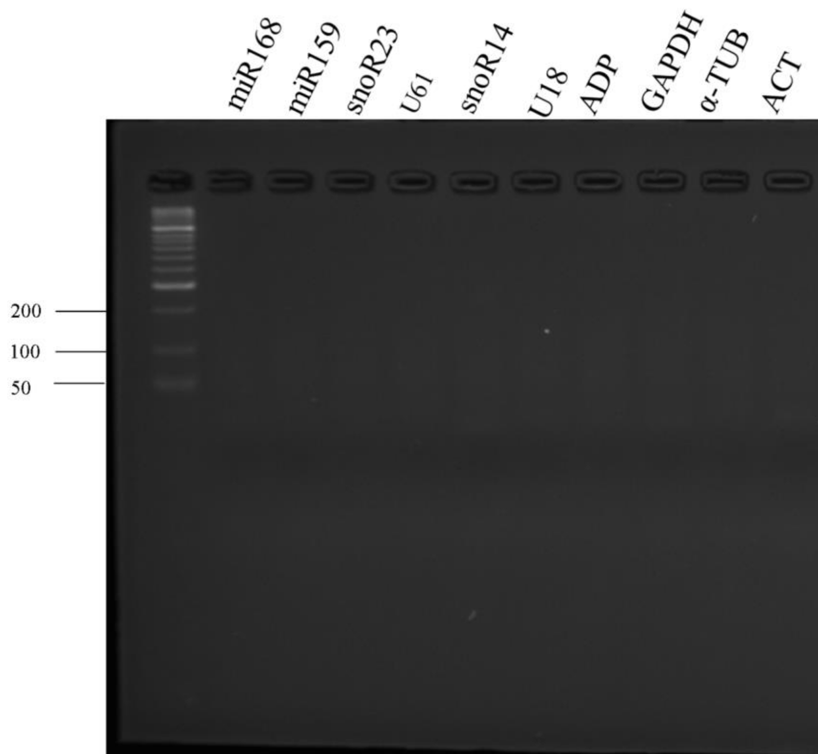
S1 Fig. Melt curves of the ten candidate internal controls.



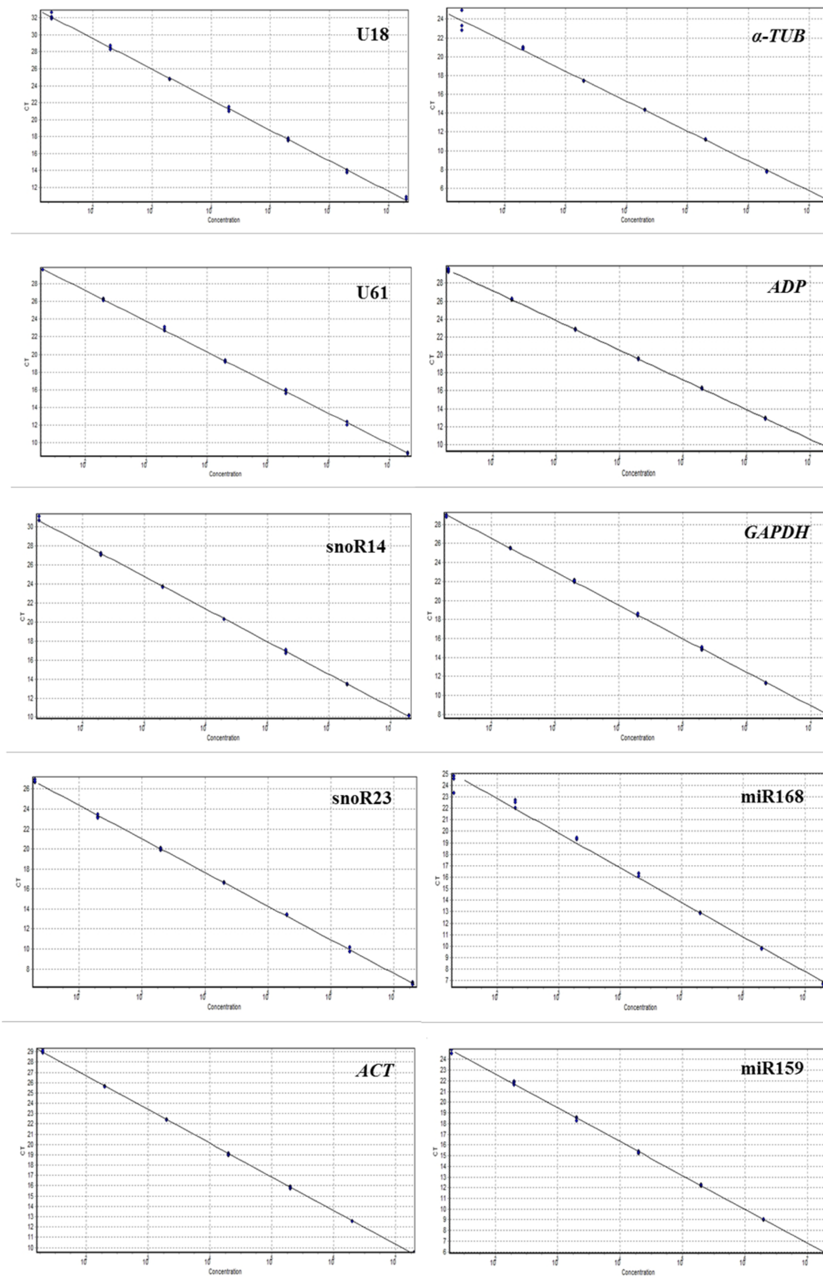
S2 Fig. Agarose gel (3%) electrophoresis showing amplification of the qPCR product of expected size for each candidate internal controls tested in this study.



S3 Fig. A polymerase chain reaction (PCR) using the mixture of RNA samples as no template controls for each primer sets.

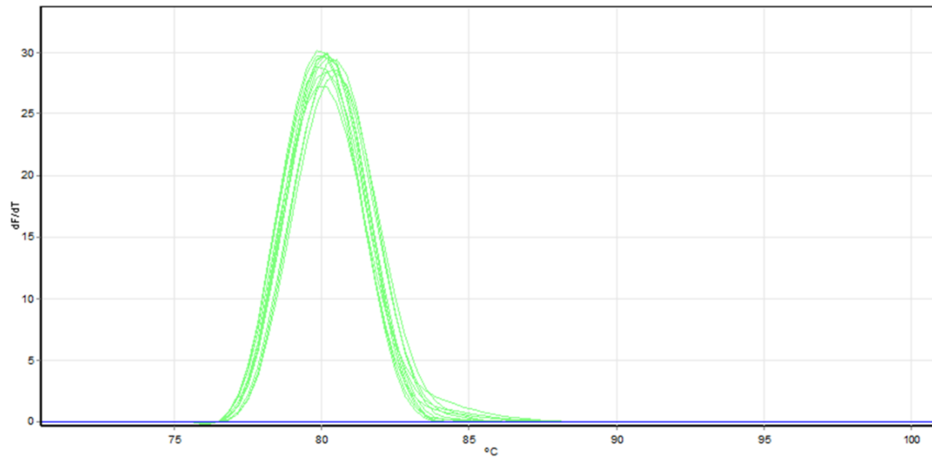


S4 Fig. Standard curves of the ten candidate reference genes.

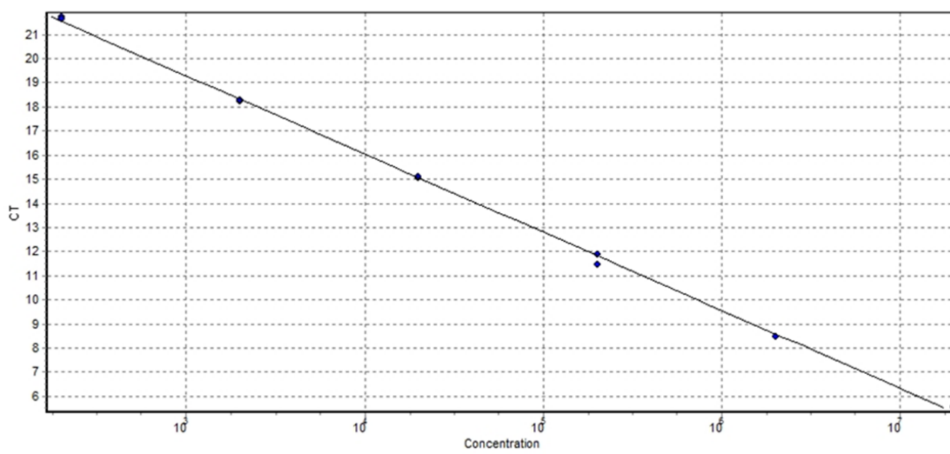


S5 Fig. Melt curve and standard curve of *SUPEROXIDE DISMUTASE*.

Melt curve for *Superoxide dismutase*

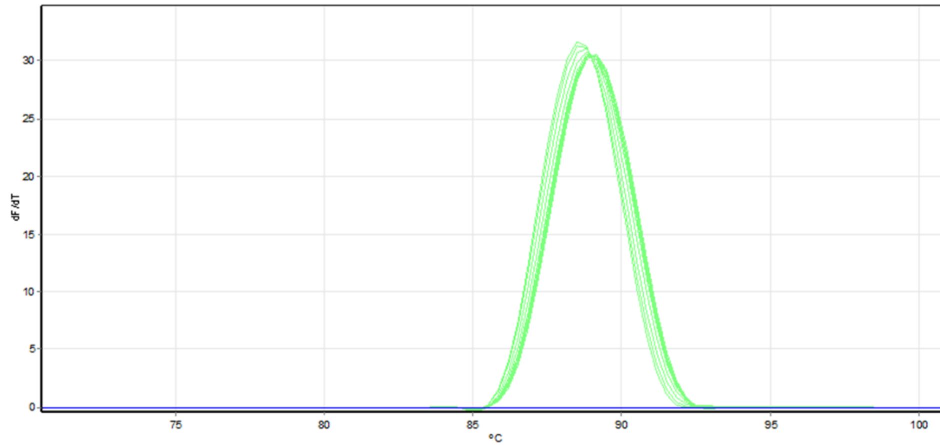


Standard curve for *Superoxide dismutase*

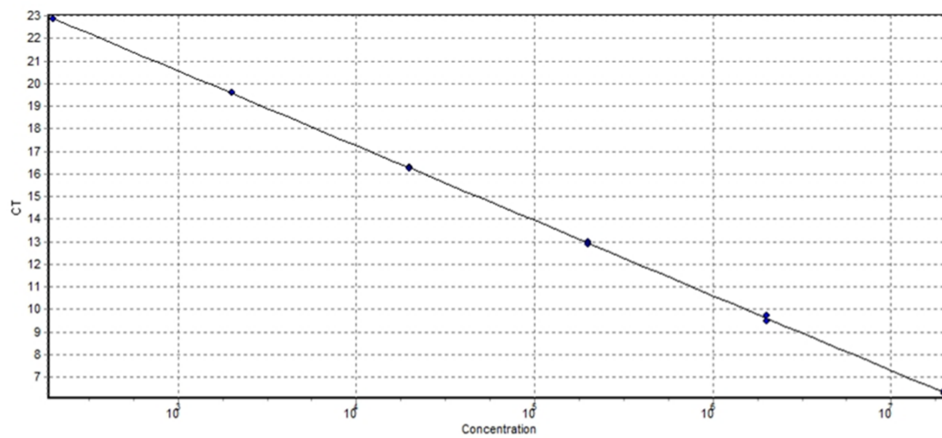


S6 Fig. Melt curve and standard curve of miR5048

Melt curve for miR5048



Standard curve for miR5048



S1 Table. Primers used for sequencing individual candidate reference genes.

Primer name	Primer sequence (5’–3’)
M13r+ SPACER + Hvu-miR168 Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGAGAGCTGGGTCCGAGGT
M13r+ SPACER + Hvu-miR159 Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGAGTGCAGGGTCCGAGGT
M13r+ SPACER + Hvu-ACT Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGACACTGAGCACGATGTTT
M13r+ SPACER + Hvu-ADP Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGAGAGACATCCAGCATCA
M13r+ SPACER + Hvu-GAPDH Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGACACATTTATTCCCATAG
M13r+ SPACER + Hvu-α-TUB Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGACGGCGGCAGATGTCAT
M13r+ SPACER + Hvu-snoR14 Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGAGTCGGGATGTATGCGTG
M13r+ SPACER + Hvu-U61 Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGAACTTCTTAGAGGGTTGT
M13r+ SPACER + Hvu-U18 Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGAAGAAGTTTATTAAGGAT
M13r+ SPACER + Hvu-snoR23 R	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGACTCAGTGGAAAGAGAA
M13r+ SPACER + Hvu-miR5048 Rev	CAGGAAACAGCTATGACCATGGATCGATCGAGATCGATCGAGTGCAGGGAGGGAGGT
M13 reverse*	CAGGAAACAGCTATGACCATG
SPACER#	GATCGATCGAGATCGATCGA

Note: Underlined sequence is a spacer sequence used for getting a complete sequence of each amplicon, and M13 reverse (M13r) primer was used to sequence all the amplicons.

S2 Table. Primers used for qPCR of barley *Superoxide dismutase* and miR5048.

Primer name	Primer sequence (5'–3')	Accession number	Amplicon (bp)	PCR efficiency	Regression coefficient (R^2)
Hvu-Superoxide dismutase forward	CTTGAAGGACACCGACTTGC	AK363344.1	141	1.03	0.999
Hvu-Superoxide dismutase reverse	CTCAAAAAGCCAAATGACAGTG				
Hvu-miR5048 forward	CGTCTTCGGTATTTGCAGGTTTTA	MIMAT0020544	65	1.0	0.999
Hvu-miR5048 reverse	GTGCAGGGAGGGAGGT				
Hvu-miR5048 stem-loop RT primer	GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACTTAGAC				

CHAPTER 4

Differential expression of microRNAs and potential targets under drought stress in barley

Statement of Authorship

Title of Paper	Differential expression of microRNAs and potential targets under drought stress in barley
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Jannatul Ferdous, Juan Carlos Sanchez-Ferrero, Peter Langridge, Linda Milne, Jamil Chowdhury, Chris Brien and Penny J. Tricker This is an experimental manuscript about drought responsive miRNA expression in different barley genotypes under drought in order to identify and validate the target genes, and to determine the cellular localization of important drought responsive miRNAs and their targets. Findings of our study suggest the involvement of miRNAs in the molecular control and genotype-specific regulation of barley under drought. This experiment is closely related to the subject matter of this thesis.

Principal Author

Name of Principal Author (Candidate)	Jannatul Ferdous		
Contribution to the Paper	Conceived and designed the experiments, performed experiments, analysed and wrote manuscript.		
Overall percentage (%)	70%		
Certification:	I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Juan Carlos Sanchez-Ferrero		
Contribution to the Paper	Analysed and validated targets in silico. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	07/01/16

Name of Co-Author	Peter Langridge		
Contribution to the Paper	Supervised designing the experiments. Collaborated with the James Hutton Institute for microRNA-target prediction. Evaluated the development of experiments, reviewed and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/16

Name of Co-Author	Linda Milne		
Contribution to the Paper	Analysed microRNA target prediction. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	11/01/2016

Name of Co-Author	Jamil Chowdhury		
Contribution to the Paper	Mentored microscopy work for in situ PCR and imaged the cellular localisation of microRNA and target expression. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	13/02/2016

Name of Co-Author	Chris Brien		
Contribution to the Paper	Designed the experiment and performed the statistical analysis. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	08/01/16

<table>
<tr><td>Name of Co-Author</td><td colspan="3">Penny J. Tricker</td></tr>
<tr><td>Contribution to the Paper</td><td colspan="3">Supervised designing and development of the experiments, data analysis and interpretation. Reviewed and edited the manuscript and acted as the corresponding author. I hereby certify that the statement of the contribution is accurate.</td></tr>
<tr><td>Signature</td><td></td><td>Date</td><td>17/02/16</td></tr>
</table>

Link to chapter 4

In order to identify and test natural variation of miRNA and target gene expression under drought, the expression levels of 11 candidate mature miRNAs was tested in the flag leaves of four drought tolerant barley genotypes by qRT-PCR. The expression of four miRNAs was differential between drought-treated and well-watered barley samples. Bioinformatic identification of the targets of these miRNAs resulted in numerous candidate targets for an individual miRNA. Therefore, we further validated the targets using two degradome libraries that enabled us to recognize miRNA mediated mRNA cleavage products from the miRNA: mRNA target interaction. Further, the spatial localization of potential drought responsive miRNAs and their targets was realised in drought treated and well-watered barley leaves. This chapter (Chapter 4) is in press as: Ferdous J., Sanchez-Ferrero J.C., Langridge P., Milne L., Chowdhury J., Brien C., & Tricker P.J. (2016). Differential expression of microRNAs and potential targets under drought stress in barley. Plant, Cell & Environment. DOI: 10.1111/pce.12764.

Differential expression of microRNAs and potential targets under drought stress in barley

Running title: miRNA expression under drought in barley

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ABSTRACT

Drought is a crucial environmental constraint limiting crop production in many parts of the world. microRNA (miRNA) based gene regulation has been shown to act in several pathways, including crop response to drought stress. Sequence based profiling and computational analysis have revealed hundreds of miRNAs and their potential targets in different plant species under various stress conditions, but few have been biologically verified. In this study, eleven candidate miRNAs were tested for their expression profiles in barley. Differences in accumulation of only four miRNAs (*Ath*-miR169b, *Osa*-miR1432, *Hv*-miRx5 and *Hv*-miR166b/c) were observed between drought-treated and well-watered barley in four genotypes. miRNA targets were predicted using degradome analysis of two, different genotypes and genotype-specific target cleavage was observed. Inverse correlation of mature miRNA accumulation with miRNA target transcripts was also genotype-dependent under drought treatment. Drought-responsive miRNAs accumulated predominantly in mesophyll tissues. Our results demonstrate genotype-specific miRNA regulation under drought stress and evidence for their role in mediating expression of target genes for abiotic stress response in barley.

Keywords: *Hordeum vulgare*, genotype, canonical cleavage, degradome, *in situ*-RT-PCR.

INTRODUCTION

In many regions of the world, crop production is constrained by prolonged dry conditions. Declining water resources and increased variation in rainfall will require the development of climate-resilient crop varieties. One of the visions of plant stress

research is to provide genetic loci as targets for enhancing stress tolerance in crop plants. A key step in the development of stress tolerant crop varieties will be understanding of the function of stress-responsive genes. Improved understanding of gene regulation during stress could aid the development of genomic tools (Langridge and Reynolds, 2015). One of the central molecules for naturally occurring regulation of gene transcription is microRNA (miRNA). miRNAs are non-coding transcripts of 18-21 nucleotides (nt) in length. Generally, plant miRNAs function in a sequence specific mode to target mRNAs based on complementary base pairing, leading to cleavage of the target mRNA (Lee *et al*., 1993; Reinhart *et al*., 2002) and it has been suggested that miRNAs play a regulatory role in activating stress defence or response gene networks (Zhu, 2002).

The identification of miRNAs is an essential precursor to understanding and quantifying their roles in regulating mRNA. Large-scale bioinformatic analysis has enabled miRNA discovery in plants such as *Arabidopsis*, *Medicago*, *Brachypodium* and, more recently, crop plants like rice, maize, wheat and barley According to miRBase (miRBase v. 20; Kozomara and Griffiths-Jones 2014), to date 7385 mature miRNA sequences from 72 plant species have been identified. Families of miRNAs may be conserved between species and taxa but unique miRNAs have also been discovered in each species analysed (Schreiber *et al*., 2011). Although many miRNAs have been identified, the regulation of miRNA expression is still poorly understood and the existence of multiple isoforms of mature miRNAs generated from a single miRNA locus complicates analysis and, hence, understanding of miRNAs' functional relevance (reviewed in Budak *et al*., 2015).

The quantification of mature miRNA expression is difficult due to their short length and absence of common sequence features (e.g. polyA) (Benes and Castoldi, 2010). Some mature miRNAs are difficult to distinguish as they differ by as few as one

or two nucleotides and technical limitations may hinder discovery when protocols are unable to distinguish between mature miRNAs and their precursors (Li *et al*., 2014), or closely related miRNAs that are very similar in sequence (Balcells *et al*., 2011). Thus expression of an abundant miRNA may mask the expression of a low abundance or very similar miRNA. Stem-loop qRT-PCR has proven a reliable technique for the detection of mature miRNAs (Chen *et al*., 2005, Varkonyi-Gasic *et al*., 2007; Shen *et al*., 2014), though the limitation of this technique is that it requires a separate reverse transcription (RT) reaction for each miRNA.

A key objective of miRNA expression profiling is to identify and validate the miRNA's target mRNAs, but this can be challenging if it depends solely on predictions using computational approaches. In plants, miRNA regulated gene expression occurs by target mRNA cleavage (Baumberger and Baulcombe, 2005; Jones-Rhoades *et al*., 2006) or by inhibition of target mRNA translation (Gu and Kay, 2010; Vazquez *et al*., 2010). miRNA directed mRNA cleavage occurs when there is perfect or near perfect Watson-Crick complementary pairing between the miRNA and target mRNA (Zhang *et al*., 2006; Krol *et al*., 2010). However, perfect pairing between the miRNA and the target is neither obvious nor sufficient for the miRNA: target interaction (Brennecke *et al*., 2005; Witkos *et al*., 2011; Künne *et al*., 2014).

The prediction of miRNA targets using computational programs based on sequence alignment has been used frequently. There are a variety of important parameters for *in silico* prediction including alignment score, maximum score, number of consecutive mismatches, number of G:U wobble pairing and number of gaps (Zhang *et al*., 2006; Xie and Zhang, 2010; Dehury *et al*., 2013). Upon base-pairing, miRNA guided mRNA cleavage occurs at its site opposite the 5' end at the 10th and 11th positions of the miRNA (Huntzinger & Izaurralde, 2011). Using these parameters, the *in silico* prediction of targets, followed by validation using a sequenced small RNA library

of degraded transcripts (degradome analysis) helps to shortlist the candidate target genes. It is worth mentioning that the targets of miRNAs may not be conserved across different plants species, although miRNAs are (Lu *et al*., 2005). Therefore, the targets of miRNAs need to be validated in individual plant species.

Despite technical hurdles to miRNA functional analysis, there is a growing body of evidence that alteration of miRNA accumulation plays an important role in reprogramming plant responses to biotic and abiotic stresses (Berger *et al*., 2009; Hackenberg *et al*., 2013). Drought stress has been revealed to alter the expression of many miRNAs. For example, differential expression of miR398a/b under drought was observed in *Medicago truncatula* in two different studies (Trindade *et al*., 2010, Wang *et al*., 2011). Members of the miRNA family miR319 were also found to be differently expressed under drought stress in rice (Zhou *et al*., 2010). miR166 was up-regulated in drought-stressed barley (Kantar *et al*., 2010) and down-regulated in wild emmer wheat (Kantar *et al*. 2011); miR171 was induced in barley (Kantar *et al*., 2010) and reduced in wheat (Kantar *et al*. 2011) under drought shock. The differential miRNA abundances under drought revealed in these studies suggest a role for miRNAs in reprogramming plant responses to drought stress, and differential miRNA expression may govern the fine tuning and control of stress signalling (Gutierrez *et al*. 2009).

Barley is not only an economically important crop, but is also well known for its genotypic variability under adverse conditions. Hence, it is an excellent model plant to study drought response and to identify and analyse functions of drought responsive miRNAs. Although deep-sequencing technology has extended the discovery of barley miRNAs (Schreiber *et al*., 2011; Hackenberg *et al*., 2015), only a limited number of studies have investigated miRNAs in barley under drought stress (Kantar *et al*., 2010, Hackenberg *et al*., 2015). It is often not clear which mature miRNAs are truly drought responsive and what is the mode of function of these miRNAs to regulate their target

gene(s). Information about the spatial patterns of drought responsive mature miRNAs and their targets could help our understanding of the molecular events involved in the drought stress response. If miRNAs are to be used in enhancing the drought tolerance of crop plants, we need to establish both function and evidence for genetic variation in their expression. Therefore, our aims in this study were to evaluate drought responsive miRNA expression in different barley genotypes under drought, to identify and validate the target genes, and to determine the cellular localization of important drought responsive miRNAs and their targets. We validated miRNAs associated with drought response in four genotypes (*Hordeum vulgare* L. 'Commander', 'Fleet', 'Hindmarsh' and breeding line WI4304) that showed similar performance in drought prone areas of Australia (Fettell, 2011; Coventry *et al*., 2012). We predicted potential miRNA targets using degradome libraries and validated both the predicted drought-responsive miRNAs and their targets using qRT-PCR. *In situ* –RT-PCR (ISRT-PCR) was performed to localize expression of selected miRNAs and their targets. Identifying miRNAs associated with drought response and recognizing their targets in different barley genotypes could help determine the potential contribution of miRNAs for the varietal selection of stress tolerant crop plants.

MATERIALS AND METHODS

Plant materials and drought treatment

Plants were grown in a growth chamber maintained at 23 °C day and 18 °C night temperatures, 12 h/12 h light/dark photoperiod, 450 µmol m^{-2} s^{-1} Photosynthetically Active Radiation and 60 % Relative Humidity. Seeds were germinated on petri dishes and seedlings that germinated at the same time and were of the same physiological stage were transplanted to pots. Twenty four pots were used where each pot contained four plants, one each of *Hordeum vulgare* L. cvs.

‘Commander’, ‘Fleet’, ‘Hindmarsh’ and breeding line WI4304, to minimize the inter pot variation. Three weeks after transplanting half the pots were subjected to drought treatment of -6 bar soil water potential, while control pots were maintained at -2.5 bar, with watering to weight for a further six weeks. The interaction between treatment and development was significant so that well-watered plants reached maturity faster (47.5 d ±1) than drought-treated plants (57.8 d ±1) (supporting information Fig. S1) so samples were collected at a defined, physiological stage (booting) from each plant. The flag leaf was harvested from the drought-stressed and well-watered plants of five replicates and immediately frozen in liquid nitrogen and stored at −80 °C. From 12 plants in each treatment per genotype, two fully expanded mature leaves (per plant) were sampled to record the leaf water potential (LWP) and the relative water content (RWC) respectively, for no more than two hours at and after solar noon. There were no significant differences in the dry weights of mature, sampled leaves between genotypes or treatments. The fresh weights of sampled leaves differed with treatment with drought-treated leaves weighing less, but there were no significant differences between the genotypes within a treatment (supporting information Fig. S2).

Leaf water potential measurement

LWP was measured using a pressure chamber (Scholander Pressure Chamber Model 3000) and the method of Boyer (1967). A fully expanded, mature leaf was cut and placed immediately through the chamber lid with the cut end of the leaf outside and the remaining part of the leaf inside the chamber. Pressure was increased slowly. A magnifying glass was used to observe the cut end of the leaf. As soon as a drop of sap appeared from the cut end of the leaf sample, the pressure shown on the chamber gauge was recorded as a measure of the LWP (Boyer, 1967).

Relative Water Content measurement

RWC was determined by the method of Barrs and Weatherley (1962). The mid-leaf section of a fully expanded leaf was weighed to determine fresh weight (FW). Then samples were re-hydrated overnight and the turgid weight was (TW) recorded. Samples were oven dried at 80°C for 24h and weighed to determine dry weight (DW). RWC (%) was calculated using the formula [(FW-DW) / (TW-DW)] x 100.

Primer designs

The selection of miRNAs used in this study was based on barley miRNAs identified by Kantar *et al*. (2010), Schreiber *et al.* (2011), Hackenberg *et al.* (2012) and Hackenberg *et al.* (2015). Selected miRNAs had previously been shown to be differential expressed under drought in leaves or shoots of different species including barley (*Hv*-miR166b/c, *Ath*-miR169b, *Osa*-miR393a, *Hv*-miR444b, *Hv*-miR5048a, *Hv*-miR171), *Medicago truncatula*, *Oryza sativa*, *Prunus persica*, *Populus euphratica* and *Triticum turgidum* ssp. *dicoccoides* (reviewed in Ferdous *et al*., 2015a) except *Hv*-miRx5 and *Ata*-miR9863a. Stem-loop reverse transcription (RT) primers were used for cDNA synthesis from mature miRNAs. Primer sequences and miRNA identities are given in Table 1. miRNA specific stem-loop RT primers, and forward and reverse primers for individual miRNAs were designed following the method established by Chen *et al.* (2005) and refined by Varkonyi-Gasic and colleagues (2007) where the last 3´ six nt of a miRNA sequence was used as the antisense overhang, and the miRNA specific forward primer was designed to contain the remaining 5´ sequences (normally 13-15 nt from the 5´ end of the specific miRNA). We considered up to three nucleotides of these remaining 5´ sequences which were not contained by the forward primer and were used as the signature nucleotides to verify the respective miRNA by sequencing.

For quantification of mRNA targets in the same RNA samples, primers were designed to span the target site; that is, including the miRNA: target pairing region and primers were *NFY-A*:

forward: 5´ CATCACGGTCACCATCTC 3´ and reverse: 5´ ATCTCTGAAGTCCTAACACG 3´; and *EF hand* containing transcript: forward: 5´ ATATCACCACAAGCGTTCAC 3´ and reverse 5´ GAGCGAGATCAGGAGAGAC 3´. Primer efficiencies and unique products were confirmed by a single, distinct peak in melt curve analysis (Supporting Information Fig. S3).

Table 1. Primers used for the miRNA stem-loop qRT-PCR

miRNA name	miRNA sequence	Primer (5′–3′)	miRBase Accession number
Hv-miR166b/c	UCGGACCAGGCUUCAUUCC	Stem-loopRT GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACGGAATG Forward AATGTTCCTTCGGACCAGG Modified Reverse GTGCAGGGAGGGAGGT	MIMAT0020737
Ath-miR169b	CAGCCAAGGAUGACUUGCCGG	Stem-loopRT GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCGGCA Forward TACACGGCAGCCAAGGATGAC Universal Reverse GTGCAGGGTCCGAGGT	MIMAT0000906
Osa-miR169n	UAGCCAAGAAUGACUUGCCUA	Stem-loopRT GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGGCA Forward GCGTGCTGTAGCCAAGAATGAC Universal Reverse GTGCAGGGTCCGAGGT	MIMAT0001059
Hv-miR171	UGUUGGCUCGACUCACUCAGA	Stem-loopRT GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACTCTGAG Forward CTGCTACTGTGTTGGCTCGACTC Modified Reverse GTGCAGGGAGGGAGGT	MIMAT0022971
Osa-miR393a	UCCAAAGGGAUCGCAUUGAUC	Stem-loopRT GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATCAA Forward ACTATGCTCCAAAGGGATCGC Universal Reverse GTGCAGGGTCCGAGGT	MIMAT0000957
Bdi-miR396b	UCCACAGGCUUUCUUGAACUG	Stem-loopRT GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGTTC Forward TTGTCAGTCCACAGGCTTTCT Universal Reverse GTGCAGGGTCCGAGGT	MIMAT0020700
Hv-miR444b	UGCAGUUGCUGUCUCAAGCUU	Stem-loopRT GTCGTATCCAGAGCTGGGTCCGAGGTATTCGCTCTGGATACGACAAGCTT Forward TCGTTCAGTTGCAGTTGCTGTC Modified reverse-2 GAGCTGGGTCCGAGGT	MIMAT0020543
Osa-miR1432	AUCAGGAGAGAUGACACCGAC	Stem-loopRT GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTCGGT	MIMAT0005966

		Forward GGTGTGGCATCAGGAGAGATG Universal Reverse GTGCAGGGTCCGAGGT	
Hv-miR5048a	UAUUUGCAGGUUUUAGGUCUAA	Stem-loopRT GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACTTAGAC Forward CGTCTTCGGTATTTGCAGGTTTTA Modified Reverse GTGCAGGGAGGGAGGT	MIMAT0020544
Ata-miR9863a	UGAGAAGGUAGAUCAUAAUAGC	Stem-loopRT GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACGCTATT Forward TCGGCGGTGAGAAGGTAGATCA Modified Reverse GTGCAGGGAGGGAGGT	MIMAT0037104
Hv-miRx5	ACUGGUUGGAUCAUGCUUCUC	Stem-loopRT GTCGTATCCAGTGCAGGGAGGGAGGTATTCGCACTGGATACGACGAGAAG Forward primer TTGCGACTGGTTGGATCAT Modified Reverse GTGCAGGGAGGGAGGT	-

miRNA and target mRNA analyses using qRT-PCR

Total RNA was extracted from leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To remove genomic DNA contamination, RNA samples were treated with DNA-free™ (Ambion, Life Technologies, Grand Island, NY, USA) twice according to the manufacturer's instructions. The concentration and integrity of the DNase treated RNA was measured with an Agilent-2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) was higher than 6 for all samples.

One µg total RNA from each sample was reverse transcribed to cDNA using SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) and gene specific primers (Table 1). cDNA synthesis for miRNAs was carried out following the method described by Varkonyi-Gasic *et al*., (2007). We used up to six stem-loop primers [1 µl of miRNA specific stem-loop RT primer each (1 µM)] in one RT reaction. The RT reaction also contained 10 µM of anti-sense primers for internal controls (Ferdous *et al*., 2015b). Gene specific primers were also used for target cDNA synthesis using SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) and following the manufacturer's instructions for the RT reaction.

miRNA and target qRT-PCR assays were carried out exactly as in Ferdous *et al.* (2015b) using the RG6000 Rotor-Gene real-time thermal cycler (Qiagen, Valencia, CA, USA) with 2 min at 95 °C, followed by 50 cycles of 1 second at 95 °C, 1 second at 60 °C, 25 seconds at 72°C, and fluorescence acquisition at 72°C. Five independent plants were used for each treatment per genotype with three technical replicates per biological replicate.

miRNA qRT-PCR products were sequenced using M13 reverse primers following the method described by Ferdous *et al*., (2015b) to verify signature

nucleotide(s) (Supporting Information Fig. S4). qRT-PCR amplicons of uncleaved targets were also verified in each genotype by Sanger sequencing.

Statistical design and analysis

A split-plot design was used where pots were the main plots and each pot was divided into four quadrants to give four subplots (Supporting Information Fig. S5). The experiment involved 24 pots arranged in four rows by six columns to give a total of 96 plants. The watering conditions were assigned to the pots using two 6 × 6 Latin squares. An analysis of variance was performed on all data variables. The blocking structure of the analyses (excluding qRT-PCR data), derived from the design, was (Rows*(PairsColumns/WithinColumns))/Quadrants. For the qRT-PCR data, five replicates were used and the only blocking structure included in the analysis was pot number. This accounted for variability between pots and similarities between qRT-PCR materials from quadrants of the same pot. The overall significance tests used an F test-statistic and tests for significance between means were conducted using a least significant difference (LSD) value at the 5% significance level.

Target prediction and validation by degradome analysis

Target prediction of the drought responsive miRNAs was first conducted *in silico*. We searched the miRNA sequences against an RNA sequencing dataset from 16 tissues and growth stages in barley from the James Hutton Institute (unpublished data) and from Ensembl Plants using psRNATarget (http://plantgrn.noble.org/psRNATarget/) and the parameters Dehury *et al*., (2013) were i) maximum expectation: 4.0 (range: 0-5.0), ii) length for complementary scoring (hspsize): 20 (range: 15-30bp), iii) target accessibility - allowed maximum energy to un-pair the target site (UPE): 25 (range: 0-100, less is better), iv) flanking length around target site for target accessibility analysis:

17 bp in upstream/13 bp in downstream, v) range of central mismatch leading to translational inhibition: 9 - 11 nt. The predicted targets of 11 miRNAs were classified based on their gene ontology (GO) and Pfam annotation.

Two barley degradome libraries were analysed: one from the cv. ‘Golden Promise’ was described by Hackenberg *et al*. (2015) and an additional cv. ‘Pallas’ was also used. Sequencing libraries were prepared from the pooled samples of leaves and roots of well-watered and drought treated plants according to the method described by Addo-Quaye *et al*. (2008). RNAs were first isolated using the Oligotex Kit (Qiagen, Santa Clarita, CA, USA) then ligated with a 5' RNA adaptor containing a MmeI restriction site using T4 RNA ligase. After reverse transcription of the ligated products, second-strand synthesis and MmeI digestion, ligation of a 3' dsDNA adaptor and gel-purification, the cDNAs were amplified by PCR and sequenced on the Illumina HiSeq platform.

To validate the targets, degradome sequence reads were mapped to the target RNA sequences using Biokanga v3.4.3 (http://sourceforge.net/projects/biokanga/) with default parameters set to obtain all perfect alignments to the cleavage product starting at the canonical (10–11 nucleotide) positions of the miRNAs. We allowed for a single nucleotide variation between varieties and 1 nt length difference between the degradome sequence and the reference mRNA sequence. The miRNAs of interest were also aligned to the reference mRNA sequences using BLAST+ v2.2.28 (Camacho *et al.*, 2009). Integrative Genomics Viewer (IGV) (Robinson *et al.*, 2011) was used to load the alignments for manual inspection.

***In situ* PCR**

Transcript localization of miRNAs and targets was performed through *in situ* PCR in formalin-fixed paraffin-embedded (FFPE) leaf sections. Sample and reagent

preparation were carried out combining the protocols described by Przybecki *et al*., (2006), Bagasra (2007), Møller *et al*., (2009) and Athman *et al*., (2014) with some modifications. Flag leaf samples from drought and well-watered conditions were fixed with fresh FAA fixative. Samples were embedded in paraffin and the Leica RM2265 Rotary Microtome (Leica Microsystems, North Ryde, Australia) was used for sectioning and subsequent treatments on slide. *In situ* reverse-transcription of miRNAs and targets was carried out in the DNase treated leaf sections using SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction except that the miRNA RT preparation and conditions were as described by Varkonyi-Gasic *et al* (2007). PCR was carried out using NEB Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with Standard Taq Buffer according to the manufacturer's instruction for a final volume of 60 µl, containing 4 µM final concentration of Digoxigenin-11-dUTP (Roche Diagnostics, Alameda, CA, USA) as an additional reagent. For the respective negative controls, the same primers and conditions were used using RNA as templates in the PCR reactions. PCR and post-PCR treatment were conducted as described by Athman *et al*. (2014). Following colorimetric detection, the sections were mounted with ImmunoHistoMount™ (Sigma-Aldrich, Castle Hill, NSW, Australia), dried and were visualized in the Zeiss Axio Imager M2 microscope (Carl Zeiss, Oberkochen, Germany) under bright field Illumination. The images were captured using the AxioCam ERc5s camera and retrieved using ZEN 2011 software (Carl Zeiss, Oberkochen, Germany) while the exposure time was adjusted to 100 ±20 ms for each specimen. The details of the *in situ* PCR method are described in the Supporting Information (File S1).

RESULTS

Response of four barley genotypes to drought treatment

Two physiological parameters, leaf water potential (LWP) and leaf relative water content (RWC) were measured under drought treatment and in well-watered conditions. The LWP dropped significantly in all genotypes under drought (Fig. 1a). Hindmarsh had higher RWC compared with the other three genotypes (Fig. 1b).

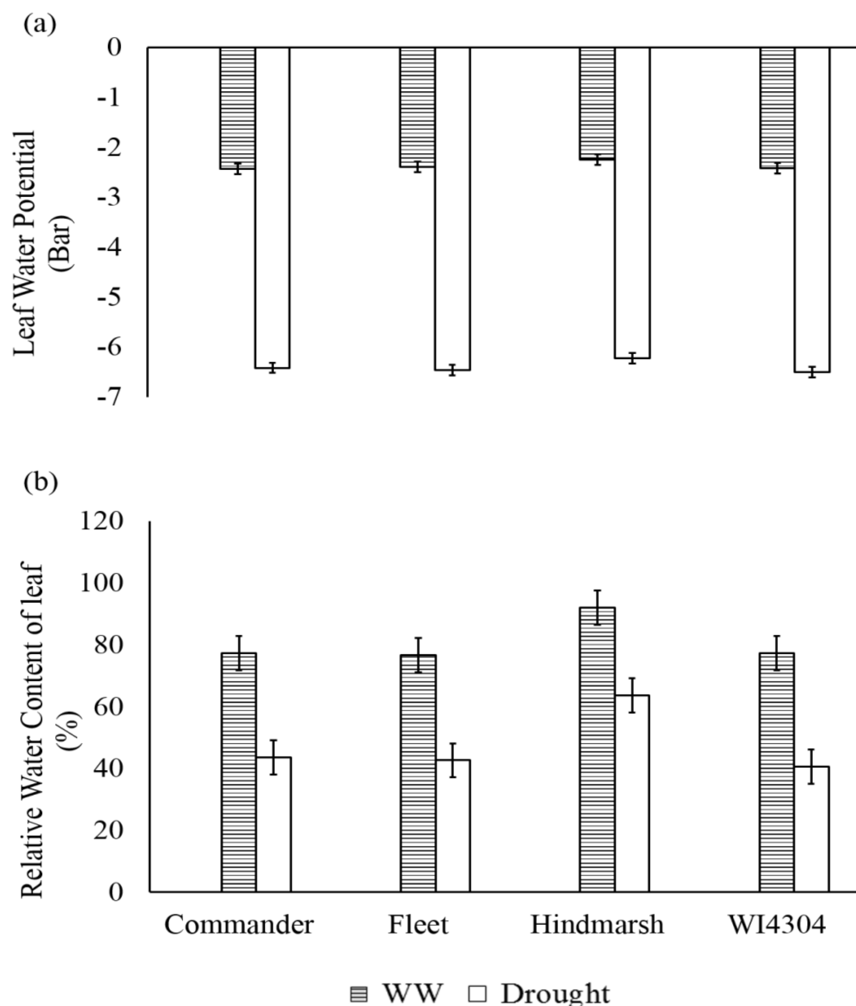

Figure 1. Response of four barley genotypes to drought. (a) Mean leaf water potential and (b) relative water content of fully expanded, mature leaves of four barley genotypes (Commander, Fleet, Hindmarsh and WI4304) at booting stage under drought and in the well-watered control (n=12). The error bars are ±0.5 lsd. The means are significantly different when the error bars do not overlap.

qRT-PCR analysis of candidate drought responsive miRNAs in barley leaves

Quantification of 11 candidate mature miRNAs was performed in five biological replicates from each genotype. Among the 11 miRNAs, four miRNAs (*Ath*-miR169b, *Osa*-miR1432, *Hv*-miRx5 and *Hv*-miR166b/c) showed differential expression under drought. The homologous miRNA *Ath*-miR169b was significantly induced under drought in Hindmarsh and WI4304 (Fig. 2a). The miRNA homologous to *Osa*-miR1432 was significantly down under drought in Commander, Fleet and Hindmarsh (Fig. 2b). Two barley miRNAs, miRx5 (Fig. 2c) and miR166b/c (Fig. 2d) were significantly reduced under drought only in Commander, but did not change between drought-treated and well-watered samples in the other three genotypes. Three miRNAs *Osa*-miR393a (Fig. 2e), *Ata*-miR9863 (Fig. 2f) and *Bdi*-miR396b (Fig. 2g) and two barley miRNAs *Hv*-miR5048 (Fig. 2h) and *Hv*-miR444b (Fig. 2i) did not show significant variation between well-watered and drought treated plants in any of the four genotypes. There were no significant differences in expression detected for miR169n (a rice homologous miRNA) and miR171 (a barley miRNA) with drought treatment, and these two miRNAs had very low abundance in the leaf tissue of the genotypes used in this study (Fig. 2j & k).

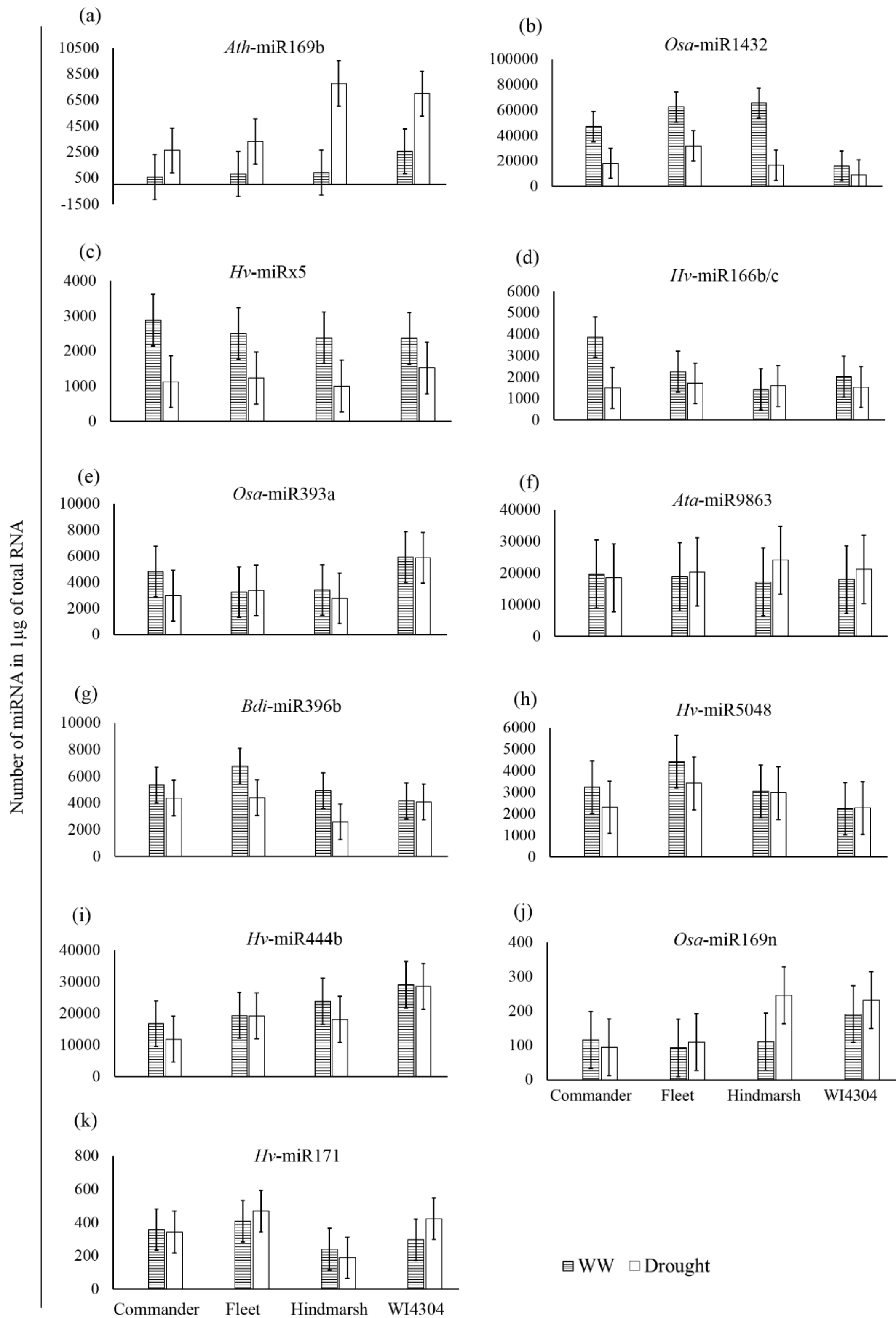

Figure 2. Absolute quantification (qRT-PCR) of the number of mature candidate miRNAs; (a) *Ath*-miR169b, (b) *Osa*-miR1432, (c) *Hv*-miRx5, (d) *Hv*-miR166b/c, (e) *Osa*-miR393a, (f) *Ata*-miR9863, (g) *Bdi*-miR396b, (h) *Hv*-miR5048, (i) *Hv*-miR444b, (j) *Osa*-miR169n, (k) *Hv*-miR171 in 1µg of total RNA in flag leaves of four barley genotypes (Commander, Fleet, Hindmarsh and WI4304) at booting stage under drought and in the well-watered control (n=5). The error bars are ±0.5 lsd. The means between watering conditions are significantly different when the error bars do not overlap.

Drought responsive miRNA targets

We detected the putative targets of the 11 chosen miRNAs using RNA sequence data. We identified ~400 putative target transcripts for 11 miRNAs (Supporting Information Table S1). The putative barley drought-responsive miRNA targets had a diverse range of functions (Supporting Information Table S1) and more than one target was predicted for each miRNA so that further experimental confirmation was required. Among the predicted targets for the 11 miRNAs, we were able to obtain 15 target sequences for nine miRNAs that had cleavage products in the degradome libraries (Table 2).

Table 2. miRNA targets confirmed through degradome analysis

Homologous miRNAs	Targets functions	MLOC numbers	Contig number (from JHI Database Barley WGS Morex Assembly v3)	Genbank accession
Hv-miR166b/c	Homeobox domain (HD)- START domain containing protein	MLOC_58644	contig_42852	AK359910.1
	HD-START domain, MEKHLA domain containing protein	MLOC_79063	contig_8318	AK365312.1
	HD-START domain, MEKHLA domain containing protein	MLOC_33978	contig_241849	AK364215.1
	HD-START domain, MEKHLA domain containing protein	MLOC_61603	contig_45665	AK362009.1
Ath-miR169b	CCAAT-binding transcription factor (CBF-B/NF-YA) subunit	MLOC_36554	contig_2546965	AK368372.1
Osa-miR169n	CCAAT-binding transcription factor (CBF-B/NF-YA) subunit	MLOC_36554	contig_2546965	AK368372.1
Osa-miR393a	Leucine-rich repeat (LRR) domain containing F-box protein	MLOC_9864	contig_1557974	AK355927.1
	Leucine-rich repeat (LRR) domain containing F-box protein	MLOC_56088	contig_40541	Not available
Bdi-miR396b	WRC and QLQ domain: DNA binding and involved in mediating protein interactions respectively	MLOC_67201	contig_52709	AK376067.1
	WRC domain: DNA binding	MLOC_66132	contig_51136	AK376404.1
Hv-miR444b	MADS-box family gene with MIKCc type-box, expressed	MLOC_61033	contig_45023	AK358388.1
Osa-miR1432	Calmodulin-related calcium sensor protein (contains EF hand domain)	MLOC_70272	contig_57713	Not available
Hv-miR5048a	Serine/threonine-protein kinase receptor precursor	MLOC_70446	contig_57988	DQ469714.1
Ata-miR9863	NB-ARC domain	MLOC_24045	contig_163538	AK372887.1
	NB-ARC domain	MLOC_21626	contig_1596863	AF427791.1

Degradome analysis demonstrated alignment positions consistent with miRNA directed cleavage of mRNA targets; that is, where the start of one or more degradome reads coincided preferentially with the canonical 5' 10-11 nt cleavage site for the mature miRNA sequence (Fig. 3a-b; Supporting Information Fig. S6 a-m). An NFY-A encoding gene was cleaved by both *Ath*-miR169b and *Osa*-miR169n, and the cleavage products were obtained in libraries from both barley cvs. Golden Promise and Pallas. Interestingly, we observed a mismatch between the miRNA and the target at the seed region (the 5′ 2-8 nt of a miRNA) which was found in the genotype Pallas (Fig. 3a and Supporting Information Fig. S6 e). An mRNA encoding a Calmodulin-related (EF hand containing) calcium sensor protein appeared to be targeted by *Osa*-miR1432 in Golden Promise (Fig. 3b). The cleavage products of mRNAs from four genes encoding homeobox START and MEKHLA domain containing proteins were the apparent targets of *Hv*-miR166b/c in Golden Promise. mRNAs from two genes encoding LRR domain containing F box proteins showed *Osa*-miR393a mediated cleavage products in both Golden Promise and Pallas. Cleavage products of mRNA targets for WRC and QLQ domain proteins were found for *Bdi*-miR 396b in Golden Promise and Pallas. An mRNA from a MADS box family gene was found to be targeted by *Hv*-miR444b both in Golden Promise and Pallas. However, the degradome product did not match the canonical site and was at the 5' 19-20 nt position opposite to the miRNA suggesting that the miRNA-mediated cleavage sites may not have been limited to 10-11 nt position. *Hv*-miR5048a was found to cleave the transcript encoding a serine/threonine-protein kinase receptor in Golden Promise. mRNAs from two genes encoding NB-ARC domain containing protein were cleaved by *Ata*-miR9863 in both libraries. We did not identify any cleavage products aligned with either degradome library for the candidate targets of miR171. For miRx5 target validation, we obtained cleavage products aligned with miRx5's predicted target *RGH1A*, starting at the 5' 5-6 nt position of the miRNA/target

duplex, opposite to the miRNA in Golden Promise (Supporting Information Fig. S6 n). This region was clearly the seed region of a miRNA. It was unlikely that target cleavage occurred on the opposite strand to the seed region of this miRNA. Moreover, the G:U wobble pairing at the 11 nt position between the miRx5 and its target might have obstructed target mRNA cleavage, thus we cannot rule out that this target might be incorrect and regulation may be via a non-cleavage mechanism, and/or this miRNA might have other as yet unknown targets.

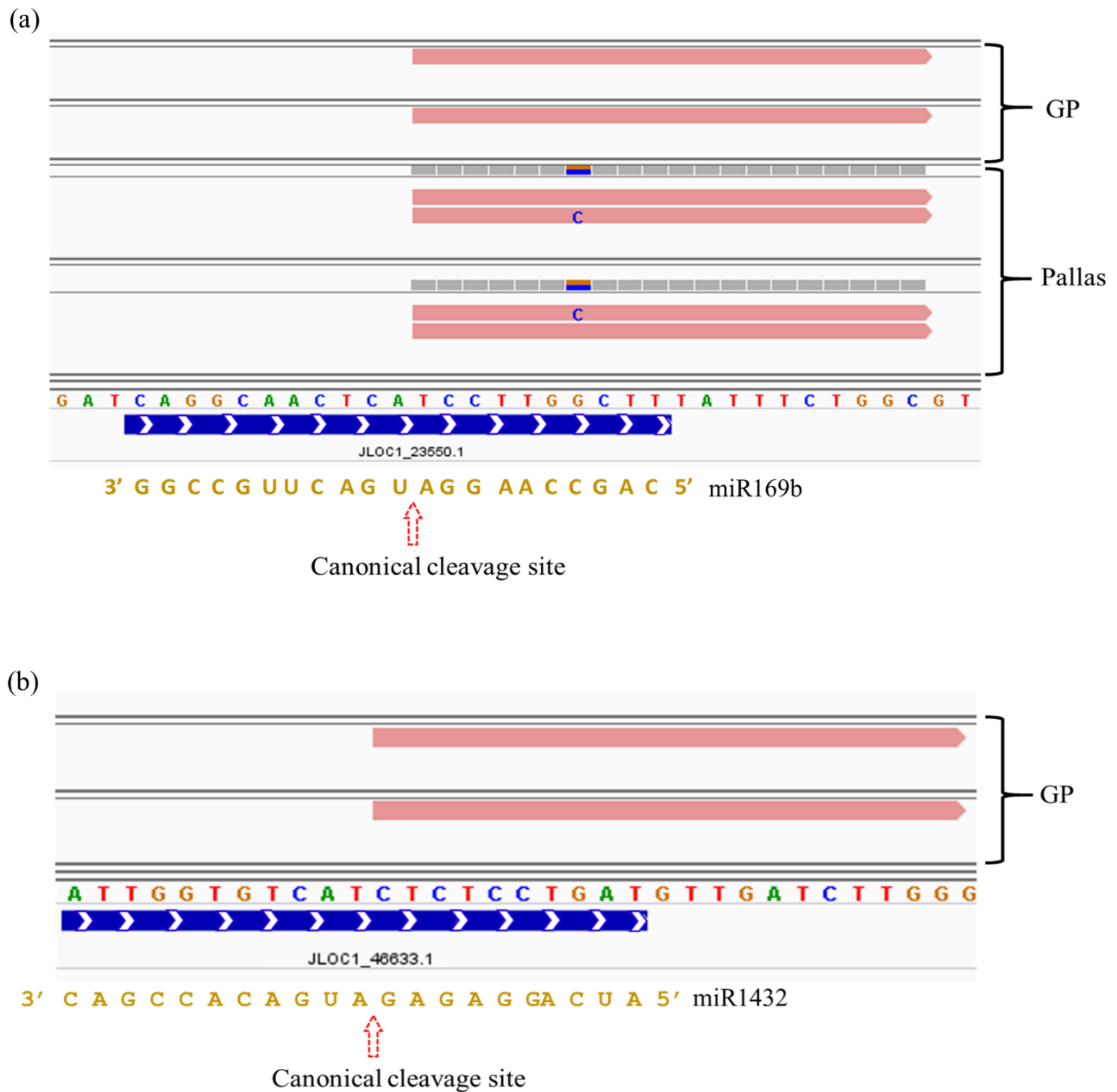

Figure 3. Validation of target transcripts encoding NFY-A (JLOC1_23550/ MLOC_36554) and Calmodulin-related (EF-hand containing) calcium sensor protein (JLOC1_46633/ MLOC_70272) through degradome analysis. Degradome sequences from two barley genotypes; Golden Promise (GP) and Pallas were aligned with the candidate target transcripts. (a) NFY-A target cleavage products were obtained for both genotypes; GP and Pallas. (b) EF-hand containing target cleavage products were obtained in one genotype, GP. Positions of cleavage products are shown in the horizontal red bars. Blue bars indicate the miRNA binding site in the target transcript in 5' -3' direction. The canonical cleavage site of the target (opposite to the miRNA) is indicated between the 5' 10 and 11 nucleotide position of the miRNA. Grey bars represent the number of nucleotides matched in the alignment between the cleavage product sequence and the reference transcript; observed mismatch is marked for the base ‘c’ in the red bar of (a).

Expression of Potential Target Genes

The expression of miRNAs' targets was quantified by qRT-PCR with the expectation that the target's expression would be inversely correlated with expression of the miRNA. We found two miRNAs, miR169b and miR1432, that were differentially expressed under drought in at least two genotypes; hence we selected *NFY-A* and an EF-hand encoding transcript, the targets of miR169b and miR1432 respectively, for expression analysis. Expression of *NFY-A* was downregulated in all four genotypes under drought (Fig. 4a), although miR169b was only significantly induced in two genotypes, Hindmarsh and WI4304 (Fig. 2a). The mRNA transcript encoding a Calmodulin-related (EF hand containing) calcium sensor protein showed an inverse correlation with miR1432 in the cv. Hindmarsh. However, we did not observe the inverse correlation of miR1432 and this target transcript in the other three genotypes (Fig. 4b), although we identified the ideal alignment of the canonical cleavage products with this transcript in one of our degradome libraries (Fig 3b).

This observation led us to investigate the sequence of miR1432 binding site within the target transcript in the four genotypes, which revealed that in Commander, Fleet, and WI4304, there was a G:U pair at position 12 nt, relative to the 5'-end of the miRNA of the miRNA: target duplex. However, in the genotype Hindmarsh, the sequence of miR1432 binding site within the target transcript had perfect complementarity with miR1432 and expression of the gene and miRNA were inversely correlated (Supporting Information Fig. S7).

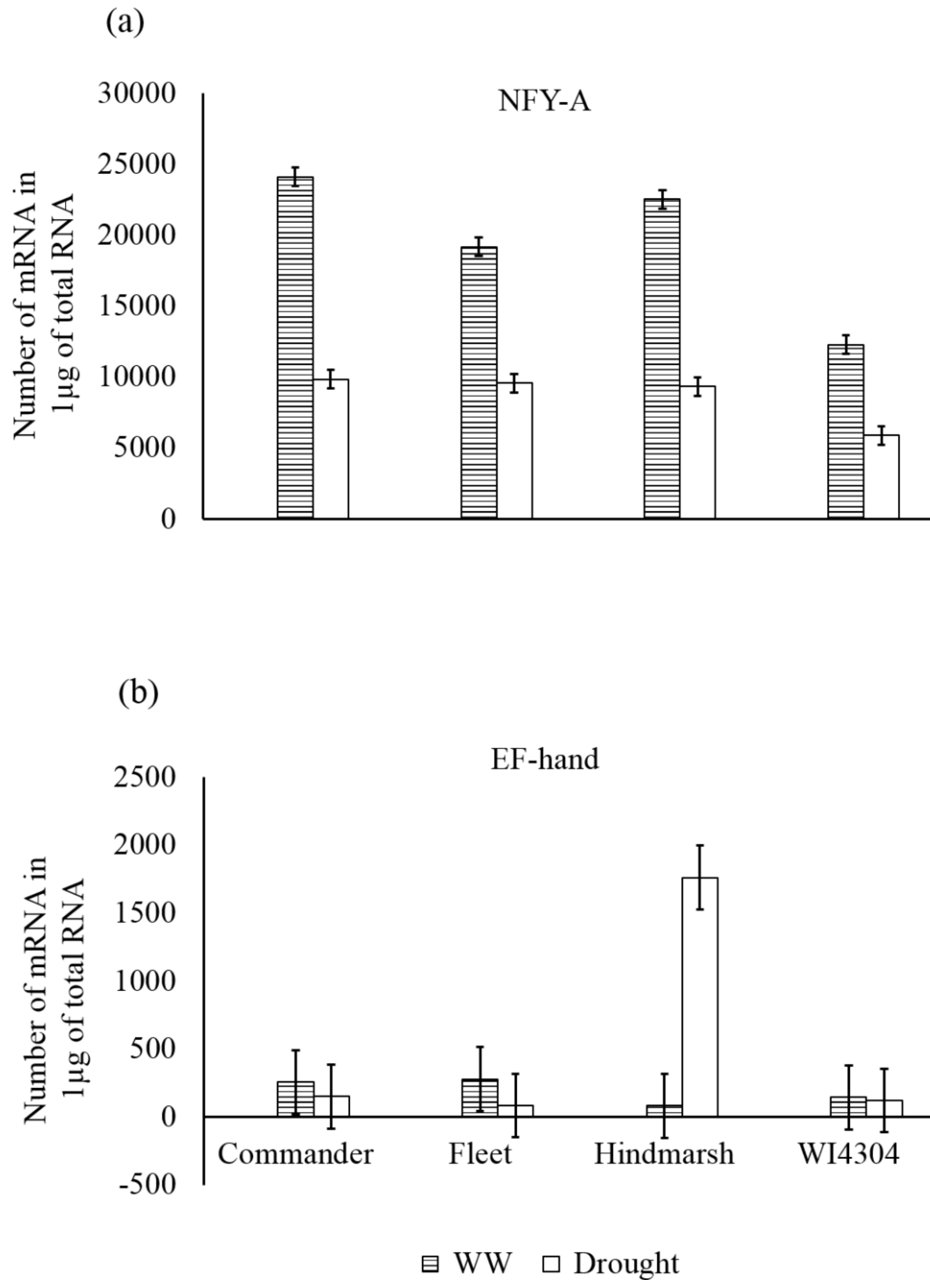

Figure 4. Absolute quantification (qRT-PCR) of the number of transcripts of putative target mRNAs. (a) NFY-A (target of miR169b) and (b) Calmodulin-related (EF hand containing) calcium sensor protein (target of miR1432) in 1µg of total RNA in flag leaves of four barley genotypes (Commander, Fleet, Hindmarsh and WI4304) at booting stage under drought and in the well-watered control (n=5). The error bars are ±0.5 lsd. The means between watering conditions are significantly different when the error bars do not overlap.

Spatial accumulation of mature miRNAs and their targets by *in situ* –RT-PCR (ISRT-PCR)

The spatial distribution of miRNAs and target co-localization were assessed by *in situ* labelling and microscopy. We selected miR169b and miR1432 and their targets for this study since these two miRNAs were differentially expressed under drought in at least two barley genotypes. Drought-treated and well-watered Hindmarsh flag leaf samples from booting stage were used for ISRT-PCR. As expected, higher accumulation of miR169b was observed in the drought-stressed leaf compared to the well-watered leaf (Fig. 5). Inversely, *NFY-A* showed lower accumulation in the drought treated compared to the well-watered sample (Fig. 5). This inverse correlation of miR169b and *NFY-A* was observed in the mesophyll tissues (Fig. 5).

Weak accumulation of miR1432 was observed under drought compared to the well-watered sample (Fig. 6); while the target of miR1432, the EF-hand encoding transcript, showed higher accumulation in the drought treated compared with the well-watered sample (Fig. 6). miR1432 and its target were also located in mesophyll tissues (Fig. 6). The *in situ* expression results for miR169b and miR1432 and the respective targets were consistent with our qRT-PCR results (Fig. 2 & 4).

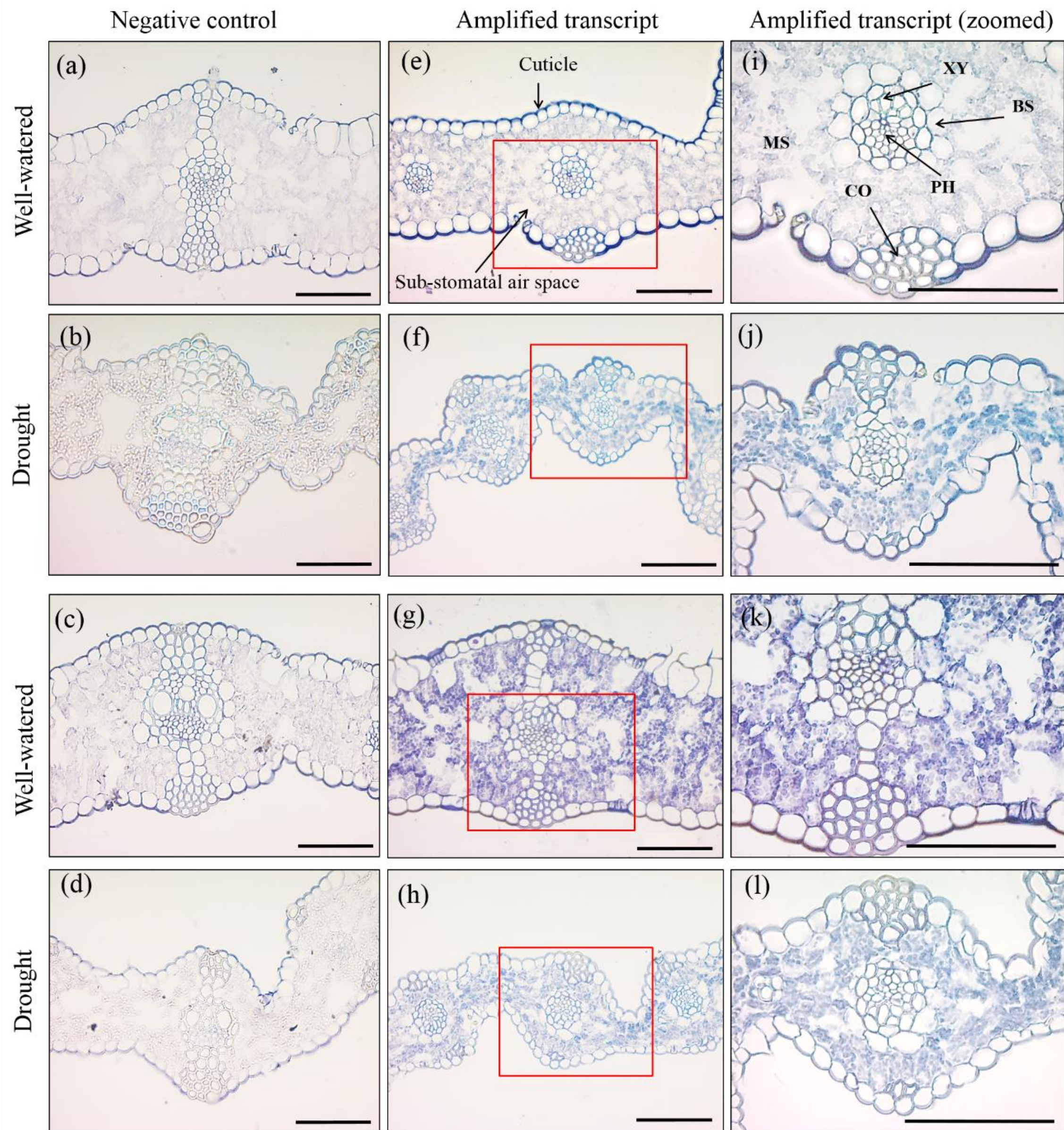

Figure 5. Light micrographs of the expression of *Ath*-miR169b and the target transcript encoding NFY-A by *in situ* PCR in Hindmarsh flag leaf sections from booting stage. Negative controls of miR169b are shown in (a) well-watered and (b) drought treated samples. Negative controls of NFY-A are shown in (c) well-watered and (d) drought treated samples. miR169b expression is shown in (e) well-watered and (f) drought treated samples. NFY-A expression is shown in (g) well-watered and (h) drought treated samples. Magnified view of the red boxed area of respective middle panel is shown the right panel (i-l). The blue stain indicates the presence of transcripts. Scale bar is 100μm. PH, phloem; XY, xylem; BS, bundle sheath; CO, collenchyma, MS, mesophyll cells.

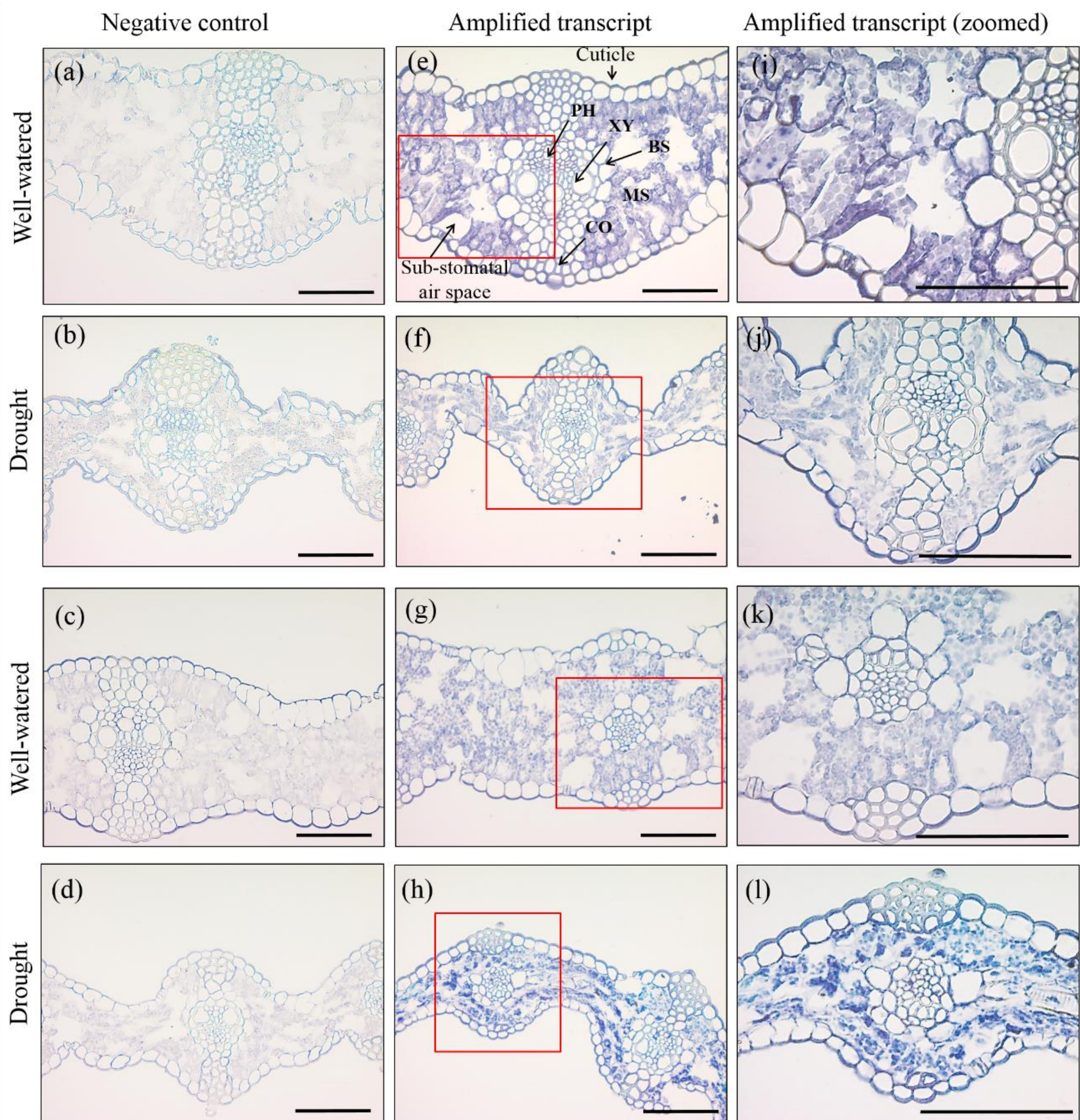

Figure 6. Light micrographs of the expression of *Osa*-miR1432 and the target transcript encoding Calmodulin-related (EF hand containing) calcium sensor protein by *in situ* PCR in Hindmarsh flag leaf sections from booting stage. Negative controls of miR1432 are shown in (a) well-watered and (b) drought treated samples. Negative controls of the target transcript are shown in (c) well-watered and (d) drought treated samples. miR1432 expression is shown in (e) well-watered and (f) drought treated samples. The target transcript expression is shown in (g) well-watered and (h) drought treated samples. Magnified view of the red boxed area of respective middle panel is shown the right panel (i-l). The blue stain indicates the presence of transcripts. Scale bar is 100µm. PH, phloem; XY, xylem; BS, bundle sheath; CO, collenchyma, MS, mesophyll cells.

DISCUSSION

Although miRNAs have diverse functions, and the regulatory roles of miRNAs are still not well understood, miRNA-based gene regulation has been implicated in several physiological pathways including drought stress response. In this study, the expression of eleven miRNAs in drought-stressed leaves was evaluated in four barley genotypes, Commander, Fleet, Hindmarsh and WI4304. Hindmarsh had a higher leaf water content under drought (and also in well-watered conditions) as compared to the other three genotypes (Fig. 1). Though all four genotypes are known to perform well in dry areas in Australia, these differences of water status among the genotypes may indicate greater water loss or reduced water uptake and higher stress levels during experimental drought treatment in the leaves of Commander, Fleet and WI4304 than in Hindmarsh.

Some of the miRNAs examined in our study showed different expression patterns compared to recent studies conducted in barley (Kantar *et al*., 2010; Hackenberg *et al.*, 2015). *Hv*-miR166 was more abundant under drought in barley leaves of Bűlbűl-89 (Kantar *et al*., 2010), Golden Promise and WI4330 (Hackenberg *et al*., 2015). In contrast, we found this miRNA was down under drought in one genotype, Commander, and was unchanged in the other three. Kantar *et al*. (2010) also found *Hv*-miR171 was upregulated in Bűlbűl-89 under drought stress, whereas it was not differentially abundant with drought in any of the genotypes we assayed. Hackenberg *et al*. (2015) found that homologous *Osa*-miR393a was down-regulated by drought in Golden Promise. However, we observed no significant differences in expression of this miRNA between the two watering conditions in any of our experimental genotypes (Fig. 2).

Differential expression of miRNAs has been observed between sensitive and tolerant genotypes of cowpea (Barrera-Figueroa *et al*., 2011), rice (Cheah *et al*., 2015)

and durum wheat (Liu *et al*., 2015) under water deficit stress where, similarly, a small number of different genotypes was studied. The distinct behaviour of miRNAs reflected the variation in response to water limitation in these species; however we observed genotype-specific miRNA responses to water limitation in four barley genotypes with similar drought tolerance in terms of yield performance in field trials. A miRNA and its target mRNA need to be co-expressed in order for the miRNA to suppress the expression of its biological target (Kuhn *et al*., 2008). The target mRNA encoding a calmodulin related calcium sensor protein, here, showed an inverse expression pattern compared to the miR1432 under drought in only one barley genotype, Hindmarsh. We observed perfect complementarity between the miRNA: target duplex in the genotype Hindmarsh (Supporting Information Fig. S7), while there was a polymorphism in the DNA sequence of the other three genotypes which could explain the lack of complementarity and correlated expression of the miRNA: mRNA in these cultivars. This finding confirmed that miRNA-mediated target regulation varied between genotypes and suggested that this depended on miRNA: mRNA sequence-specific binding. Although some, single miRNA mediated mRNA cleavage products were aligned ideally with separate target sequences in degradome libraries from two different genotypes, Golden Promise and Pallas (Table 2), for others (for *Osa*-miR1432, *Hv*-miR166b/c and *Hv*-miR5048a) the cleavage products were only obtained in Golden Promise, but not in Pallas, again suggesting genotype-specific target binding was important for miRNA-mediated regulation.

Nonetheless, we observed that the same transcript encoding NFY-A could be the target of both *Ath*-miR169b and *Osa*-miR169n in barley, although these two miRNAs differ at four nucleotides. Our result indicated that this target could still be cleaved by these miRNAs despite seed region mismatches (Fig. 3a and Supporting Information Fig. S6e). It has been suggested that there is the possibility of mismatch between the miRNA

and the target in the seed region and, although seed region mismatches are uncommon, imperfect seed region pairing can be overcome by 3′ compensatory pairing (Witkos *et al.*, 2011). The compensatory pairing in the 3′ of miRNA could reduce the seed pairing requirement to as little as four base pairs (Brennecke *et al.*, 2005; Künne *et al.*, 2014).

We found inversely correlated expression between *Ath*-miR169b and *NFY-A* (Fig. 2a & 4a) which was consistent with previous studies (Zhao *et al.* 2011; Li *et al.*, 2008). We observed up-regulation of *Ath*-miR169b in Hindmarsh and WI4304 under drought (Fig. 2a) and *NFY-A* was down-regulated in all four barley genotypes under drought (Fig. 4a) so that the miRNA seed region match appeared to be less important than the genotypic target sequence for miRNA-mediated cleavage, at least at this target. Nuclear factor Y (NFY), a CCAAT box-binding transcription factor, is composed of three subunits: NFY-A, NFY-B, and NFY-C (Baxevanis *et al.*, 1995). NFY family members are reported to play roles in the molecular control of flowering, seed development, photosynthesis and improved tolerance to abiotic stresses such as drought (Qu *et al.*, 2015; references therein). Although *Ath*-miR169b and *NFY-A* expression were inversely correlated in two genotypes under drought, there was no correlation with the abundance of *Osa*-miR169n that could also cleave NFY-A transcripts and no significant up-regulation of *Ath*-miR169b under drought in two of four genotypes. This suggested that *NFY-A* down-regulation under drought was mediated by multiple mechanisms including miR169b mediation. In Hindmarsh under drought conditions, both the up-regulated (miR169b) and down regulated (miR1432) mature miRNAs were observed in the mesophyll cells (Figs. 5&6). As expected the NFY-A and the calmodulin related calcium sensor transcripts were also found preferentially in the same cellular compartment in leaf tissue showing inverse expression compared with their regulatory miRNAs under drought (Figs. 5&6). Information on the tissue localization of barley drought responsive miRNAs has not been reported previously. The differential

expression of these miRNAs in the mesophyll cells under drought could provide useful information for further elucidating the role of these miRNAs under stress.

miRNAs that are down-regulated under drought are expected to have targets that are positive regulators of stress responses (Wei *et al*., 2009). In our study, miR1432 was significantly down under drought in three genotypes (Fig. 2b). miR1432 was previously predicted to target the mRNAs of genes encoding EF-hand proteins in rice (Sunkar, 2008). In our degradome library, we found miR1432 mediated cleavage products ideally aligned with the target transcript encoding a calmodulin related calcium sensor protein which has EF-hand domains, and the target cleavage occurring in the canonical cleavage site (Fig. 3b). EF-hand domains contain a helix-loop-helix structural motif reported to bind with calcium (Ca^{2+}) (Cheng *et al.*, 2002). It appears that miR1432 targets EF-hand domain containing transcripts and thus indirectly contributes to calcium signalling, a vital signalling mechanism involved in various physiological processes in plants (Ni *et al.* 2009). In our study, down-regulation of miR1432 under drought appeared to mediate increased expression of its target transcript localized in mesophyll cells (Fig. 6). However, we observed up-regulation of this target transcript under drought only in Hindmarsh and not in the other three genotypes (Fig 4b) underlining the importance of functional experimentation, rather than correlative studies, with both miRNAs and their targets for understanding their roles in regulation.

Despite the majority of cleavage products here being ideally derived from canonical cleavage, for the miR444b target, a MADS-box family gene conserved in monocots (Sunkar *et al.*, 2005), we observed that target cleavage might occur between the 19th and 20th nt position (opposite to the miRNA) in both Golden Promise and Pallas (Supporting Information Fig. S6j). This suggested that miRNA-mediated cleavage sites might not be limited to the 10–11 nt position. The possibility of non-canonical cleavage was previously suggested by Hackenberg *et al.* (2015). However we were unable to find

any other studies that showed target cleavage at the 19th and 20th nt position. MADS-box family mRNAs were reported to be targeted by miR444b in rice where the cleavage products were obtained from the canonical cleavage site (Sunkar *et al.*, 2005). Our results demonstrated that, although numerous targets were predicted for the examined drought responsive miRNAs, only a few were regulated by miRNA mediated cleavage and these were genotype-specific. Target recognition also appeared to be genotype-specific and miRNA mediated cleavage sites varied within the miRNA: target binding region in barley.

In this study, we examined the expression of eleven miRNAs, nine of which were previously reported to be drought responsive, in four barley genotypes adapted to low-rainfall environments. Only four of 11 studied miRNAs had significant expression differences under drought in barley, and of these only two miRNAs, miR169b and miR1432, had a consistent expression pattern in more than one genotype. Bioinformatic analysis predicted numerous targets of the candidate miRNAs. Although bioinformatic analysis has been widely used for the discovery of miRNAs and prediction of targets in recent years, there is little information available on the expression of mature candidate miRNAs and biological validation of their targets. The present study provides an important glimpse into miRNA expression, target prediction and validation and the difficulty of relying on bioinformatic predictions. Localisation of miRNAs and their targets may help improve our understanding of the involvement of miRNAs in plant drought stress responses. Though drought tolerance is a complex trait, miRNA mediated differential expression of target genes that are positive regulators of stress responses could help us to identify potential biomarkers in different genotypes under drought that would be of interest for research. Additional evidence will be required to confirm miRNA-based regulation of important genes in drought stress-responsive networks and

the genotype specificity of the regulation. However, we have identified useful targets for the additional studies and evidence for genotypic variation.

ACKNOWLEDGEMENTS

Our grateful thanks to Dr. Bu-Jun Shi for initiating the project; Dr. Gwenda M Mayo and Dr. Takashi Okada for supporting tissue fixation, embedding and sectioning; Asmini Athman for training for the *in situ* PCR; Yuan Li and Hui Zhou for performing the qRT-PCR; Janine Jones for some statistical analysis; and Dr. Runxuan Zhang for early discussion about miRNA target prediction.

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SUPPORTING INFORMATION

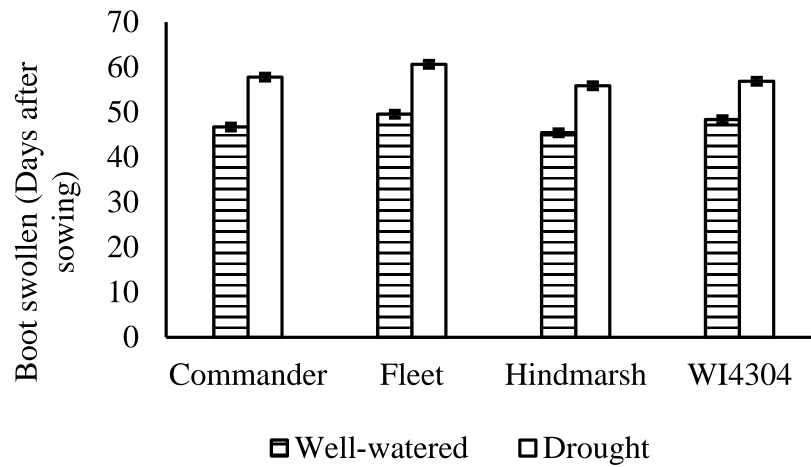


Figure S1. Time to boot swollen (days from sowing) of four barley genotypes (Commander, Fleet, Hindmarsh and WI4304) under the experimental conditions. The error bars are ±0.5 lsd. The means (n=12) are significantly different when the error bars do not overlap.

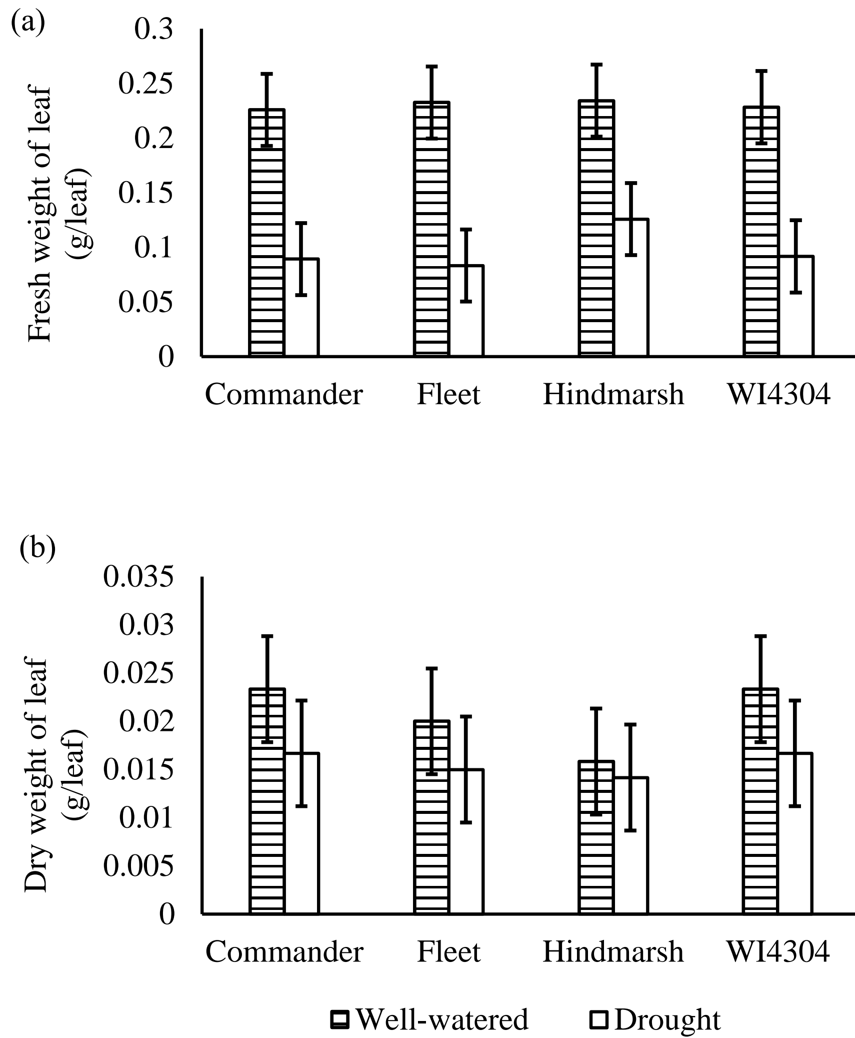


Figure S2. Leaf biomass of four barley genotypes (Commander, Fleet, Hindmarsh and WI4304) under the experimental conditions. (a) Fresh weight and (b) Dry weight of fully expanded, mature leaves at booting stage under drought and in the well-watered control. The error bars are ±0.5 lsd. The means (n=12) are significantly different when the error bars do not overlap.

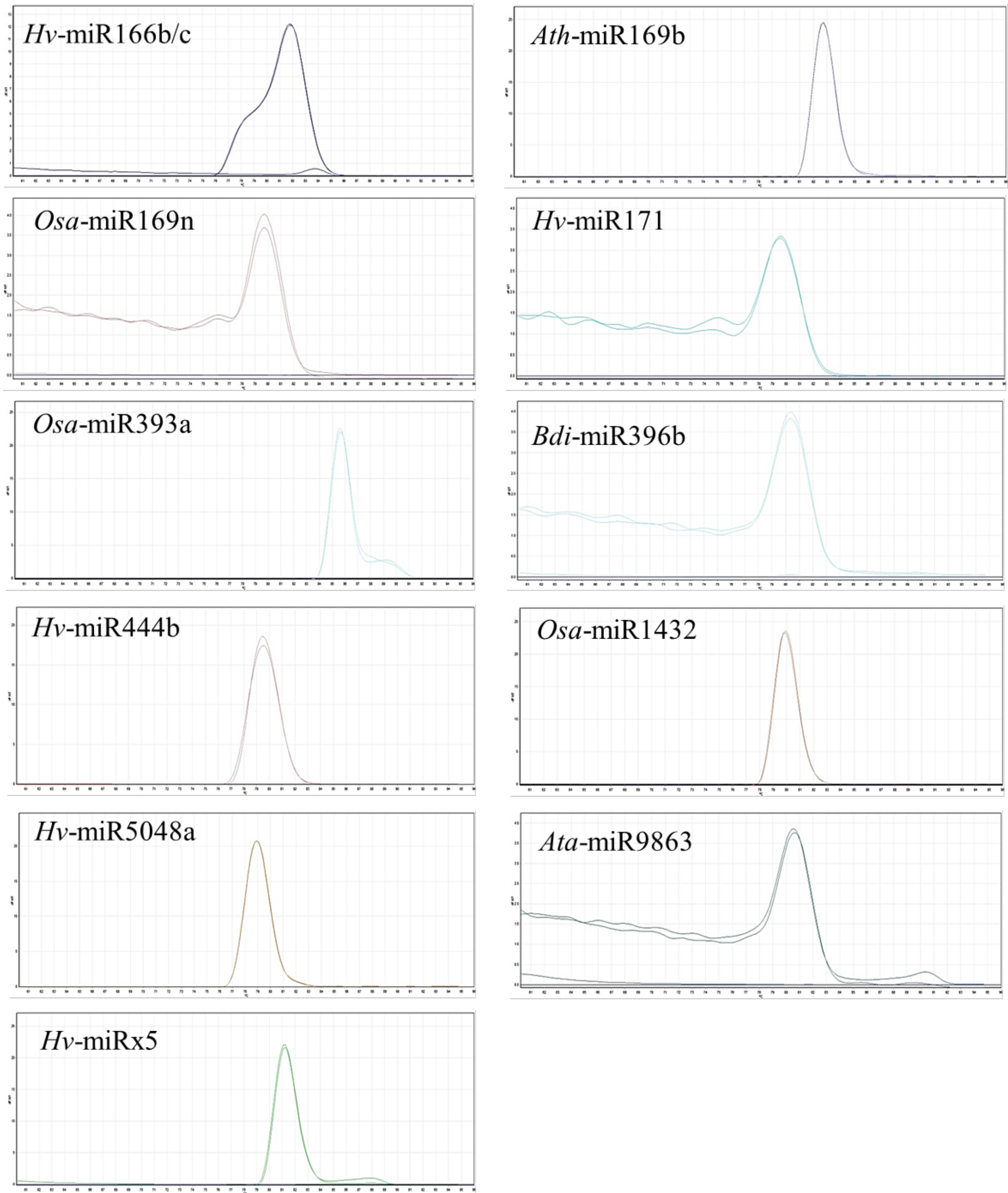
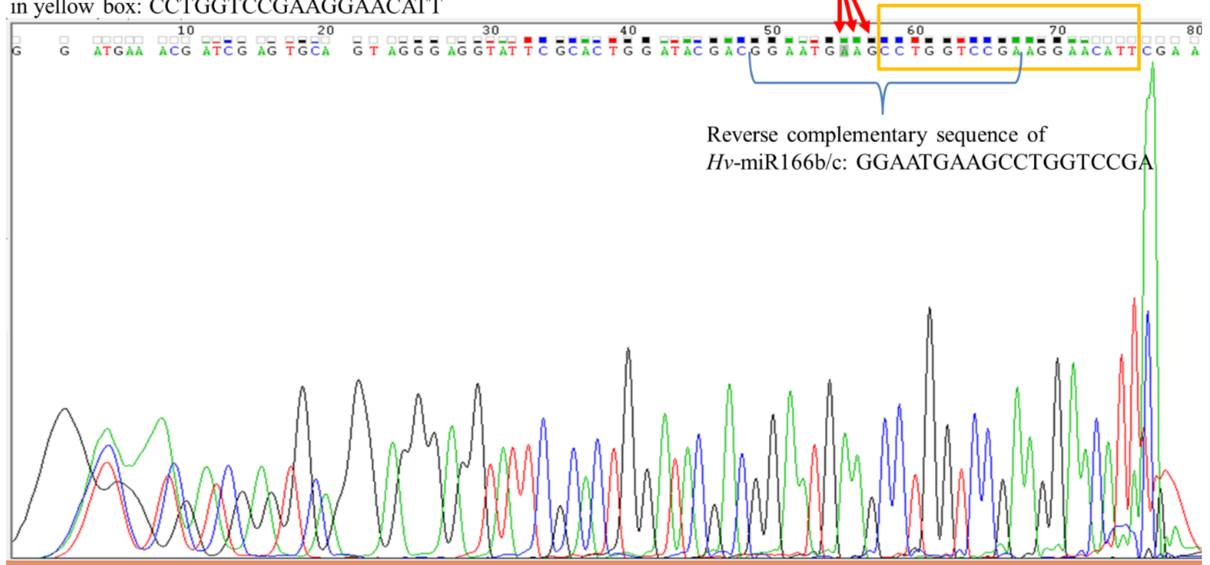


Figure S3. Melt curve analysis of the mature miRNAs for qRT-PCR.

(a) *Hv*-miR166b/c

Signature nucleotides verified in the sequencing to detect the miRNA

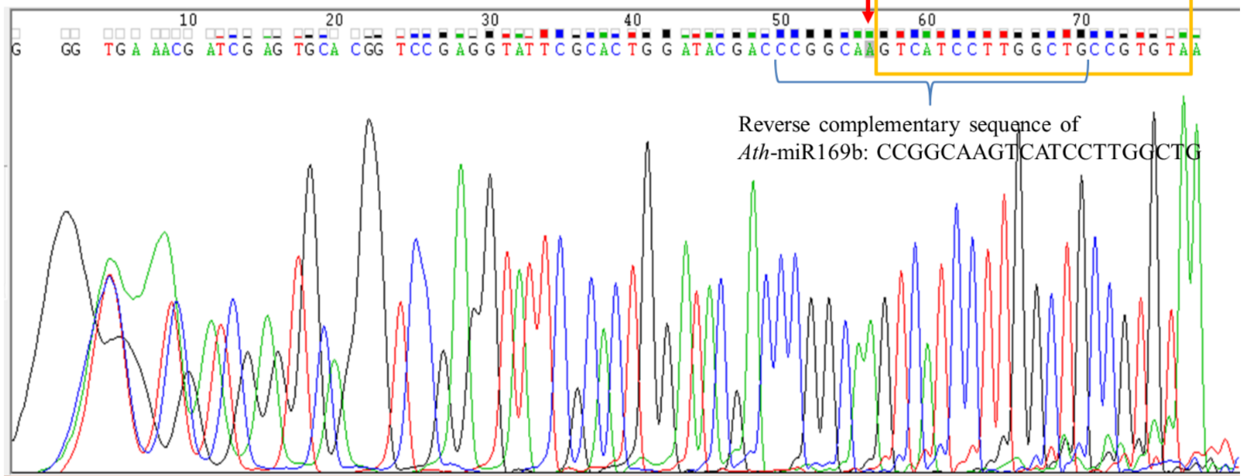
Reverse complementary of the forward primer of *Hv*-miR166b/c in yellow box: CCTGGTCCGAAGGAACATT



(b) *Ath*-miR169b

Signature nucleotide verified in the sequencing to detect the miRNA

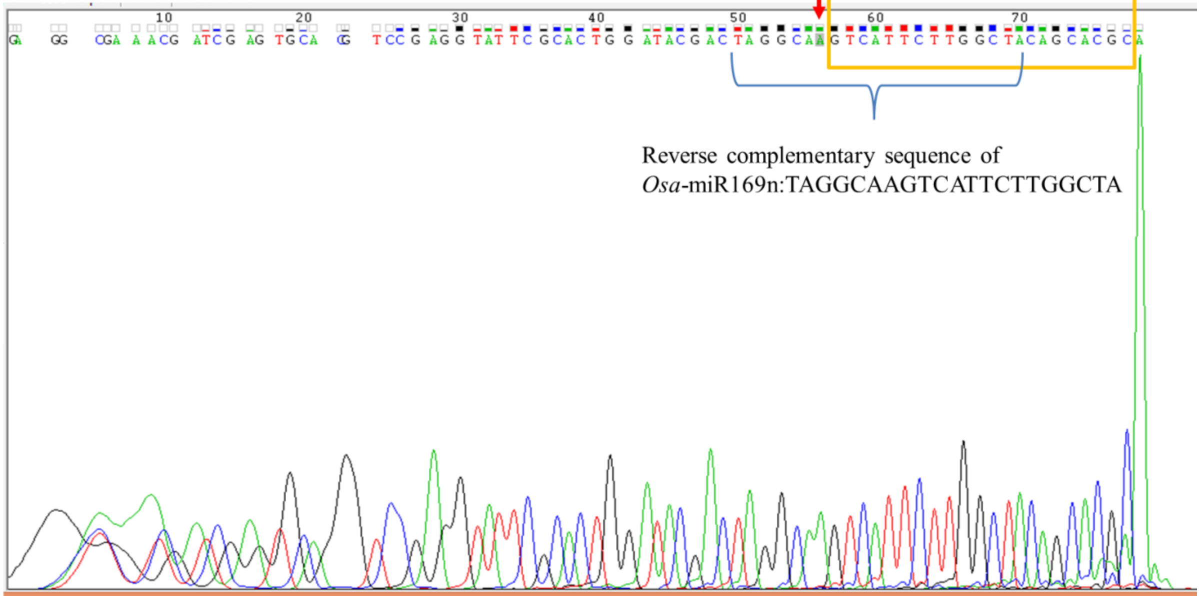
Reverse complementary of the forward primer of *Ath*-miR169b in the yellow box: GTCATCCTTGGCTGCCGTGTA



(c) *Osa*-miR169n

Signature nucleotide verified in the sequencing to detect the miRNA

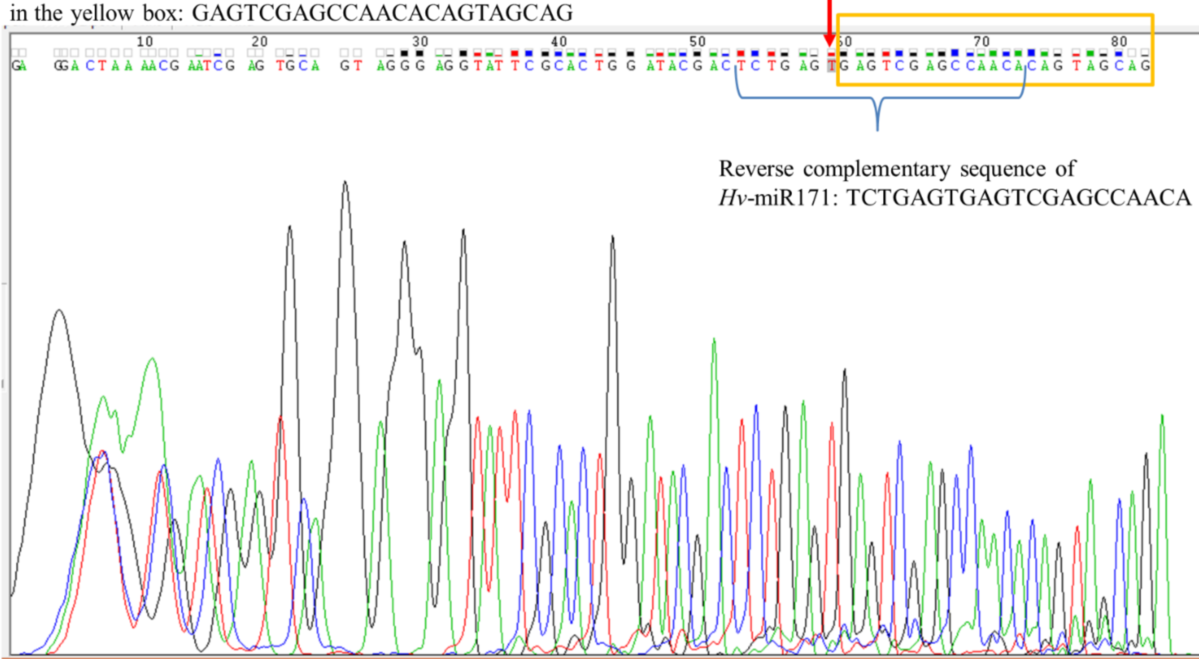
Reverse complementary of the forward primer of *Osa*-miR169n in the yellow box: GTCATTCTTGGCTACAGCACGC



(d) *Hv*-miR171

Signature nucleotide verified in the sequencing to detect the miRNA

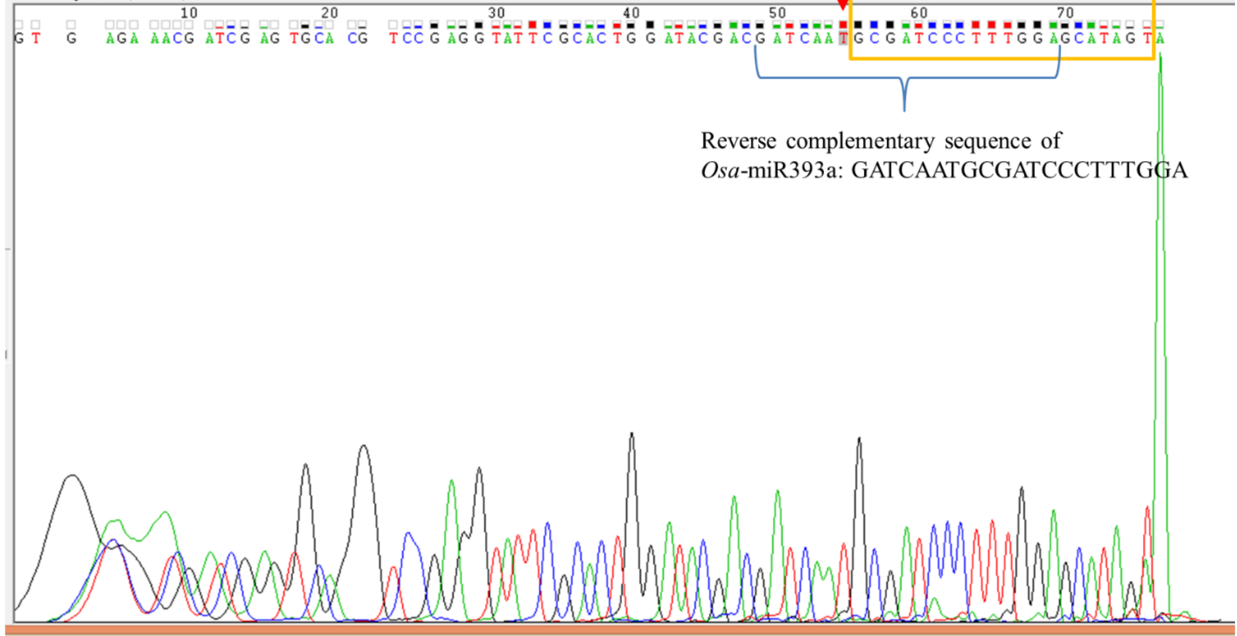
Reverse complementary sequence of the forward primer of *Hv*-miR171 in the yellow box: GAGTCGAGCCAACACAGTAGCAG



(e) *Osa*-miR393a

Signature nucleotide verified in the sequencing to detect the miRNA

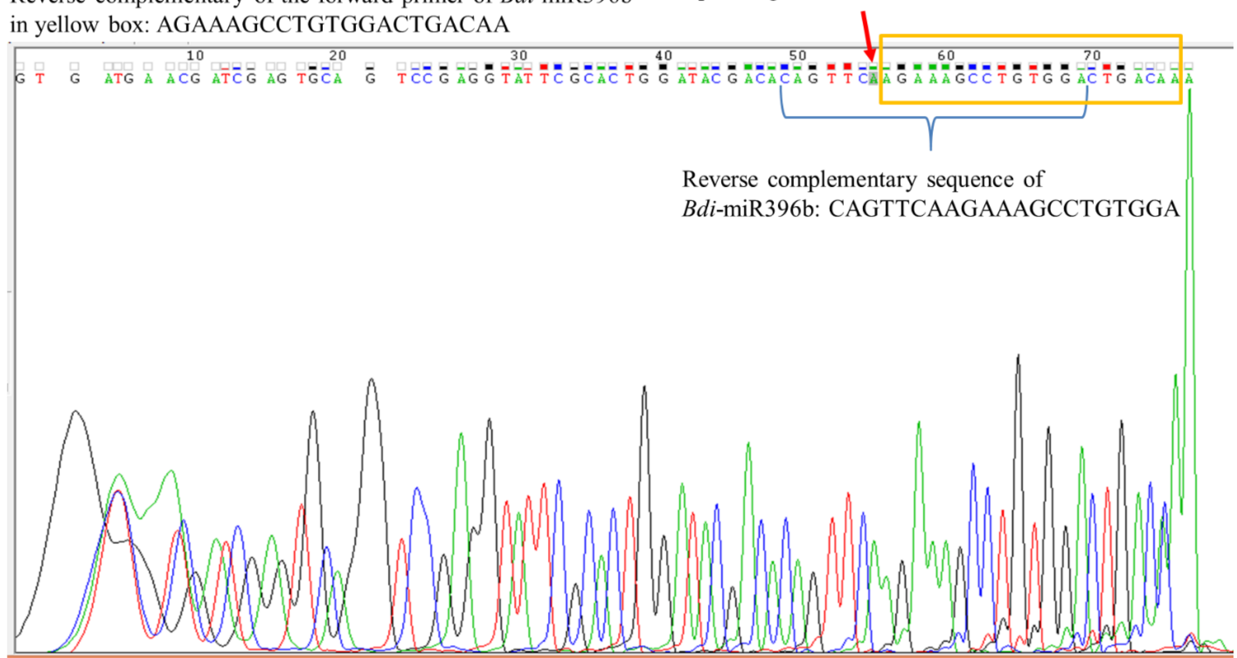
Reverse complementary of the forward primer of *Osa*-miR393a in the yellow box: GCGATCCCTTTGGAGCATAGT



(f) *Bdi*-miR396b

Signature nucleotides verified in the sequencing to detect the miRNA

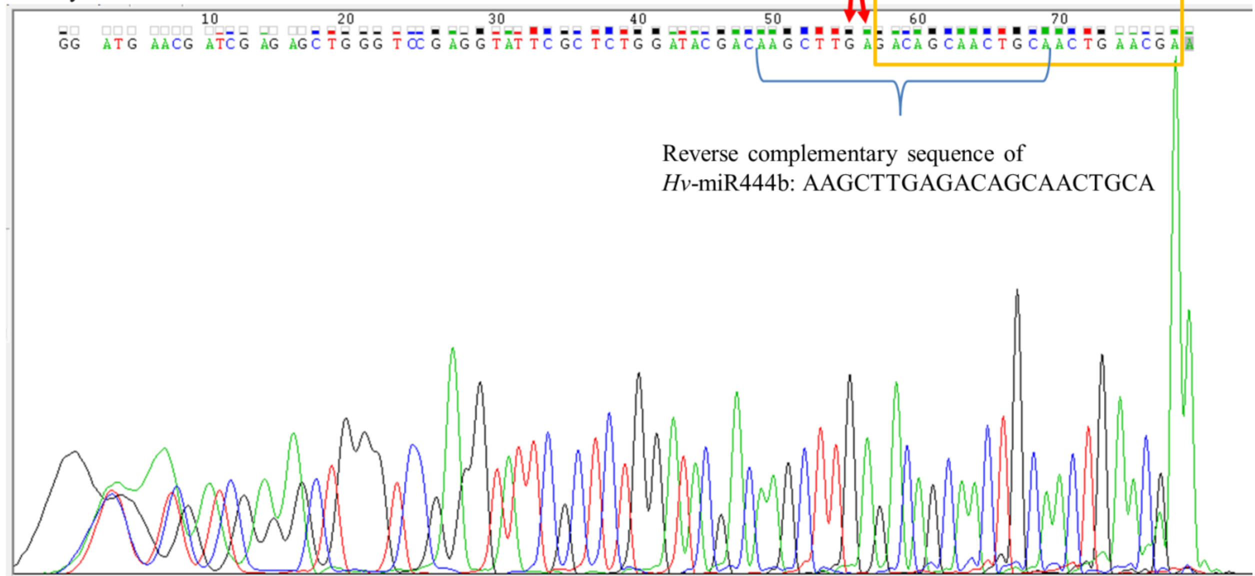
Reverse complementary of the forward primer of *Bdi*-miR396b in yellow box: AGAAAGCCTGTGGACTGACAA



(g) *Hv*-miR444b

Signature nucleotides verified in the sequencing to detect the miRNA

Reverse complementary of the forward primer of *Hv*-miR444b in the yellow box: GACAGCAACTGCAACTGAACGA



(h) *Osa*-miR1432

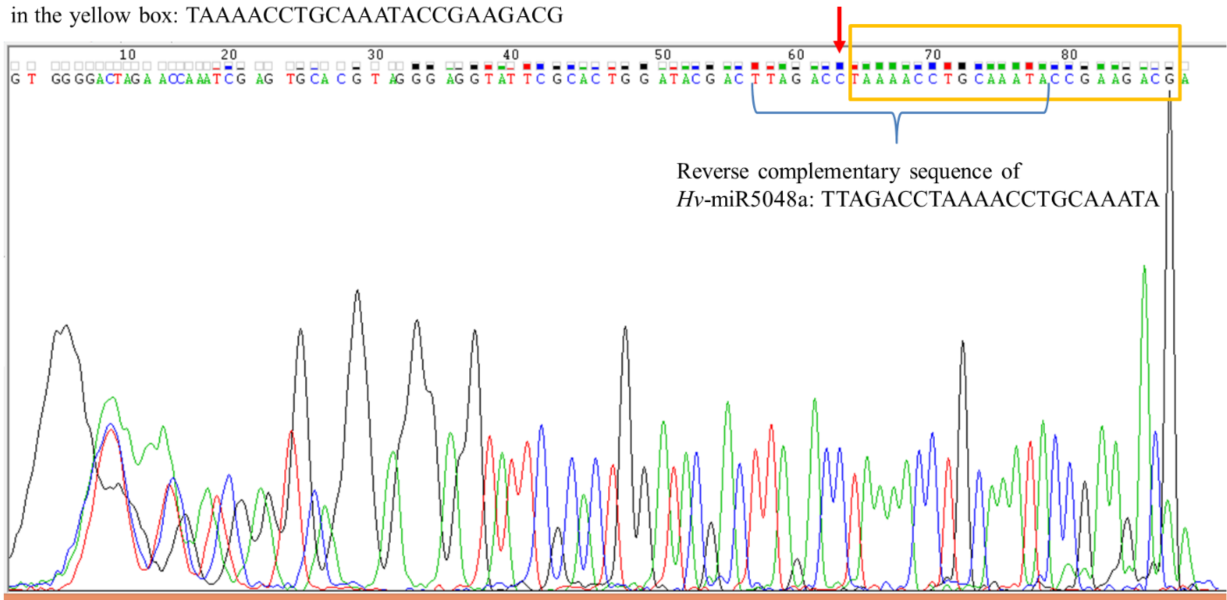
Signature nucleotides verified in the sequencing to detect the miRNA

Reverse complementary sequence of the forward primer of *Osa*-miR1432 in the yellow box: CATCTCTCCTGATGCCACACC

(i) *Hv*-miR5048a

Reverse complementary of the forward primer of *Hv*-miR5048a in the yellow box: TAAAACCTGCAAATACCGAAGACG

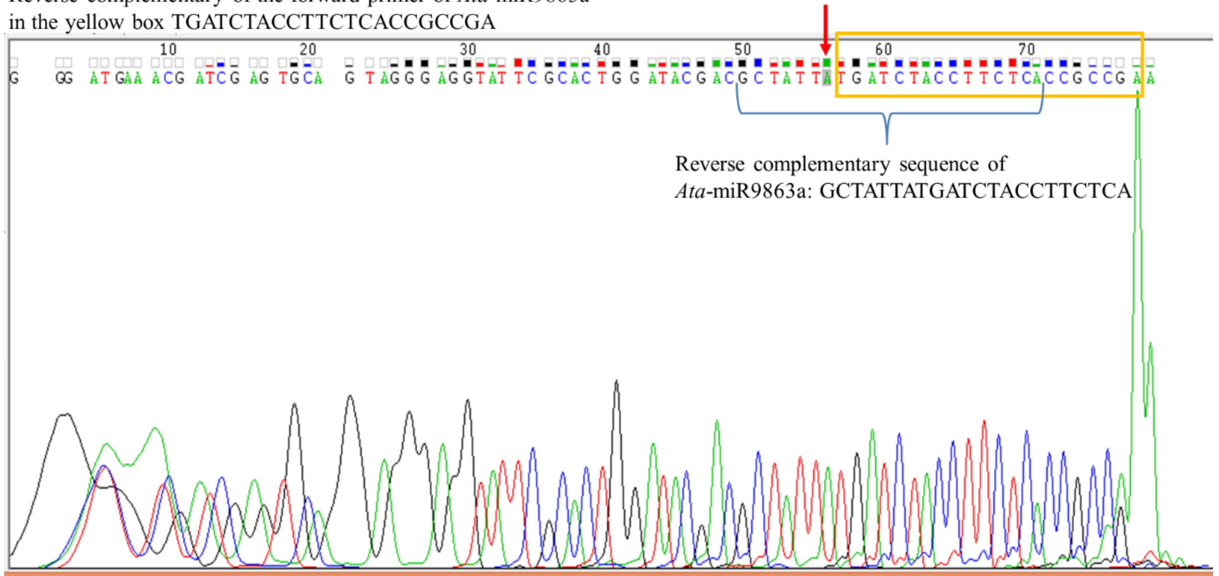
Signature nucleotide verified in the sequencing to detect the miRNA



(j) *Ata*-miR9863a

Reverse complementary of the forward primer of *Ata*-miR9863a in the yellow box TGATCTACCTTCTCACCGCCGA

Signature nucleotide verified in the sequencing to detect the miRNA



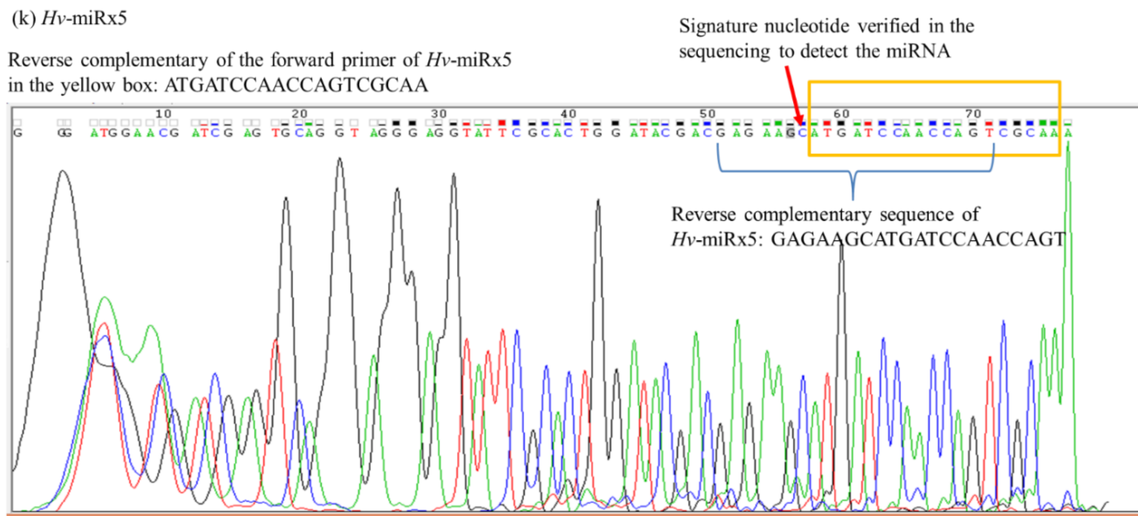


Figure S4. The sequencing verification of miRNA stem-loop qRT-PCR products (a-k). Reverse complimentary sequence of the forward primers for respective miRNA is shown in the yellow box. M13 reverse primer was used for sequencing to verify the miRNA sequence. The nucleotide sequence which is/are not contained by the forward primer was considered as signature nucleotide(s), and verified through sequencing (indicated by red arrow in each figure).

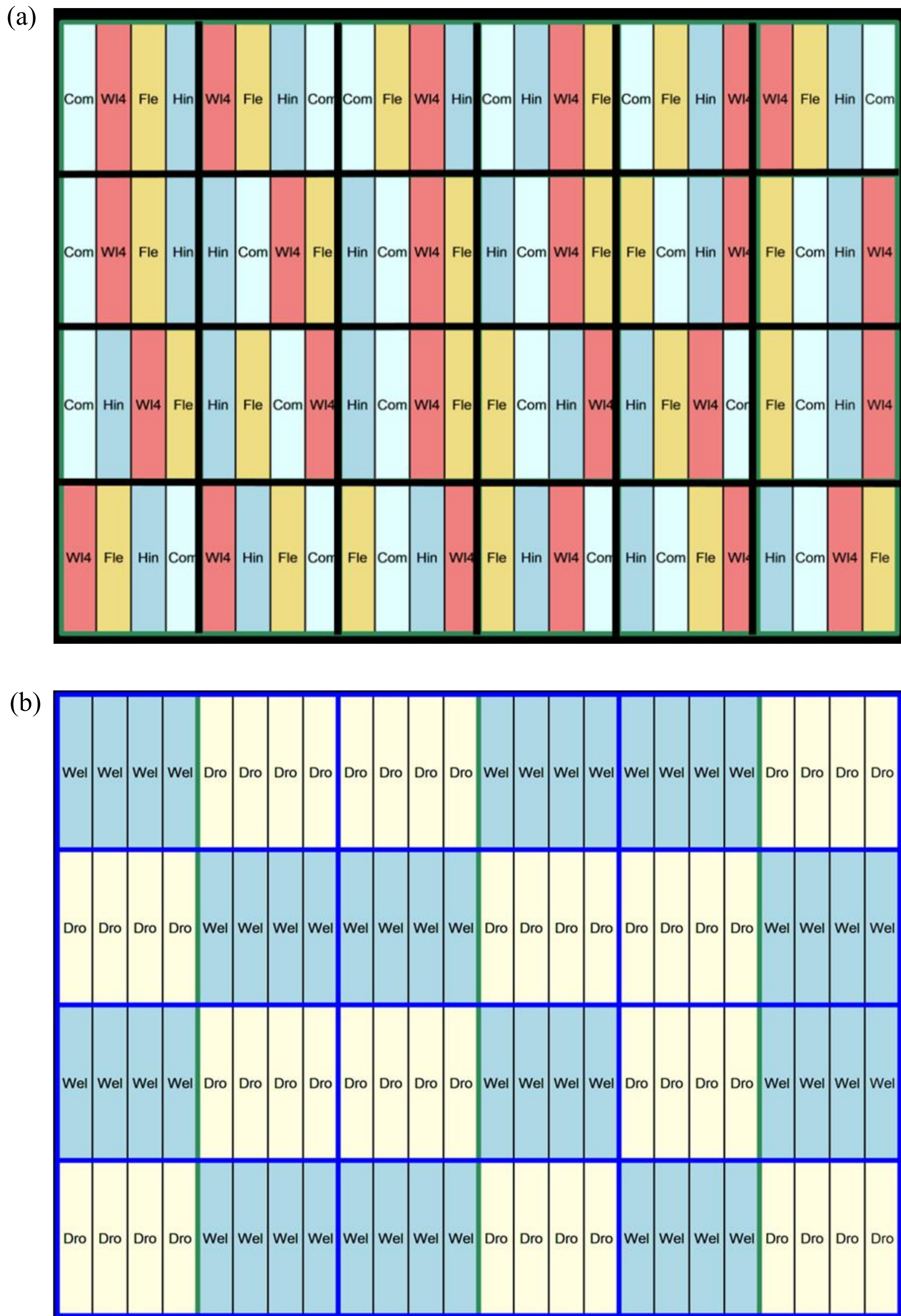
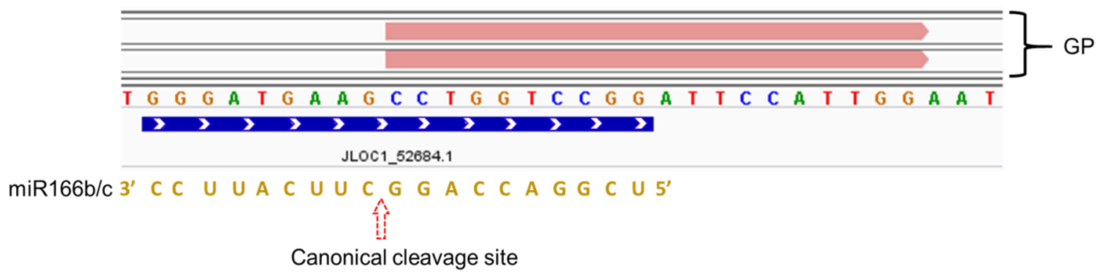


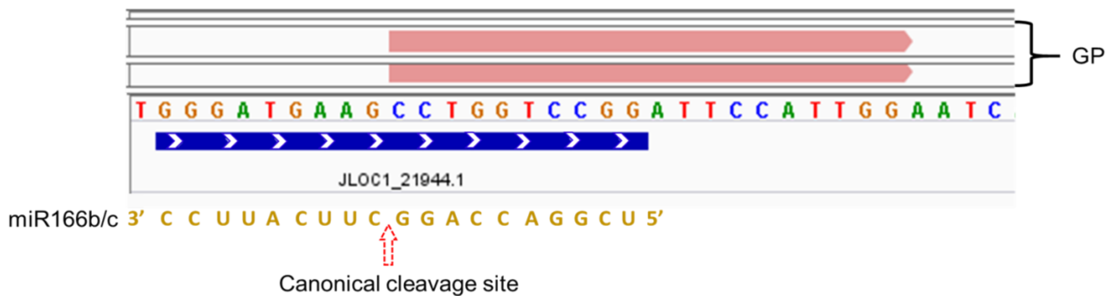
Figure S5. Experimental design of genotypes and treatments. a. Layout of genotypes. Black borders indicate pots. The genotypes are coded; Com = Commander, Fle = Fleet, Hin = Hindmarsh, WI4 = WI 4304. b. Layout of the watering conditions of the respective pots, Dro = Drought treated pot, Wel = Well-watered pot.

(a) miR166b/c target: Cleavage products aligned with JLOC1_38121/ MLOC_58644

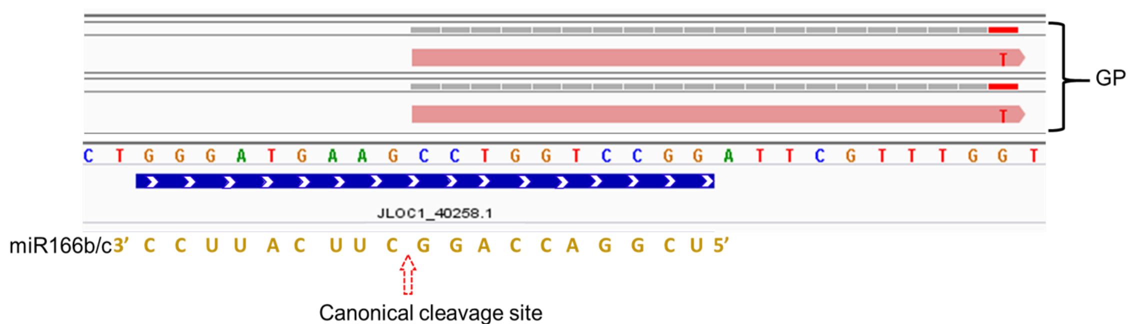
(b) miR166b/c target: Cleavage products aligned with JLOC1_52684/ MLOC_79063



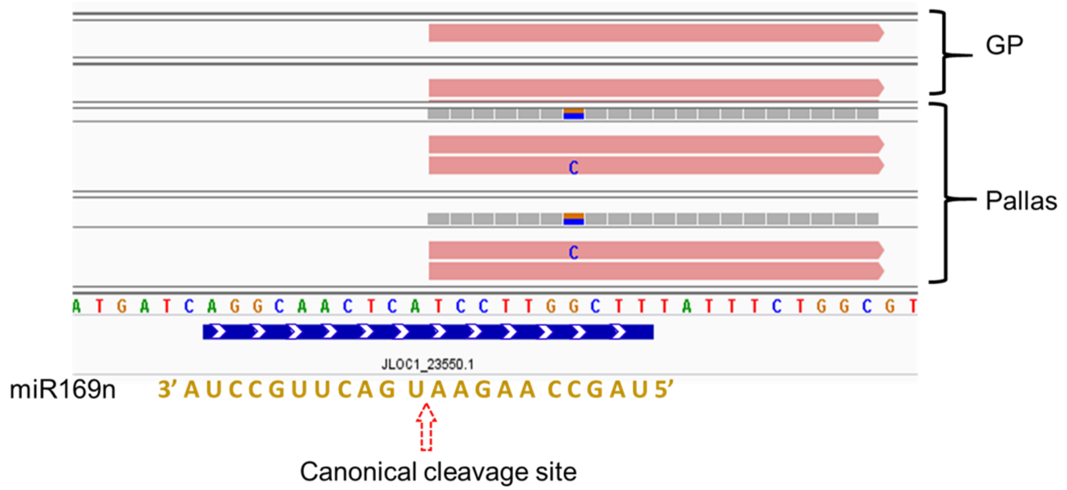
(c) miR166b/c target: Cleavage products aligned with JLOC1_21944/ MLOC_33978



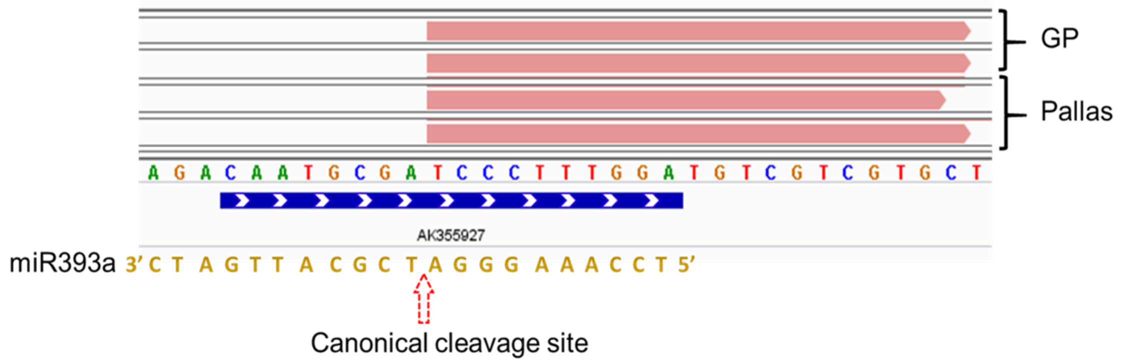
(d) miR166b/c target: Cleavage products aligned with JLOC1_40258/ MLOC_61603



(e) miR169n target: Cleavage products aligned with JLOC1_23550/ MLOC_36554

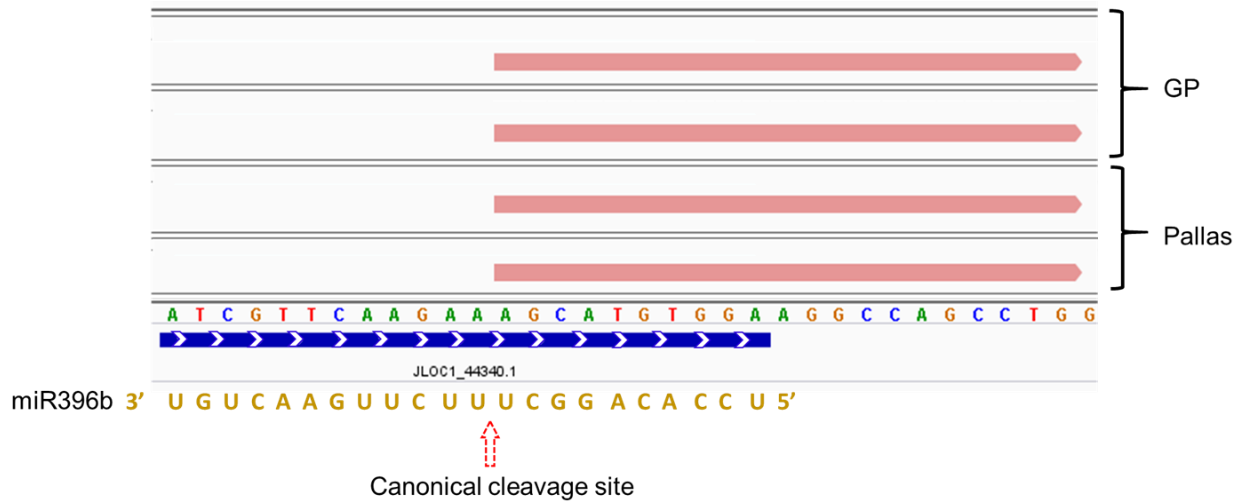


(f) miR393a target: Cleavage products aligned with AK355927/ MLOC_9864

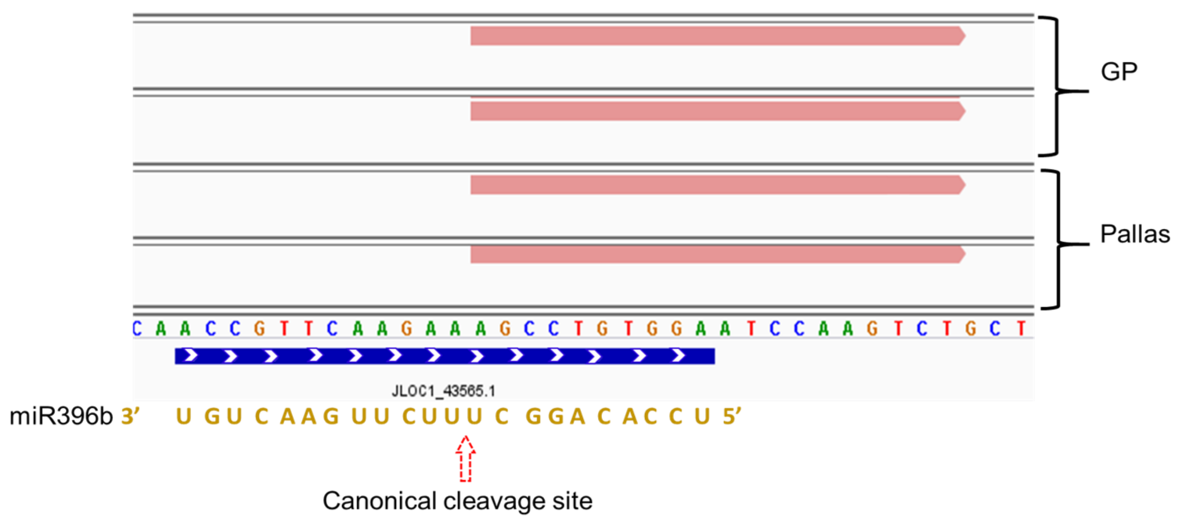


(g) miR393a target: Cleavage products aligned with JLOC1_36251/ MLOC_56088

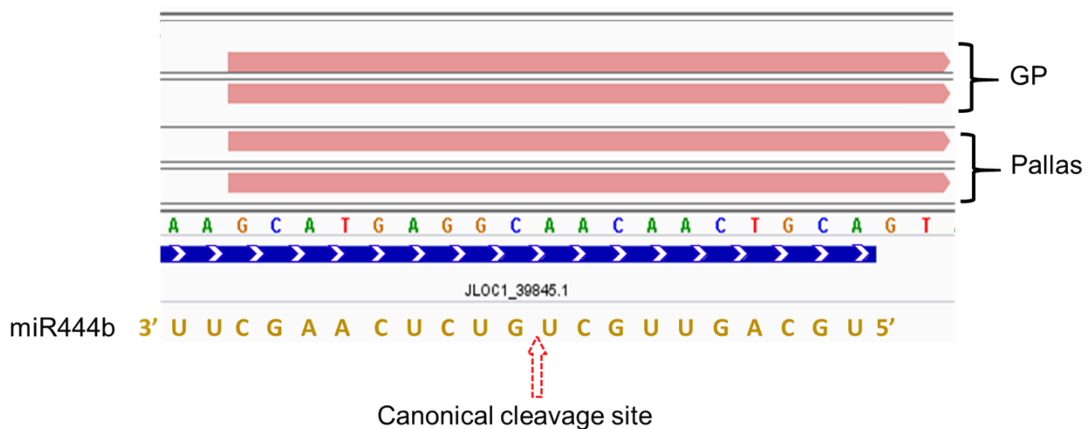
(h) miR396b target: Cleavage products aligned with JLOC1_44340/ MLOC_67201



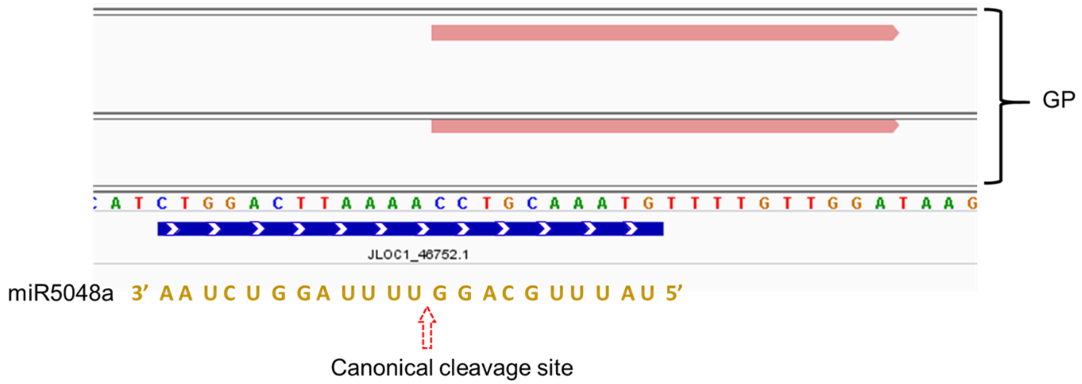
(i) miR396b target: Cleavage products aligned with JLOC1_43565/ MLOC_66132



(j) miR444b target: Cleavage products aligned with JLOC1_39845/ MLOC_61033

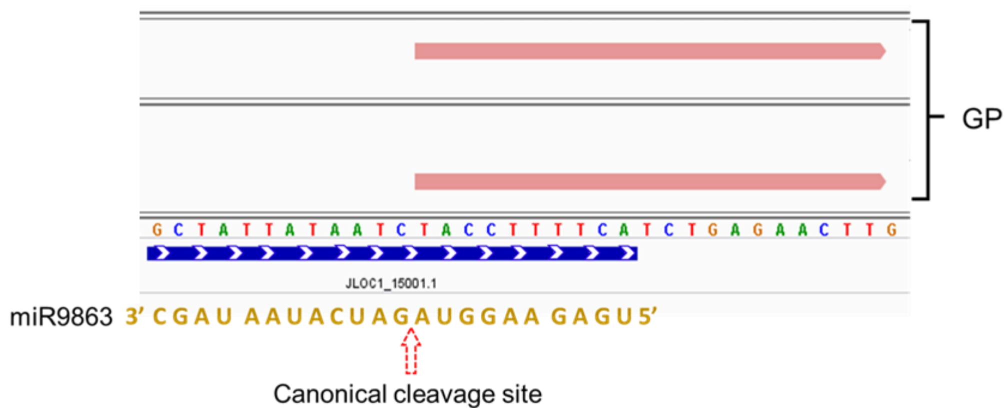


(k) miR5048a target: Cleavage products obtained for JLOC1_46752/ MLOC_70446



(l) miR9863 target: Cleavage products aligned with JLOC1_16566/ MLOC_24045

(m) miR9863 target: Cleavage products aligned with JLOC1_15001/ MLOC_21626



(n) miRx5 target: Cleavage products aligned with JLOC1_39449/ MLOC_60488

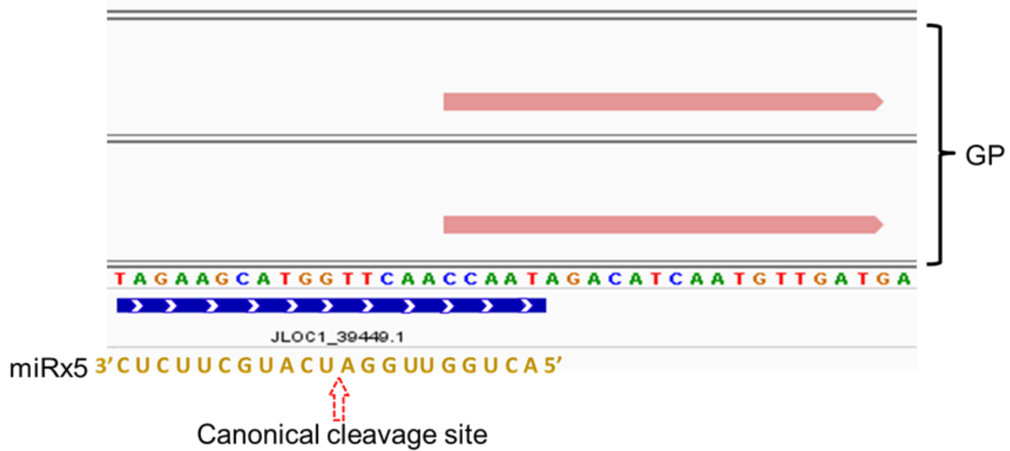


Figure S6. Degradome results for miRNA target validation (a-n). Cleavage products of two degradome libraries developed from two barley genotypes; Golden Promise (GP) and Pallas aligned with the candidate target transcripts. Some target cleavage products obtained either in GP (a, b, c, d, k and m) or in Pallas (l). Some target cleavage products were obtained in both genotypes (e, f, g, h, i, j). Positions of cleavage products are shown in the horizontal red bars. The blue bar indicates the miRNA binding site of the target transcript (5' -3' direction). The canonical cleavage site of the target (opposite to the miRNA) is indicated between the 5' 10 and 11 nucleotide position of the miRNA. Grey bar is shown to represent the number of nucleotides matched in the alignment between the cleavage product sequence and the reference transcript, while there is any mismatch observed.

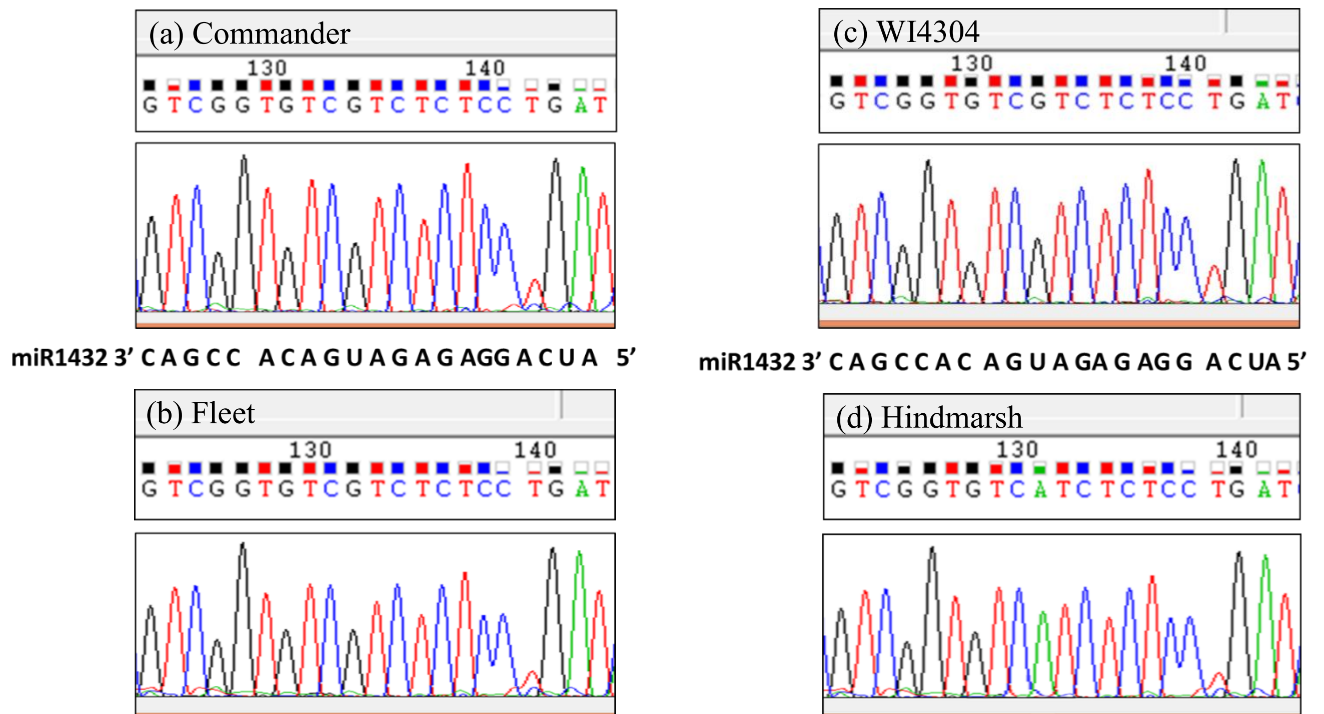


Figure S7. miR1432 Target site sequence of the four barley genotypes. G:U pair at the position 12 nt (from 5'-end of the miRNA) in the miRNA: target duplex in genotype (a) Commander, (b) Fleet, and (c) WI4304. (d) Perfect complementary pairing in the miRNA: target duplex in genotype Hindmarsh.

File S1

***In situ* PCR (ISPCR) of miRNAs and targets**

An RNase-free environment should strictly be maintained until the reverse transcription process. Tissues were fixed in fresh FAA fixative (50% [v/v] absolute ethanol, 5% [v/v] Glacial acetic acid, 25% [v/v] Paraformaldehyde [16%, EM grade] and 0.1 % [v/v] Tween 20). Vacuum infiltration was carried out as soon as possible after sample harvest at ~25 mm Hg pressure; the solution was replaced with fresh FAA fixative, and was kept at 4°C overnight. The fixative was then replaced with wash buffer 1 (50% ethanol, 5% acetic acid) and incubated twice for 10 minutes each. The samples were subjected to dehydration through graded ethanol series (v/v) 50%, 60%, 70%, 85%, 95%, 100% and dehydrated ethanol (dehyd EtOH) for 30-45 min each. Samples were kept in fresh dehyd EtOH overnight. Clearing and infiltration was carried out in 75% dehyd EtOH + 25% Histolene (Thermofisher); 50% dehyd EtOH + 50% Histolene; 25% dehyd EtOH + 75% Histolene; 100% Histolene in the fume hood for 45 minutes each at RT. For paraffin embedding, the sections were subjected to 100% Histolene, ¼ volume paraffin chips for overnight incubation at RT.

On the following day, the samples/ tubes were placed in the 60°C incubator until the wax chips have completely melted. After several hours the wax/histolene solution was replaced with 100% molten wax in the fume hood and immediately replaced in the oven. The sections were embedded in paraffin blocks. The blocks were preserved in 4°C until use for ISPCR.

For ISPCR, the desired paraffin block was first trimmed with a single-edged razor blade to remove the excess wax from the edges of the paraffin block to get a pyramid shape structured block. Then the desired block was placed in a proper orientation in the Leica RM2265 Rotary Microtome (Leica Microsystems, North Ryde, Australia). A 10µm thin ribbon section was cut from the block, and the sections were placed on slides (Superfrost

polysine coated white with Cellophane, 25 X 75 mm, Thermo Fisher Scientific, Waltham, MA, USA; Cat. # MENSF41296PL). The slides were left on the hot plate at 42°C overnight to dry. The slides were used on the following day for deparaffinization and Rehydration to proceed for ISPCR.

For deparaffinization and rehydration, first, the slide-mounted sections were immersed in Histolene twice for 10 minutes each using two separate glass slide jars. Deparaffinized sections were then rehydrated in the graded ethanol series using fresh solutions, avoiding carry-over of solvents as follows: incubation in 100% absolute ethanol for ten minutes followed by 95%, 90% absolute ethanol for 2 minutes. Then the slides were incubated in 80% EtOH+ 20% saline (NaCl), 60% EtOH+ 40% saline (NaCl), 30% EtOH+ 70% saline (NaCl), 20% EtOH+ 80% saline (NaCl) and 100% saline solution (NaCl) for two minutes each followed by incubating the slides in 1 X PBS for five minutes. The slides were then transferred into the slide mailer containing DEPC treated water for 2-3 minutes incubation. During this time the slide mailer needs gentle rotation manually. The samples were then incubated in 2x SSC for 30 minutes replacing the DEPC treated water from the slide mailer. Excess 2x SSC from the slide surface was removed and slides were placed into the plastic humidified box for Proteinase k treatment (Przybecki *et al*., 2006).

Proteinase k treatment is a crucial step to allow the digestion of proteins and appropriate penetration of the DNase treatment, reverse transcription and PCR reagents. After deparaffinization and rehydration, the leaf sections were treated with 2µg/mL final concentration of proteinase K (Sigma-Aldrich, Castle Hill, NSW, Australia, Cat. # P2308) at 37°C for 20 minutes [Proteinase k digestion was optimized in our study for barley leaf tissues before starting the ISPCR following the method described by Bagasra (2007)]. The reaction was stopped by using 2% glycine in 1X PBS for two minutes followed by two washes with 1X PBS for 5 minutes each (Przybecki *et al*., 2006). DNase treatment was carried out

overnight at 37°C to remove the genomic DNA contamination following the method described by Przybecki *et al*., (2006) with the setup of frame seal slide chamber (Bio-Rad Laboratories, Hercules, CA, USA; Cat. # SLF0601).

After the DNase treatment the section contained slides were washed with 0.5 M EDTA, 2X SSC, 1X SSC, 0.5X SSC and DEPC treated water (Przybecki *et al*., 2006). For a detailed preparation of reagents and storage, readers are referred to the study by Przybecki *et al*., (2006). Just after the DNase treatment, in a humidified box, the negative control slide was put in the freeze without any added reagents. The other slides were subjected to the reverse-transcription process. miRNA and target reverse transcription were carried out using SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction except the miRNA reverse transcription preparation and conditions were followed according to the protocol described by Varkonyi-Gasic *et al* (2007). The final volume of reaction was 60 µL per slide chamber. The reaction was assembled in the GS1 thermal cycler (Applied Biosystems, Foster, CA, USA) on *in situ* block for glass slides.

After the reverse transcription, the sections were washed with ice-cold sterile water to remove the reverse transcription reagents. Then on each slide (including the negative control) PCR master mixture was added to the sections using NEB's Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with Standard Taq Buffer (M0273) according to the manufacturer's instruction for a final volume of 60 µl, in which 4 µM final concentration of Digoxigenin-11-dUTP (Roche Diagnostics, Alameda, CA, USA Cat # 11093088910) was added as an additional reagent (Athman *et al*., 2014). It is worth mentioning that in our study, the number of PCR cycles followed for the respective miRNA/target was optimized. To increase the possibility of differentiating the abundance of PCR product, minimum cycle number is suggested (Athman *et al*., 2014).

Anti-DIG antibody incubation, washing and colourimetric detection of DIG labeled PCR products were carried out exactly as in Athman *et al*., (2014) on slide. BM Purple AP Substrate (Roche, cat. no. 11442074001) was used for colourimetric detection of DIG labeled PCR products. Once purple blue signal appeared in the sections, the BM purple was removed from the sections. The sections were washed with Wash Buffer 2 (0.1 M Tris-Cl, 0.15 M NaCl, pH 9.5, kept at 4°C) thrice, five minutes for each time followed by a wash with 100 µl sterile water (Athman *et al*., 2014). For a detailed preparation of reagents, timing, and critical steps, readers are referred to the study by Athman *et al*., (2014).

The sections were then mounted with an aqueous-based mounting medium, ImmunoHistoMount™ (Sigma-Aldrich, Castle Hill, NSW, Australia; Cat. No. I1161) and allowed it to dry overnight and were visualized in the Zeiss Axio Imager M2 microscope (Carl Zeiss, Oberkochen, Germany) under the bright field Illumination. The images were captured using the AxioCam ERc5s camera and retrieved using ZEN 2011 software (Carl Zeiss, Oberkochen, Germany) while the exposure time was adjusted to 100 ±20 millisecond for each specimen.

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Bagasra O. (2007) Protocols for the *in situ* PCR-amplification and detection of mRNA and DNA sequences. *Nature Protocols* **2**, 2782–2795.

Przybecki Z., Siedlecka E., Filipecki M. & Urbańczyk-Wochniak E. (2006) *In situ* reverse transcription PCR on plant tissues. *Methods in Molecular Biology* **334**,181–197.

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CHAPTER 5

Drought-inducible expression of miR827 enhances drought tolerance in transgenic barley

Statement of Authorship

Title of Paper	**Drought-inducible expression of miR827 enhances drought tolerance in transgenic barley**
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Jannatul Ferdous, Peter Langridge, Martin Nguyen, Chris Brien, Penny J. Tricker This is an experimental manuscript about phenotyping and functional analysis of transgenic barley (Cv. Golden Promise) expressing *Ath*-miR827 and *Hv*-miR827 under the control of *CaMv: 35S* and *Zm-Rab17* promoter. Here, we conducted experiments to analyse of the miR827 transgenic barley plants to investigate the role of miR827 for drought tolerance in barley. This chapter is written in a manuscript format and is intended for submission to a peer reviewed journal. This experiment is closely related to the subject matter of this thesis.

Principal Author

Name of Principal Author (Candidate)	Jannatul Ferdous		
Contribution to the Paper	Conceived and designed the experiments, performed experiments, analysed and wrote manuscript.		
Overall percentage (%)	70%		
Certification:	I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-author: Ryan Whitford

Contribution to the paper: Developed the transgenic plants, reviewed and edited manuscript. I hereby certify that the statement of the contribution is accurate.

Signature: Date: 19/05/2016

Name of Co-Author	Peter Langridge		
Contribution to the Paper	Supervised designing the experiments. Collaborated with the James Hutton Institute for microRNA-target prediction. Evaluated the development of experiments, reviewed and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/16

Name of Co-Author	Chris Brien		
Contribution to the Paper	Designed the experiment and performed the statistical analysis of the phenotyping experiment for digital non-destructive imaging in the Plant Accelerator, Adelaide. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	08/01/16

Name of Co-Author	Martin Nguyen		
Contribution to the Paper	Performed the statistical analysis for digital non-destructive imaging in the Plant Accelerator, Adelaide. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	07/01/16

Name of Co-Author	Penny J. Tricker		
Contribution to the Paper	Supervised designing and development of the experiments, data analysis and interpretation. Reviewed and edited the manuscript and will be acted as the corresponding author. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	17/02/16

Link to chapter 5

miR827 is reported to confer drought tolerance in transgenic *Arabidopsis* (Aukerman and Park, 2009). Plants overexpressing *Ath*-miR827 constructs showed improved drought tolerance by maintenance of rosette leaf area under drought conditions and ABA hypersensitivity of germination inhibition in transgenic *Arabidopsis* plants when compared to the control (Col-0) (Aukerman and Park, 2009). It was suggested that the recombinant DNA constructs of miR827 could be used for any plant for the identification of the regulatory role of miR827 for drought tolerance (Aukerman and Park, 2009). Additionally, a number of promoters could be used for the development of recombinant DNA constructs for miR827 depending on the desired outcome (Aukerman and Park, 2009).

On this basis transgenic barely plants over-expressing miR827 were developed in the barley cultivar 'Golden Promise' (unpublished data of Ryan Whitford). Golden Promise is a drought sensitive genotype (Wendelboe-Nelson and Morris, 2012). We hypothesized that the transgenic barley lines would show enhanced drought tolerance compared with the control plants. Here, we conducted phenotyping and functional analyses of the miR827 transgenic barley plants to investigate the regulatory role of miR827 for drought tolerance in barley. This chapter (Chapter 5) is written in a manuscript format and will be submitted to a peer reviewed journal.

References:

Aukerman M. & Park W. (2009), Drought tolerant plants and related constructs and methods involving genes encoding mir827. Patent application number: 20090165168.

Wendelboe-Nelson C. & Morris P.C. (2012) Proteins linked to drought tolerance revealed by DIGE analysis of drought resistant and susceptible barley varieties. Proteomics 12(22), 3374-3385.

Drought-inducible expression of miR827 enhances drought tolerance in transgenic barley

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Abstract

Drought is one of the major abiotic stresses reducing crop yield. Since the discovery of plant microRNAs (miRNAs) considerable progress has been made in clarifying their role in plant responses to abiotic stresses, including drought. miR827 was previously reported to confer drought tolerance in transgenic *Arabidopsis*. We examined barley (*Hordeum vulgare* L. ssp. *Vulgare* Cv. 'Golden Promise') plants over-expressing miR827 for plant performance under drought. Transgenic plants constitutively expressing *CaMV-35S*::*Ath*-miR827 and drought inducible *Zm-Rab17*::*Hv*-miR827 were phenotyped by non-destructive imaging for growth and whole plant water use efficiency (WUE_{wp}). We observed that the growth, WUE_{wp} and reproductive performance of transgenic barley plants expressing *CaMV-35S*::*Ath*-miR827 were negatively affected in both well-watered and drought-treated growing conditions compared with the wild type plants. In contrast, transgenic plants over-expressing *Zm-Rab17*::*Hv*-miR827 showed improved WUE_{wp} with no growth or reproductive penalty compared with the wild type plants. The recovery of *Zm-Rab17*::*Hv*-miR827 over-expressing plants also improved following severe drought stress. Our results suggest that *Hv*-miR827 has the potential to improve the performance of barley under drought and that the choice of promoter to control the timing and specificicty of miRNA expression is critical.

Keywords

microRNA. Transgenic barley miRNA. Non-destructive imaging. *Zm-Rab17*. Phenotyping. Drought.

Introduction

A number of abiotic stresses affect crop yields. Drought is one of the major stresses constraining plant growth and crop productivity in many parts of the world (Boyer and Westgate, 2004). With the expected increase in world population and declining water resources for crop production, it is imperative that new strategies for enhancing food production are explored. The development of drought tolerant varieties will be particularly important for increasing food production given the expected climate changes and understanding plant drought tolerance mechanisms will be crucial (Lawlor, 2013). However, the genetic mechanisms of drought response and tolerance are highly complex involving multiple pathways and large suites of genes (Nevo and Chen, 2010; Blum, 2011). Conventional breeding for the development of drought tolerant varieties has been hindered by low trait heritability and the lack of cost-effective phenotypic selection strategies (Kirigwi *et al.*, 2004). Molecular tools and transgenic approaches for the manipulation of genes have been applied towards elucidating the mechanisms of drought tolerance (Zhang *et al.*, 2004; Shinozaki and Yamaguchi-Shinozaki, 2007; Nakashima *et al.*, 2009, Morran *et al.*, 2011). Over the past decade the involvement of regulatory molecules in plant drought stress response is gradually being unveiled. However, considerable additional work will be needed to identify the master regulators and their signalling networks required to improve plant performance under drought.

microRNAs (miRNAs) are an important class of regulatory molecules. Mature miRNAs are non-protein coding, approximately 18-21 nucleotide (nt), single-stranded, highly conserved RNA sequences (Axtell and Bartel, 2005; Zhang *et al.*, 2006; Jones-Rhoades *et al.*, 2006; Sunkar *et al.*, 2012). In association with Argonaute (AGO) proteins, mature miRNAs recognize target mRNAs based on sequence complementarity and, via target cleavage, function as negative transcriptional/translational regulators across multiple regulatory networks, both in plants and animals (Bartel, 2009).

Several studies report the involvement of plant miRNAs in various abiotic stress responses (Sunkar *et al*., 2007; Lu and Huang, 2008; Li *et al.*, 2008; Trindade *et al.*, 2010; Wei *et al*.,2009; Hackenberg *et al*., 2015). Research groups have investigated transcription profiles of miRNAs upon exposure to drought stress for several plant species including *Arabidopsis*, maize, tobacco, poplar, soybean, wheat, sorghum, rice, barley (Sunkar and Zhu, 2004; Liu *et al*., 2008; Wei *et al*., 2009, Frazier *et al*., 2011; Qin *et al*., 2011; Kulcheski *et al*., 2011; Kantar *et al*., 2011; Katiyar *et al*., 2015; Hackenberg *et al*., 2015; Cheah *et al*., 2015; Ferdous *et al*., 2016). Although small RNA sequencing from barley has resulted in a surge of genomic resources leading to discoveries of conserved and novel miRNAs (Schreiber *et al*., 2011; Hackenberg *et al*., 2015), the functional analysis of these miRNAs in this species has been lacking.

Previous testing of constitutively expressed miRNA, *Ath*-miR827, increased drought tolerance in *Arabidopsis* (Aukerman and Park, 2009). In the present study, barley plants were genetically modified to express either the precursor miRNA (pre-miRNA) of *Ath*-miR827 or its cereal pre-miRNA orthologue *Hv*-miR827. *Ath*-miR827 was constitutively expressed using *CaMV-35S* whilst *Hv*-miR827 expression was drought inducible through the action of the *Zm-Rab17* promoter. The aim of the present study was to evaluate miR827 function in transgenic barley plants expressing either *Ath*-miR827 or *Hv*-miR827 and to test plant performance upon exposure to drought stress.

Materials and methods

Generation of transgenic barley expressing *Ath*-miR827 and *Hv*-miR827

Barley (*Hordeum vulgare*, cv. 'Golden Promise') was transformed with the precursor miRNA827s (pre-miR827) from *Arabidopsis* and barley under the control of the constitutive promoter *CaMV-35S* or the drought-inducible promoter *Zm-Rab17* respectively, with the *Nos* terminator. The transformation method, selection, and

regeneration procedures were as described in Morran *et al*., (2011). In brief, the pre-miR827 was isolated from *Arabidopsis* and cloned into the pMDC32 vector (Curtis and Grossniklaus, 2003). The pre-miR827 from barley was cloned into another pMDC32 vector in which the *CaMV-35S* promoter was excised using *Hind*III-*Kpn*II restriction sites and replaced with a *Zm-Rab17* promoter (634 bp fragment) (Busk *et al*., 1997). Both of the constructs were transformed into barley genotype 'Golden Promise' using *Agrobacterium*-mediated transformation. T_0 seedlings/events were obtained for the transgenic plants containing constructs of *CaMV-35S*::*Ath*-miR827 and *Zm-Rab17*::*Hv*-miR827. Here the term 'event' denotes the group of plants from each respective (*CaMV-35S*::*Ath*-miR827 and *Zm-Rab17*::*Hv*-miR827) transformation episode that regenerated from tissue culture. Plantlets were then transferred to coco-peat medium in pots under greenhouse conditions. Genomic DNA was extracted from mature leaf tissue of wild type (WT), transgenic lines and null plants following the protocol of Edwards *et al*. (1991). The presence or absence of the transgenic fragment DNA was confirmed in *CaMV-35S*::*Ath*-miR827 over-expressed (OX) plants and in *Zm-Rab17*::*Hv*-miR827 drought inducible (DI) plants by PCR amplification using one μl of genomic DNA with *CaMV-35S* or *Zm-Rab17* promoter specific forward primers (*CaMV-35S* forward: 5′-TTCATTTCATTTGGAGAGGACCTCGACT-3′;
Zm-Rab17 forward: 5′-CGGGCTGGTATTTCAAAACTAT-3′); and *Nos* terminator specific reverse primer (5′-AACCCATCTCATAAATAACGTCATGCA-3′)
for the respective construct. PCR amplification was carried out in a DNA Engine Tetrad Peltier Thermal Cycler (Bio-Rad Lab, Hercules, CA, USA) using IMMOLASE™ DNA Polymerase according to the manufacturer's instructions. Each 20 μl PCR reaction consisted of 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 1X immolase buffer, 1.25 units immolase DNA polymerase (Bioline, Sydney, Australia), 0.5 μM of each primer, and 1 μl (50 ng) DNA template. The thermal cycler was programmed for an initial step at 95°C for

10 min followed by 35 cycles with denaturation for 30 s at 95°C, annealing for 30 s and extension for 30 s at 72°C. The reaction was terminated with a final extension at 72°C for 10 min. For annealing temperature, 56° and 55°C were used for the aforementioned primer pairs of OX and DI lines respectively. Barley S-adenosylmethionine decarboxylase (*Hv-SAMDC*) gene was used as a control gene for PCR (forward 5′-CTCAAACTGCAACAATGGCCG-3′ and reverse 5′-ACAGACGGAACAGCGACAGC-3′). The annealing temperature of 58 °C was used for *Hv-SAMDC* primer pair. Here the events regenerated from each respective transformation through tissue culture lacking the transgene are called 'null' events. Southern blotting was carried out in order to select single copy transgenic plants following the method described in Shi *et al*., 2010. Single copy homozygous lines were derived from self-pollination of low copy number plants from the T_0 and the subsequent generations. Thus, we obtained three single copy, homozygous events for *CaMV-35S*::*Ath*-miR827 and four single copy, homozygous events for *Zm-Rab17*::*Hv*-miR827 constructs.

Gene expression analysis

Total RNA was extracted from fully expand mature leaves of wild type (WT), transgenic lines and null plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To remove genomic DNA contamination, RNA samples were treated with DNA-free™ reagents twice (Ambion, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. For semi quantitative RT-PCR, one µg of total RNA from the WT, transgenic lines or nulls were reverse transcribed with random hexamer priming (100ng) using SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Expression of the pre-*Ath*-miR827 was confirmed in the T_1 generation using precursor-*Ath*-miR827 specific forward (5′-TTAGATGACCATCAACAAACT-3′) and *Nos*-terminator specific

reverse (5′-AACTAGTTAATTAAGGAATTATCGAA-3′) primers in the *CaMV-35S*::*Ath*-miR827 lines. Precursor-*Hv*-miR827 specific forward (5′-TTAGATGACCATCAGCAAACA-3′) and *Nos*-terminator specific reverse (5′-AACTAGTTAATTAAGGAATTATCGAA-3′) primers were used to assess the expression of pre-*Hv*-miR827 in the *Zm-Rab17*::*Hv-miR827* lines. The expression of barley glycolytic glyceraldehyde-3- phosphate dehydrogenase (*Hv-GAPDH*, accession number: *X60343.1*) gene provided an internal control (forward 5′-GCCAAGACCCAGTAGAGC-3′ and reverse 5′-CACATTTATTCCCATAGACAAAGG-3′).

Determination of drought treatment using transgenic barley plants expressing a *Zm-Rab17* controlled *GUS* gene

To determine the timing and strength of our drought inducible promoter, we conducted an experiment using *Zm-Rab17* controlled expression of *GUS* in transgenic barley (Cv. Golden Promise) plants, and observed the drought inducible expression during the progression and recovery from water deficit stress. Seeds were sown in UC (University of California) mix: coco peat: clay loam= 1:1:1 in a growth chamber at 23 °C day and 18 °C night temperature, 12 h/12 h light/dark photoperiod, 450 μmol m^{-2} s^{-1} photosynthetically active radiation and 60 % relative humidity, and plants were well-watered for four weeks. Four weeks after germination, watering was stopped to provide the drought treatment for 14 days. At 0 (65% gravimetric water content (GWC)), 3 (45% GWC), 5 (28% GWC), 7 (18% GWC), 9 (16% GWC), 12 (12% GWC), and 14 (10% GWC) days after water withdrawal, and 3 (59% GWC), 6 (60% GWC), 9 (61% GWC) days after re-watering, leaves were collected for GUS staining. The drought-treated pots were weighed at each time point of sample collection to determine the GWC at each sampling stage. GWC was calculated using the formula: GWC (%) = [(wet soil weight /

dry soil weight) – 1] x100 where wet soil weight is the soil weight during sample collection and dry soil weight is the oven dried soil weight. Six individual plants were used for this experiment. The following drought phenotyping experiments were carried out under mild (18% and 16% GWC) and severe (12% and 10% GWC) drought treatments.

Experimental layout and statistical analysis

The experimental design for non-destructive imaging considered 10 genotypes (DI L-1, DI L-2, DI L-3, DI L-4 of *Zm-Rab17*:*Hv*-miR827 transgenic and respective null plants; OX L-1, OX L-2, OX L-3 of *CaMV-35S*:*Ath*-miR827 transgenic and respective null plants and the wild type 'Golden Promise') under two watering conditions (drought or well-watered). The design employed was a split-plot design with main plots being two consecutive pots/carts. The experiment involved 160 pots arranged in eight lanes by 20 positions. Eight biological replicates were used for each treatment. The lines were assigned to pairs of pots using a nearly-trend-free, randomized complete-block design. The water conditions were randomly assigned to the two consecutive pots/carts within each main plot. The design was generated in the R statistical software environment (R Development Core Team, 2014) using the Digger and dae packages (Coombes, 2009; Brien, 2011). The layout for the experiment is given in Fig. S1.

The analysis-of-variance model for this experiment was:

E[Y] = xMainplot + Lines * Condition

var[Y] = Lanes/Positions; where, xMainplot was a numeric variable indexing the mainplots and allowed for a linear trend across the mainplots. The overall significance tests used an F test-statistic and tests for significance between means, which was conducted using a least significant difference (LSD) value at the 5% significance level. A mixed model analysis was performed on the 'shoot area' and the 'whole plant water use efficiency' using

Asreml-R (Butler *et al.*, 2010), a package for the statistical computing environment R (R Development Core Team, 2015). The mixed model allowed for (i) differences amongst the varieties and water conditions and (ii) for variability within the smarthouse between the two halves, between lanes, between pairs of carts that formed main plots and between individual carts.

Mild drought phenotyping experiment for non-destructive imaging

Seeds of single copy T_3 transgenic barely lines were surface sterilized (using 5% sodium hypochloride for 30 minutes), then sown in cellular plug trays. After confirming the presence of transgene by PCR, 10 day old seedlings at the same physiological stage were transplanted to sealed white pots (19.46 cm height x 14.94 cm diameter, Berry Plastics, item number T51386CP) filled with potting mix, as before. One seedling was transplanted per pot. White marble chips (National Terrazzo & Cement Works Pty Ltd in North Plympton, 8294 1233, 3mm size) covered the soil surface to minimise evaporation and the residual soil evaporation was calculated gravimetrically in pots with no plants. Twenty days after transplanting the pots were loaded onto a fully automated conveyor system in a temperature-controlled Smarthouse in The Plant Accelerator® facility (Honsdorf *et al.*, 2014). The plants of constitutively over-expressed (OX) lines, drought inducible (DI) lines, WT and null plants were subjected to a constant water deficit treatment maintaining 18% GWC, while control pots were maintained at 45% GWC. Watering was at automatic watering stations programmed to water each pot to weight (Bizerba, Balingen, Germany). The temperature of the Smarthouse maintained was 15ºC night / 22ºC day. Plants were grown in natural light.

Non-destructive imaging for shoot area

With the onset of the drought treatment, control and drought treated plants were imaged in The Plant Accelerator® facility using a LemnaTec 3D Scanalyzer (LemnaTec, GmbH, Germany). Each day, high-resolution visible light (RGB) digital images were taken, including two side and one top view for each plant, for 30 days. The projected shoot area of an individual plant was calculated as in Honsdorf *et al*., 2014. To separate the plant tissue area from the background, background-foreground separation was applied. Kilopixels per image per plant were estimated, and the sum of kilopixels from the three images was the projected shoot area. Eight independent plants were assessed per genotype per treatment.

Physiological measurements

WUE_{wp} was determined as the ratio of shoot biomass at the end of the imaging period against the amount of water (g/ml/plant) supplied during the imaging period. Anthesis time (d) and grain weight per plant (g/plant) were recorded during anthesis and after harvesting, respectively. Upon harvesting, the above ground shoot material was collected and oven (Contherm Scientific Ltd, Wellington, New Zealand) dried for a week at 60 °C for dry biomass measurements. Eight independent replicates were used per genotype and treatment.

Drought tolerance following mild and severe periodic drought stress

To further understand the drought performance of the *Zm-Rab17* controlled *Hv*-miR827, the T4 generation of DI lines and the controls (wild type and null) plants were grown in the growth chamber with conditions as before. Eight independent plants from each event were grown as one plant per pot. Four week old (at stem elongation stage) plants were first subjected to drought treatment avoiding inter-pot variation by maintaining watering to weight for 9 days until the pots reached 16% GWC. At the end of day 9 (16%

GWC), re-watering started for all the plants. Four days after re-watering, the plants were harvested and fresh weight of above ground biomass (n=8) was measured.

Subsequently, the T4 generation of DI lines and the controls (wild type and null) were grown for four weeks maintaining regular watering in the above-mentioned growth chamber conditions. Four week old (at stem elongation stage) plants were subjected to drought treatment avoiding inter-pot variation by maintaining watering to weight for 14 days until the pots reached 10% GWC. At day 12 after drought inception (12% GWC), photosynthetic assimilation (A) and stomatal conductance (g_s) were measured on a fully expanded, mature leaf (n=3) at photosynthetically active radiation inside the chamber (PARi) 1500 µmol $^{m-2\ s-1}$ using the LI-6400XT Portable Photosynthesis System (Li-Cor, Lincoln, Nebraska, USA). Fully expanded mature leaf blades (five independent plants, three leaves from each plant) were used for measuring the relative amount of chlorophyll (per 2x3 mm area of leaf) using SPAD-502 Chlorophyll Meter (Konica Minolta, Australia) at days 0, 6, 9 and 12 after drought inception. At day 12 after drought inception (12% GWC), fully expanded mature leaf blades (n=3) were also collected from well-watered and drought-treated plants of control and DI lines to isolate RNA for qRT-PCR.

After the severe drought episode, at day 14 after drought inception (10% GWC), re-watering started to observe the recovery and survival rate of the plants. Plant survival was determined at five weeks after re-watering.

Target prediction and validation by degradome analysis

Target prediction and validation through degradome analysis was conducted exactly as in Ferdous *et al*. (2016) except that the degradome library from only one genotype 'Golden Promise' was used in this experiment.

Expression of miR827 and its putative target

Hv-miR827 specific stem-loop reverse transcription primer (5´-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACTGTTTG-3´) and appropriate forward (5´-TCTGACGACGTTAGATGACCATC-3´) and reverse (5´-GTGCAGGGTCCGAGGT-3´) primers were designed following the method described by Chen *et al*., (2005) and Varkonyi-Gasic *et al*. (2007). We designed the primers for the putative target, an *SPX* (SYG1/PHO81/XPR1) domain encoding transcript, spanning the miRNA binding site (forward: 5´-GCACCTGGAGCCATCGTC-3´ and reverse 5´-TTCACCATCTTGCTGTTTCTACC-3´) using AlleleID software (Premier Biosoft International, Palo Alto, CA, USA). All oligo primers used in this study were synthesized by Sigma-Aldrich (Sydney, Australia).

Total RNA extraction and removal of genomic DNA were conducted exactly as before. The concentration and integrity of the DNA free RNA was determined by Agilent-2100 Bioanalyzer using RNA 6000 NanoChips (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) for the experimental samples ranges from 5.7 to 7.1. Two µg of total RNA from the well-watered and drought treated samples were reverse transcribed with *Hv*-miR827-specific stem-loop reverse transcription primer using SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) by the pulsed RT method (Varkonyi-Gasic *et al*., 2007). For target qRT-PCR, two µg of total RNA from the same experimental samples were reverse transcribed by random hexamer (100ng) using SuperScript® III RT (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. For qRT-PCR, three biologically independent plants were used for each treatment per line with three technical replicates per biological replicate. qRT-PCR was carried out exactly as in Ferdous *et al.* (2015) using the RG6000 Rotor-Gene real-time thermal cycler (Qiagen, Valencia, CA, USA). Mature miRNA qRT-PCR product was sequenced using the M13 reverse primer following the method described by

Ferdous *et al.*, (2015). The sequence of a qRT-PCR amplicon of the un-cleaved target was also verified by Sanger sequencing. The melt curve plots for miRNA and mRNA qRT-PCR are shown in Fig. S2.

Results

Generation of transgenic barley constitutively over-expressing (OX) *Ath*-miR827 or expressing *Hv*-miR827 under the control of a drought inducible promoter (DI)

Transgenic barley expressing *Ath*-miR827 under the control of constitutive *CaMV-35S* promoter and *Hv*-miR827 under the control of *Zm-Rab17* promoter were generated via *Agrobacterium*-mediated transformation. Three representative single copy lines for *CaMV-35S*::*Ath*-miR827 (OX L-1, OX L-2 and OX L-3) and *Zm-Rab17::Hv*-miR827 (DI L-1, DI L-2 and DI L-3) were selected based on Southern blot analysis (Fig. S3). Transgene presence in these lines was confirmed using transgene specific primers designed to amplify either a 765 bp (OX plants) or 1111 bp (DI plants) fragment spanning the promoter through to the terminator (Fig. S4a & b). Additionally, transgene specific semi-quantitative RT-PCR confirmed pre-miRNA expression for both OX (282 bp amplicon) and DI (101 bp amplicon) transgenic lines. These amplicons were not detected in either the WT or null segregants (Fig. S4c & d). Primers designed to flank an intron in the endogenous gene *Hv-GAPDH* was used as an internal control to detect cDNA amplification (78 bp amplicon) control (Fig. S4c & d).

Drought inducibility of *Zm-Rab17*

The pattern of gene expression under the control of the *Zm-RAB17* promoter was assessed using transgenic barley plants with *Zm-Rab17* driving *GUS* gene expression. GUS staining was first detected at 28% GWC (Fig. 1). As time progressed and water deficit increased, GUS expression was enhanced until re-watering when GUS expression was then reduced. By comparing the *Zm-Rab17* driven GUS detection (Fig. 1) with the plant

phenotype, we determined that expression started with ‘mild’ drought stress (18% and 16% GWC) and was strongest under ‘severe’ drought (12% and 10% GWC). The promoter was turned off after rewatering.

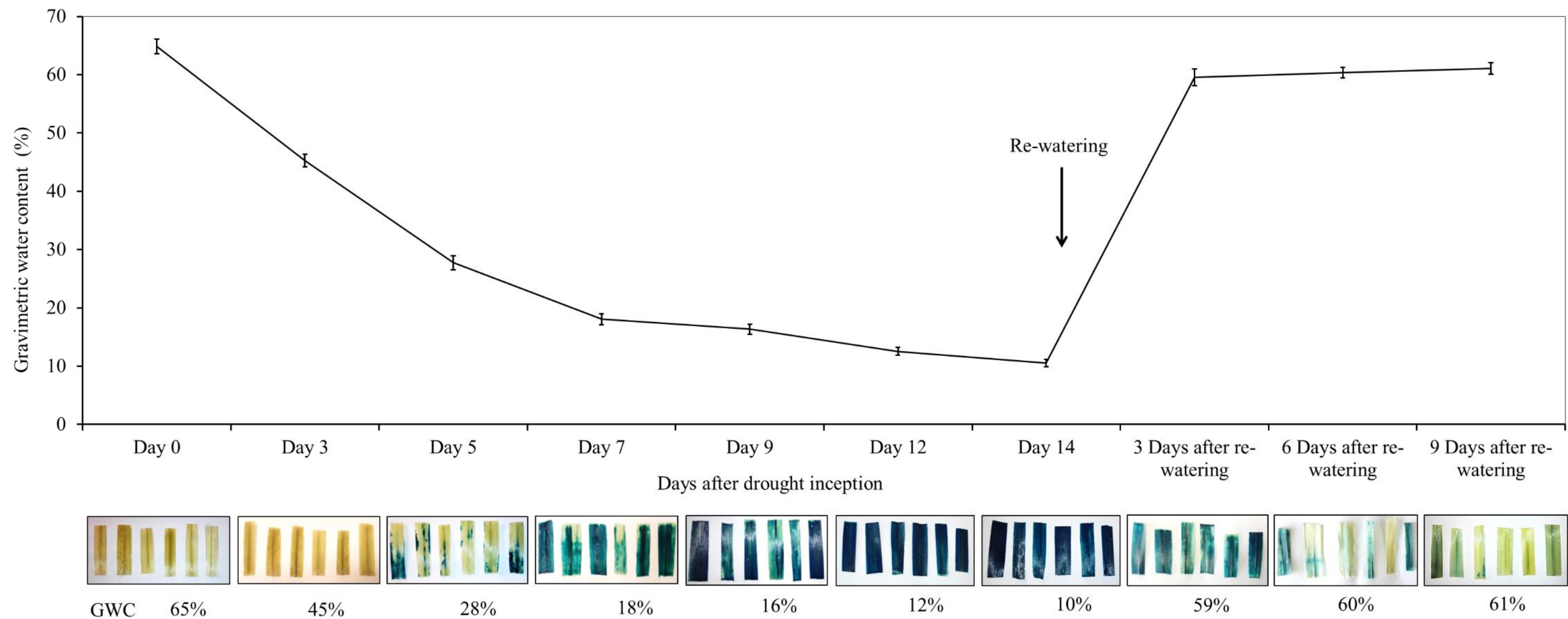
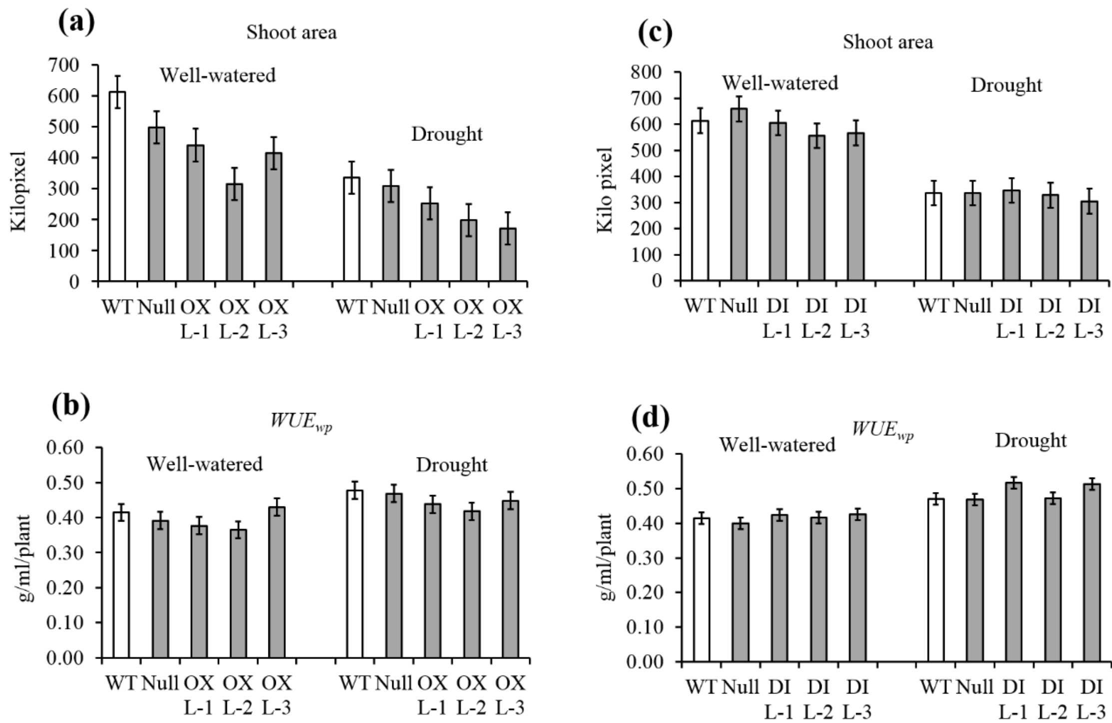


Figure 1. Expression of *GUS* gene in *Zm-Rab17*:*GUS* transgenic plants to observed the drought inducible expression of *GUS* gene during the progression of water deficit and upon re-watering.

Shoot area and WUE_{wp} of the OX and DI transgenic plants

Digital non-destructive imaging of plants showed that the projected shoot area (kilo pixel) of the three independent transgenic barley lines expressing *Ath*-miR827 (OX L-1, 2 and 3) either decreased or remained the same compared to non-transgenics, both under well-watered and drought (18% GWC) conditions (Fig. 2a). The WUE_{wp} of OX L-1 and OX L-3 was not significantly different compared to the WT plants under both watering regimes, whereas WUE_{wp} of OX L-2 displayed a significant reduction compared to WT plants for both treatments (Fig. 2b). Imaging drought inducible transgenic plants expressing Hv-miR827 driven by *Zm-Rab17* (DI L-1, 2 and 3) showed that the three independent transgenic barley lines had no significant reduction in projected shoot area compared to non-transgenic controls for both treatments (Fig. 2c). Additionally, no significant difference in WUE_{wp} was observed for DI L-1, 2 and 3 transgenic plants relative to WT controls under well-watered conditions. However, two of these lines (DI L-1 and 3) showed significantly higher WUE_{wp} compared to the non-transgenic controls under drought (Fig. 2d).



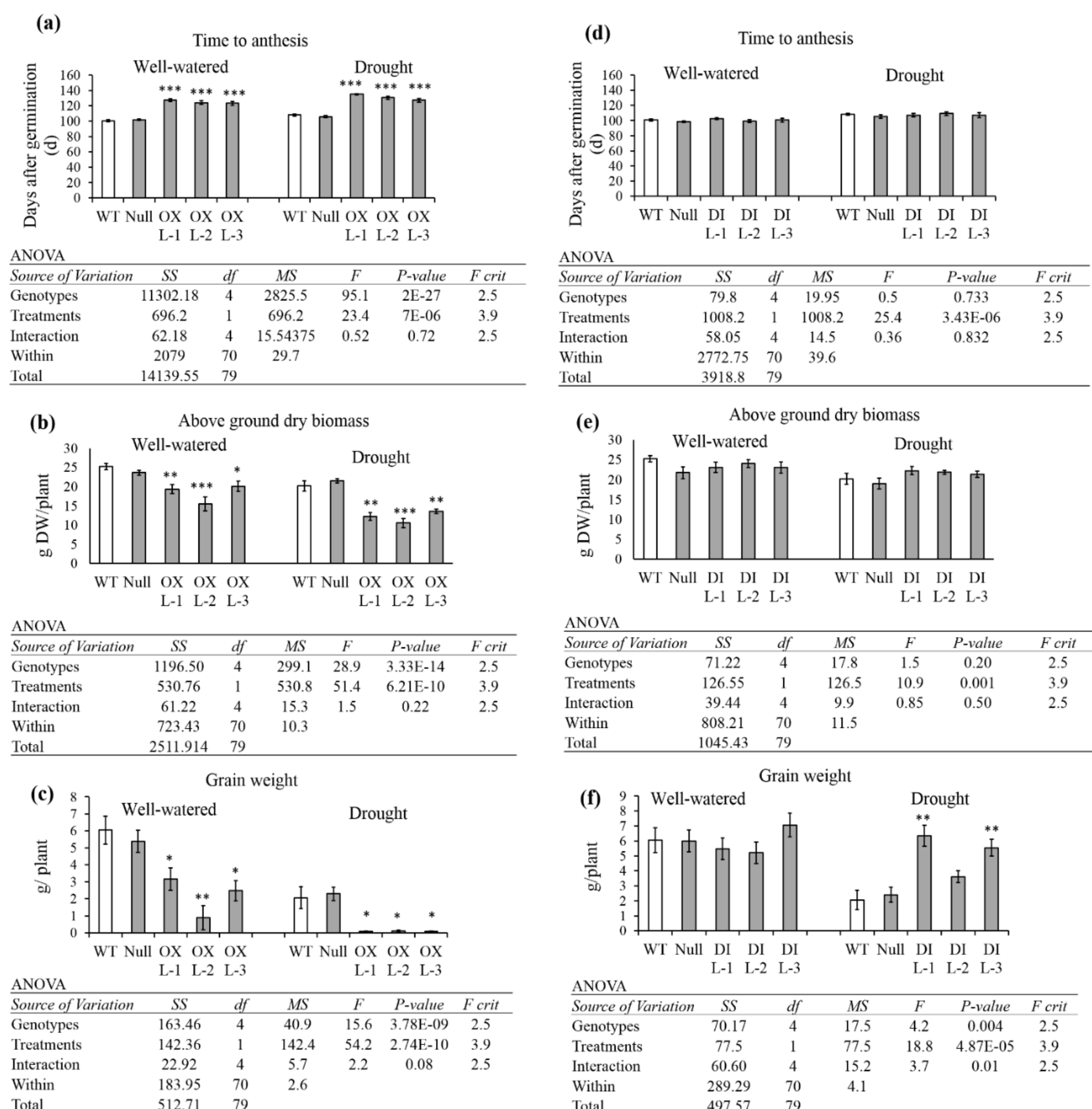
Summary result from statistical analysis	
Genotypes	**Shoot Area p-values**
DI lines	
Treatment	<0.001
Genotype	0.420
Treatment:Genotype	0.380
OX lines	
Treatment	<0.001
Genotype	<0.001
Treatment:Genotype	0.073
Genotypes	**WUE_{wp} p-values**
DI lines	
Treatment	<0.001
Genotype	<0.001
Treatment:Genotype	0.168
OX lines	
Treatment	<0.001
Genotype	0.046
Treatment:Genotype	0.159

Figure 2. Phenotyping parameters of non-transgenic plants and T_3 transgenic plants from the non-destructive imaging in the smarthouse green house condition. (a) Projected shoot area and (b) water use efficiency of well-watered and drought treated plants constitively expressing from *CaMV-35S*::*Ath*-miR827 (OX lines). (c) Projected shoot area and (d) water use efficiency of well-watered and drought treated *Zm-Rab17*::*Hv*-miR827 transgenic showing drought inducible expression (DI lines) and non-transgenic plants. Drought treatment was maintenance at 18% GWC of soil.

Figure 2. Legend cont.
Projected shoot area (kilopixel) was derived from high resolution visible light (RGB) plant images. Wild type 'Golden Promise' and null segregants (of the respective construct) are used as the non-transgenic controls. Three independent events of the over-expressing lines are depicted by OX line-1, 2 and 3, and drought inducible lines are depicted by DI line 1, 2 and 3. The error bars are ±0.5 lsd (n= 8). The means are significantly different when the error bars do not overlap. A summary of the analysis of variance is shown below the graphs.

Anthesis, shoot biomass and grain weight of the transgenic plants

Comparisons of non-transgenic control plants to T_3 progenies of constitutively over-expressed lines revealed a significant delay of anthesis under both control and drought treatment (18% GWC) (Fig. 3a). OX lines' above ground shoot dry biomass and grain weight significantly differed from non-transgenic controls under both watering regimes (Fig. 3b & c). However, for either watering regime, no differences in time to anthesis were observed for DI lines when compared with non-transgenics (Fig. 3d). Average above-ground shoot dry biomass of DI lines was similar to non-transgenics for both watering regimes (Fig. 3e). Two of the DI lines; DI L-1 and L-3 maintained grain weight under drought treatment, whilst grain weight of DI L-2 was similar to non-transgenics upon drought (Fig. 3f).



(a) ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Genotypes	11302.18	4	2825.5	95.1	2E-27	2.5
Treatments	696.2	1	696.2	23.4	7E-06	3.9
Interaction	62.18	4	15.54375	0.52	0.72	2.5
Within	2079	70	29.7			
Total	14139.55	79				

(d) ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Genotypes	79.8	4	19.95	0.5	0.733	2.5
Treatments	1008.2	1	1008.2	25.4	3.43E-06	3.9
Interaction	58.05	4	14.5	0.36	0.832	2.5
Within	2772.75	70	39.6			
Total	3918.8	79				

(b) ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Genotypes	1196.50	4	299.1	28.9	3.33E-14	2.5
Treatments	530.76	1	530.8	51.4	6.21E-10	3.9
Interaction	61.22	4	15.3	1.5	0.22	2.5
Within	723.43	70	10.3			
Total	2511.914	79				

(e) ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Genotypes	71.22	4	17.8	1.5	0.20	2.5
Treatments	126.55	1	126.5	10.9	0.001	3.9
Interaction	39.44	4	9.9	0.85	0.50	2.5
Within	808.21	70	11.5			
Total	1045.43	79				

(c) ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Genotypes	163.46	4	40.9	15.6	3.78E-09	2.5
Treatments	142.36	1	142.4	54.2	2.74E-10	3.9
Interaction	22.92	4	5.7	2.2	0.08	2.5
Within	183.95	70	2.6			
Total	512.71	79				

(f) ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Genotypes	70.17	4	17.5	4.2	0.004	2.5
Treatments	77.5	1	77.5	18.8	4.87E-05	3.9
Interaction	60.60	4	15.2	3.7	0.01	2.5
Within	289.29	70	4.1			
Total	497.57	79				

Figure 3. Phenotyping parameters of non-transgenic plants and the three independent events of over-expressers (OX lines) and drought inducible expressers (DI lines) T_3 transgenic plants. (a) Time to anthesis, (b) above ground dry biomass and (c) grain weight of well-watered and drought treated OX lines (*CaMV-35S*::*Ath*-miR827). (d) Time to anthesis, (e) above ground dry biomass and (f) grain weight of well-watered and drought treated DI lines (*Zm-Rab17*::*Hv*-miR827). Drought treatment was achieved by maintenance of stress at 18% GWC of soil. Values are the mean ± standard error of mean (s.e.m) at $P < 0.05$ by t test (n= 8). Significant difference compared to the wild type is depicted by asterisk (*). ANOVA tables from two-way ANOVA are also shown below the corresponding graphs.

Based on phenotyping results from non-destructive imaging, we observed several unexpected phenotypes; that is, reduced shoot area, WUE_{wp}, delayed anthesis, grain weight per plant and reduced shoot biomass of OX lines compared with non-transgenics upon both drought and under well-watered conditions. DI lines did not exhibit growth penalties or altered time to anthesis compared to non-transgenics, but showed higher WUE_{wp} compared with non-transgenics exposed to drought. Therefore, DI lines were further evaluated.

DI lines recovered from mild drought stress

Assaying recovery from mild drought stress, in which the GWC declined from 65% to 16%, upon re-watering, the DI lines performed better relative to controls (Fig. 4). Additionally, DI lines had a faster recovery in terms of rescue from wilting (Fig. 4) and significantly higher fresh biomass, compared to the non-transgenic plants (Fig. S5).

Figure 4. Behaviour of non-transgenic and transgenic (T4) plants with drought-inducible expression of *Hv*-miR827 (*Zm-Rab17*::*Hv*-miR827) under progression and recovery from water deficit. Re-watering started when GWC declined from 65% to 16%. Three independent events of the *Zm-Rab17*::*Hv*-miR827 plants are depicted by DI lines (DI L-1, 2 and 3). Images of non-transgenic and DI lines after drought inception and re-watering (n= 8).

DI lines exhibit improved physiological response and survival rate during severe drought stress

Under severe drought stress, when GWC declined from 65% to 10% (Fig. S6a) before re-watering, a higher percentage of DI L1 and DI L3 survived relative to non-transgenic plants, however the survival rate for DI L-2 did not differ from non-transgenics (Fig. S6b and c).

The same two DI lines (DI L-1 and DI L-3) showed higher instantaneous leaf level stomatal conductance (g_s) and photosynthetic assimilation (A) relative to non-transgenic plants at this time point (Fig. 5a & b). Changes in leaf chlorophyll content (SPAD units/ per 2 x 3 mm area of leaf) were measured in fully expanded leaf blades from transgenic and control plants at 0, 6, 9 and 12 days post drought inception. At 12% GWC, DI L-1 and DI L-3 exhibited higher leaf chlorophyll content relative to non-transgenics, with DI L-2 showing a similar leaf chlorophyll content to non-transgenics (Fig. 5c). Additionally, DI L-2 showed similar stomatal conductance (g_s) and photosynthetic assimilation (A) compared to non-transgenics at 12% GWC (Fig. 5a & b).

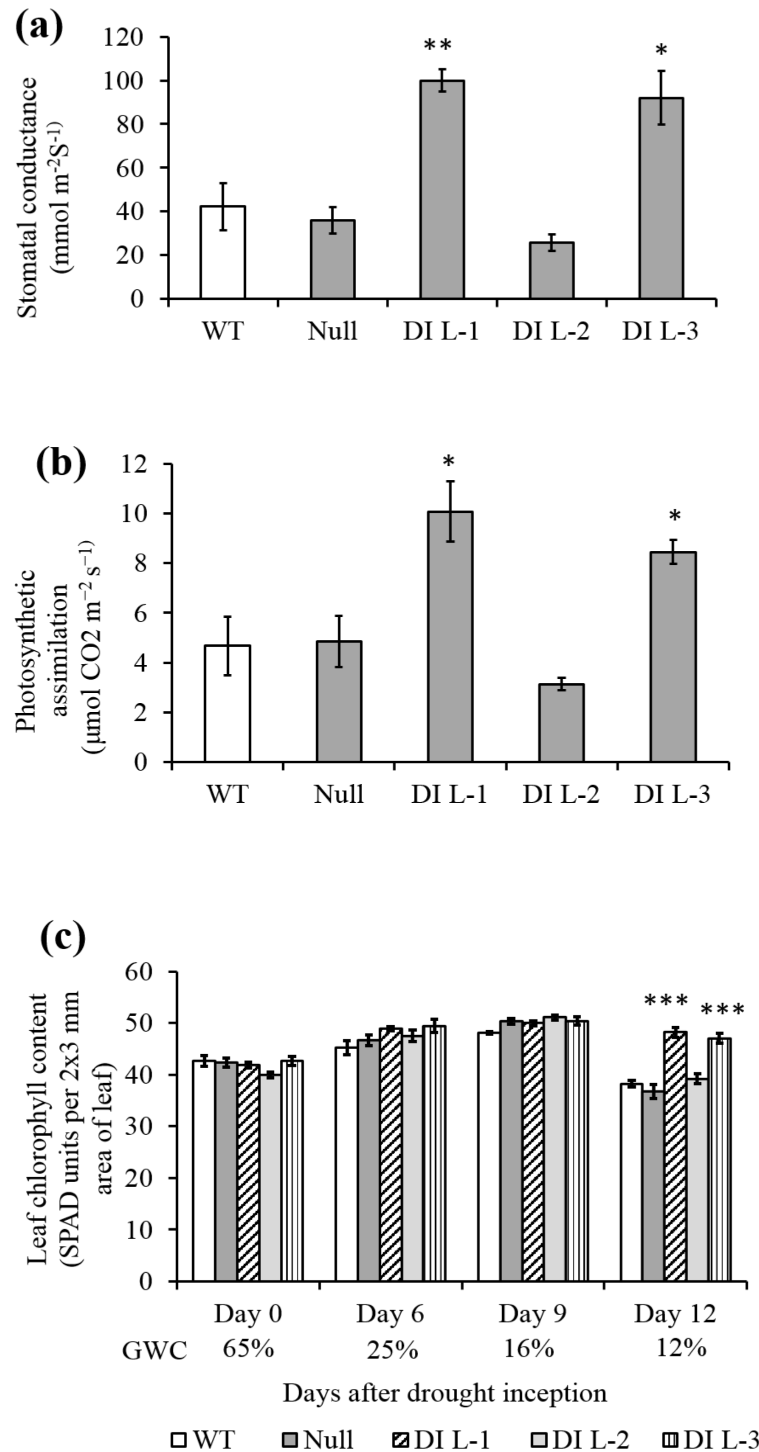
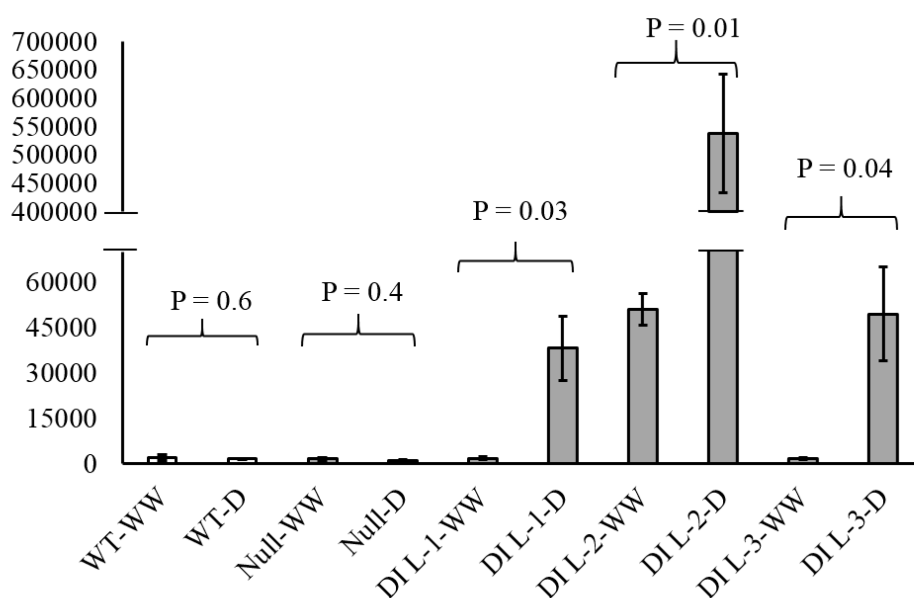


Figure 5. Gas exchange and leaf chlorophyll content of non-transgenic plants and T4 generations of the three independent events of DI lines under severe drought stress. (a) Stomatal conductance (n= 3), (b) photosynthetic assimilation (n= 3) and (c) leaf chlorophyll (n=5), were measured from the leaf samples of severe water deficit (when GWC declined from 65% to 12%). Values are the mean ± s.e.m at $P < 0.05$ by t test. Significant difference compared to the wild type is depicted by asterisk (*).

Zm-Rab17 driven expression of *Hv*-miR827 in DI lines

Both endogenous and transgene derived *Hv*-miR827 transcriptional changes in leaf tissue induced by *ZmRab17* in response to drought for the DI lines were assayed and compared to non-transgenics under both drought and well-watered conditions. The detectable levels of mature *Hv*-miR827 in the three DI lines reflected the drought inducibility of the *ZmRab17* promoter (Fig. 6). DI L-1 and DI L-3 showed a similar level of induction under drought , whereas DI L-2 was significantly stronger ($P = 0.01$) (Fig. 6). Endogenous expression of *Hv*-miR827 in non-transgenics was below detection limits and no change could be detected between treatments. The drought inducible expression from the *ZmRab17* promoter, was confirmed in the expression patterns in DI L-1 and DI L-3 where barely detectable expression was seen under the well-watered treatment. However, DI L-2 showed higher levels of transgene derived *Hv*-miR827 expression relative to non-transgenics under both watering regimes indicating that the *Zm-Rab17* promoter showed abnormal expression or was highly leaky in this line (Fig. 6).



ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Genotype	3.85057E+11	4	96264367626	19.00	1.37E-06	2.86
Treatments	97528376032	1	97528376032	19.25	0.0003	4.35
Interaction	2.63552E+11	4	65887908817	13.00	0.00002	2.86
Within	1.01296E+11	20	5064808187			
Total	8.47434E+11	29				

Figure 6. Quantification of mature miRNA *Hv*-miR827 in non-transgenic and T4 transgenic plants (*Zm-Rab17*::*Hv*-miR827). Level of expression of *Hv*-miR827 was detected by qRT-PCR in 2µg of total RNA from well-watered and drought treated (at 12% GWC of soil) samples. Mature leaf blade of non-transgenic and DI line plants were used (n=3). Well-watered plants are depicted as 'WW' and drought treatment plants are depicted as 'D'. Values are the mean ± s.e.m at $P < 0.05$ by t test. ANOVA table from two-way ANOVA is shown below the graph.

Putative targets of *Hv*-miR827 and expression analysis of target genes in DI lines

From the *in silico* analysis, we obtained numerous predicted targets of *Hv*-miR827 (Supplementary Table 1). The putative targets included *SPX* encoding transcripts, nucleotide-binding site–leucine-rich repeat (NBS-LRR) proteins, caseinolytic protease (Clp) amino terminal domain, aberrant pollen transmission 1 (*APT1*) and TCP (TB1, CYC and PCFs) family transcription factors. Experimental confirmation of these targets was undertaken by interrogation of the degradome library to identify cleavage products in leaf tissue derived from the pooled leaves and roots of well-watered and drought treated plants. However, no cleavage products were identified for any of the putative targets. Previously, *Osa*-miR827 was reported to target two genes encoding *SPX*, with mRNA cleavage of both targets at the predicted canonical site being confirmed in rice (cv. Nipponbare) (Lin *et al*., 2010). To get an indication whether *SPX* was a likely target for *Hv*-miR827 in barley, we determined whether there was an inverse correlation in *SPX* abundance (JLOC1_37332/ MLOC_57566) relative to *Hv*-miR827 at the transcript level. For this purpose, we used transgenic and non-transgenic plants differing in total *Hv*-miR827 abundance. We did not observe an inverse correlation between *Hv*-miR827 and *SPX* in either the DI lines or non-transgenic controls (Fig. S7). Interestingly, we observed increased *SPX* transcript abundance under drought for DI L-1 and DI L-3. For DI L-2, the expression of the *SPX* transcript did not significantly differ between the two watering regimes.

Sequencing revealed that there are three and two nucleotides mismatches between the complementary pairing of *Ath*-miR827: target site and *Hv*-miR827: target site, respectively (Fig. S8).

Discussion

The availability of barley deep sequencing datasets and the application of bioinformatics tools have facilitated the identification of both conserved and novel miRNAs (Schreiber et al., 2011). Such bioinformatics analyses have revealed a number of conserved and novel miRNAs differentially expressed in barley under drought stress (Hackenberg et al., 2015). However, functional analysis of miRNAs and their cognate targets is challenging as methodologies for analysis typically require mutants or the generation of transgenics. Because mutant and other genomics resources in barley are not as well established as for the model crops *Arabidopsis* and rice, we took a transgenic approach towards functional validation of barley miR827. To date, stable genetic modification (GM) of a miRNA in barley has not been reported. We used the barley transgenic plants to determine the phenotypic effect of both transgenic and cisgenic miR827 on plant performance under drought, given that miR827 was reported to enhance wilting avoidance in the model species *Arabidopsis* (Auckerman & Park, 2009). Phenotyping of *Ath*/*Hv*-miR827 in transgenic barley indicated that this miRNA could play a regulatory role leading to improved plant performance under drought.

One of the first reactions of plants to abiotic stress is reduced growth (Boyer, 1970). Growth reduction allows plants to minimise water consumption (Morran *et al.*, 2011). In this study we observed reduced shoot area of the *Ath*-miR827 over-expressers (OX lines). Other observed phenotypic differences included, WUE_{wp}, time to anthesis, shoot biomass and grain weight per plant , which were negatively affected compared to the WT plants under both watering regimes (Fig. 2a-b, 3a-c). Delayed anthesis may have affected grain weight of the OX lines. These findings are somewhat unexpected considering these abnormal growth and development phenotypes were not reported in the model species *Oryza* (Lin *et al.*, 2010). This indicated that miR827 derived from the dicot *Arabidopsis* may not function normally in barley. One plausible explanation, is

that *Ath*-miR827 over-expression causes aberrant down-regulation of its cognate mRNA targets, resulting in perturbed growth and development. Predicted targets for *Ath*-miR827 in barley include membrane protein SPX, NBS-LRR domain containing proteins, aberrant pollen transmission 1 (*APT1*), seven in absentia (*SINA*) family proteins, protein kinase domain containing proteins and F-box/LRR-repeat proteins (Table S1). *SPX* was negatively regulated by *Osa*-miR827 and has been reported to function in phosphate (Pi) sensing in rice (Lin *et al.*, 2010). Maize *APT1* was previously reported to be involved in pollen tube growth (Xu and Dooner, 2006). *APT1* is also a member of a conserved protein family vital for cell elongation in higher plants (Xu and Dooner, 2006). Another putative target of *Ath*-miR827, *SINA*, was reported to promote drought tolerance in *Arabidopsis* in an ABA-dependent manner (Bao *et al.*, 2014). However, when aligned to the degradome sequences of 'Golden Promise', no cleavage products were obtained for any of the putative target sequences. This observation suggests that *Ath*-miR827 mediated target gene regulation might not be through post-transcriptional cleavage or that *Ath*-miR827 might have other yet unknown target(s) in barley. It is possible that the target genes are involved in development or flowering. This might have caused disruption of genes related to phenology affecting the combined phenotypes such as shoot area, anthesis time, biomass and grain yield in the *Ath*-miR827 over-expressers plants compared to that of the non-transgenic plants.

We tested the expression of mature *Hv*-miR827 and a predicted target *SPX* encoding transcript in the DI lines and found no inverse correlation of *Hv*-miR827 and *SPX* transcripts under drought (Fig. 6 & Fig. S7). Interestingly, we observed drought induced expression of this target transcript in DI L-1 and DI L-3 compared to the non-transgenic plants (Fig. S7). We also examined the miR827 target site sequence in 'Golden Promise' which revealed that there were mismatches between the miRNA: target duplex including at the 5′ 9th nt position of both *Ath*-miR827 and *Hv*-miR827

(Fig. S8). While, typically extensive complementarity (≤ 5 mismatches) is required to ensure functional targeting, base pairing between the 5′ 2 to 13 nt positions of a miRNA with its target is critical for miRNA-mediated target suppression (Liu *et al*., 2014). Additionally, base pairing at the central positions, the 9 to 11 nt of miRNA:target sites, are particularly important for miRNA and the target pairing in the vicinity of the AGO-catalyzed slicing site (Parizotto *et al*., 2004; Schwab *et al*., 2005; Liu *et al*., 2014). Liu *et al*. (2014) demonstrated that a single nucleotide mismatch between miRNA and the target at positions 9 and 10, as well as combinations of mismatches at positions 9-11, resulted in complete elimination of the responsiveness of a miR164-targeted sensor sequence in *Nicotiana benthamiana*.

Constitutive over-expression of a transgene can result in a different degrees of growth retardation and delayed flowering in plants (Oh *et al*., 2007). It has been suggested that the use of stress-inducible promoters can minimize the undesirable phenotypes observed under the constitutive expression of a transgene (Kasuga *et al*., 1999; Morran *et al*., 2011). The *Zm-Rab17* promoter is reported to be responsive to abscisic acid (ABA) and water stress (Busk *et al*., 1997; Lopato *et al*., 2012). In our experiment, plants expressing *Hv*-miR827 under the control of the drought inducible (DI) promoter *Zm-Rab17* showed several promising phenotypes under drought treatment. The higher WUE_{wp} of the two DI lines, DI L-1 and DI L-3 (Fig. 2d), and higher grain weight per plant of DI L-1 and DI L-3 (Fig. 3f) indicated that these two lines required less water than other genotypes to provide the same shoot area and better maintenance of grain weight under drought than the other genotypes.

In this study, efforts were made to ensure that all the factors other than the desired treatments (mild and severe drought) were non-limiting during the experimental period. Our observations suggest that the extent of water deficit might result in variation in the phenotypic performance of the drought induced lines. In a mild drought

experiment, all plants of the tested DI lines and the non-transgenic plants recovered after the drought period. However, the transgenic plants showed faster recovery compared to the non-transgenic plants (Fig. 4). Additionally, the increased plant biomass of the transgenics upon re-watering (Fig. S5) suggested that drought induced expression of *Hv*-miR827 improved the ability of barley plants to recover from mild drought stress. Under severe drought stress (when the GWC declined from 65% to 10%, differences in both plant mortality and in the speed of recovery among the DI lines were observed (Fig. S6a & b) with DI L-1 and DI L-3 plants' higher survival indicating their better performance than the other genotypes (Fig. S6c). Under severe drought stress, the super-abundant accumulation of mature *Hv*-miR827 in DI L-2 (Fig. 6) led to high mortality of this line under severe drought stress relative to the other transgenics. Under mild drought stress, this was not the case indicating that recovery after re-watering depends on drought intensity and duration (Xu *et al*., 2010). This observation suggests that the level of drought-induced accumulation of miR827 has an impact on the phenotypic performance of the transgenic plants. These observations again suggest that the high level of expression of *Ath*/*Hv*-miR827 might not be effective for improving the performance of barley plants under drought.

In our study, as one of the early observations of DI L-1 and DI L-3 was improved WUE_{wp} under mild drought, we expected that the main driver of increased water-use efficiency in these two DI lines would be decreased transpirational water loss through low stomatal conductance compared to the non-transgenic plants. However, we still observed higher stomatal conductance, photosynthetic assimilation, and relative leaf chlorophyll content of the DI L-1 and DI L-3 compared to the non-transgenic plants under severe drought stress (Fig. 5a-c), whereas DI L-2 behaved similarly to the non-transgenic plants under severe drought stress (Fig. 5a-c). These observations suggest that higher photosynthetic assimilation in these two lines could potentially contribute to

the increased WUE_{wp}. The ability of DI L-1 and DI L-3 to maintain key physiological processes, such as photosynthetic assimilation during severe drought treatment is indicative of the potential of these lines to support productivity under water deficit (Centritto *et al*. 2009). A preliminary field trial with the three DI lines, WT and null plants was conducted at a single site in 2015 and showed encouraging result (data not shown). However, extensive field trials under drought at multiple sites and in large plots will be necessary to confirm the yield benefits seen in the preliminary field.

Conclusion

In this study, drought induced expression of *Hv*-miR827 improved whole plant water use efficiency of transgenic barley under drought. The drought induced expression of *Hv*-miR827 influenced grain weight per plant, and enabled plants to recover after drought treatment. Our findings suggested that unlike the drought inducible expression of *Hv*-miR827, constitutive over-expression of *Ath*-miR827 had a negative effect on growth, time to anthesis and grain weight of transgenic plants. It was clear that the phenotypic performance of transgenic barley plants expressing *miR827* could depend on the nature of promoter. These findings suggest the possibility of creating water use efficient transgenic barley utilising *Hv*-miR827 under the control of the *Zm-Rab17* promoter.

Acknowledgements

This research was supported by the Australian Centre for Plant Functional Genomics (ACPFG), funded through a grant from DuPont/Pioneer (USA). Our grateful thanks to Patricia Warner and ACPFG Transformation Group for barley transformation; Margaret Pallotta and Suzanne Manning for their assistance with Southern Blotting, Dr. Sergiy Lopato and Dr. Ainur Ismagul for producing the *Zm-Rab17*::*GUS* transgenic barley germplasm. We specially thank the team of The Plant Accelerator for technical support in running the experiment and conducting the image analysis. The Plant

Accelerator, Australian Plant Phenomics Facility, is funded under the National Collaborative Infrastructure Strategy. We are thankful to Dr. Ursula Langridge, Alex Kovalchuk and Yuri Onyskiv for their assistance with growing plants at different phases of this experiment, and Yuan Li and Hui Zhou for performing the qRT-PCR. We also thank Dr. Bu-Jun Shi for his advice at the early stage of this experiment.

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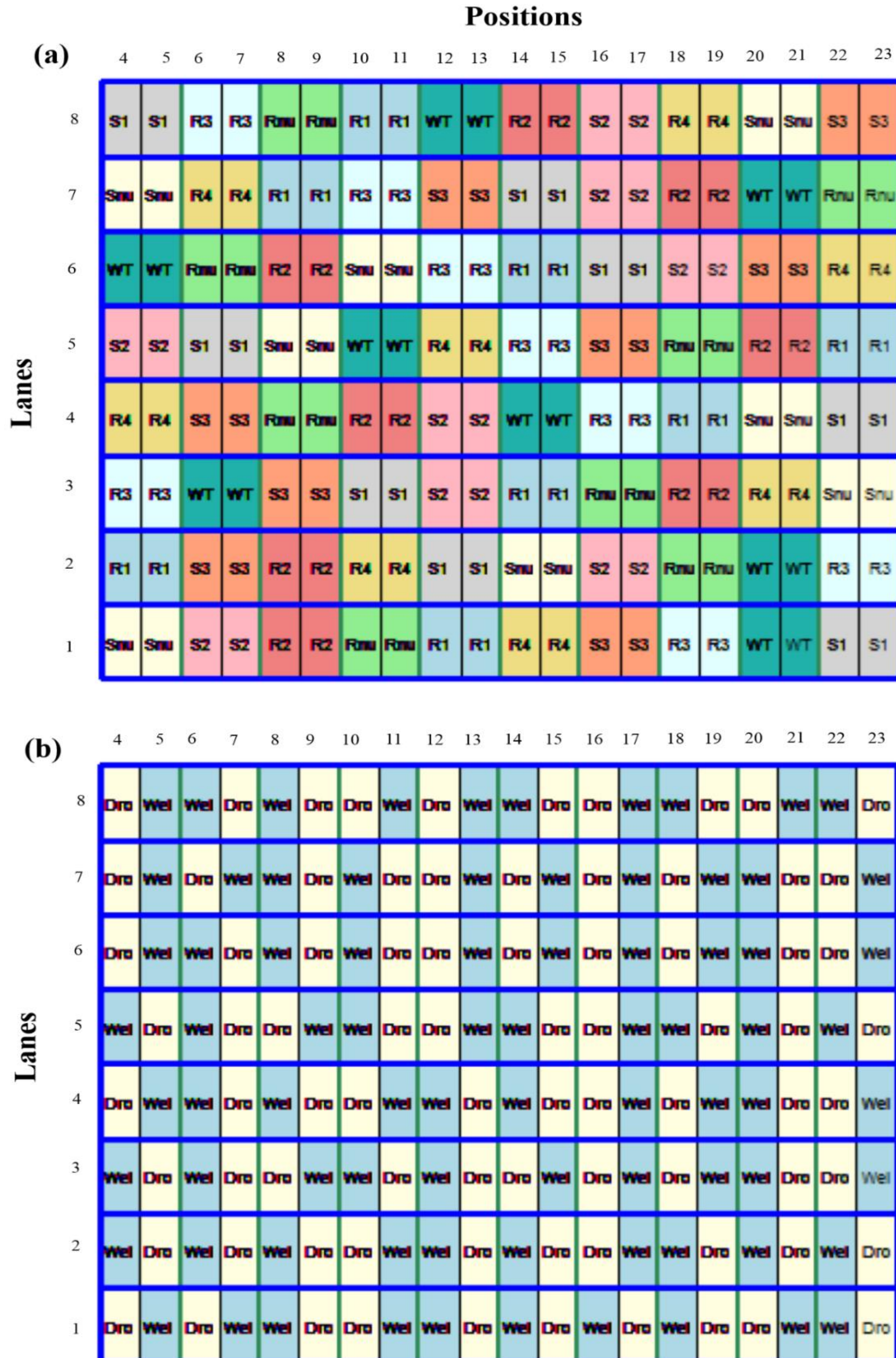
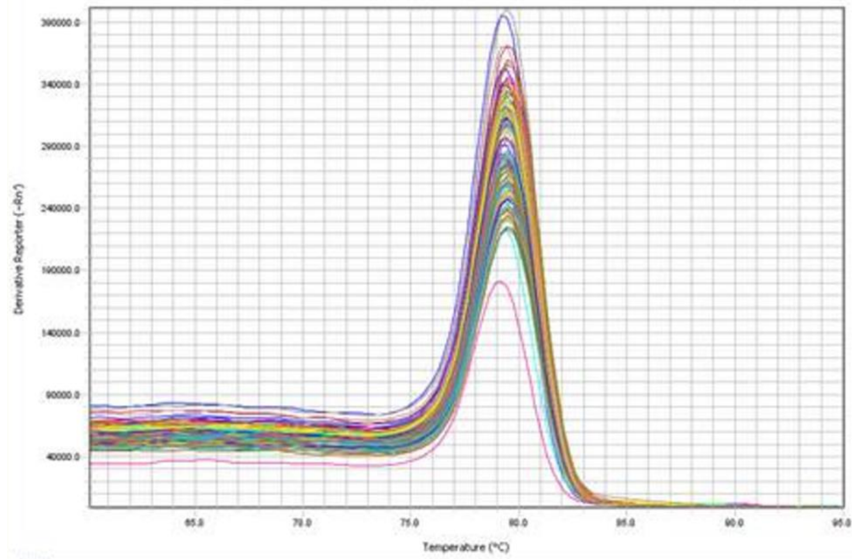
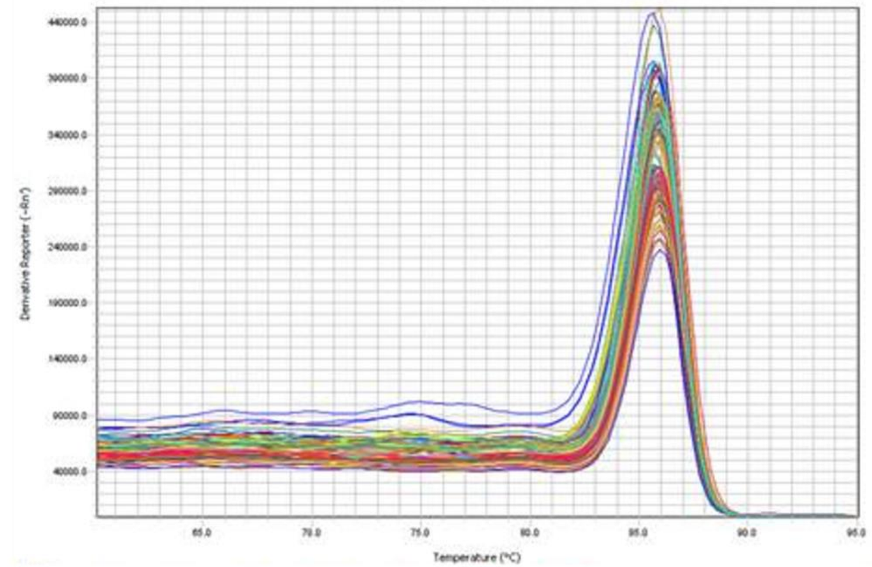


Supplementary Figure 1. Spatial design for smart house experimentation. (a) Conveyor layout of genotypes. Green border indicates pots. The independent events of the *CaMV-35S*::*Ath*-miR827 transgenic barley are coded as S1, S2, S3. The independent events of the *Zm-Rab17*::*Hv*-miR827 transgenic barley are coded as R1, R2, R3, R4. 'Snu' are null plants from *CaMv-35S*::*Ath*-miR827 construct. 'Rnu' are null plants for the *Zm-Rab17*::*Hv*-miR827 construct. 'WT' is wild type barley cv. Golden Promise. (b) Conveyor layout for watering depicting respective lanes and positions. Green border indicates pots and blue border indicates replicates. 'Dro' and 'Wel' represent drought and well-watered respectively.

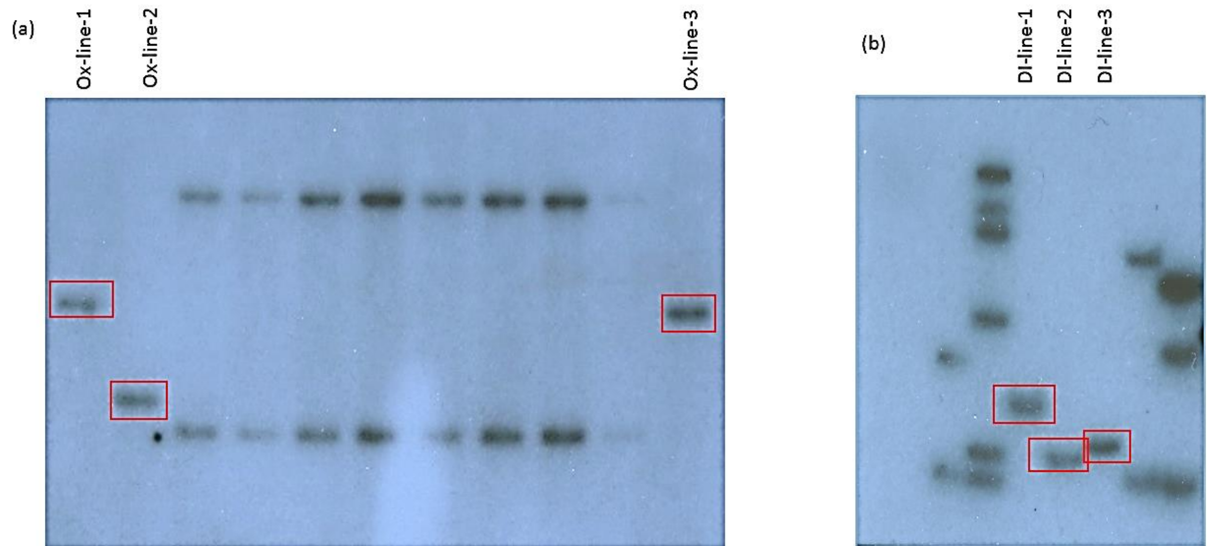
Hv-miR827



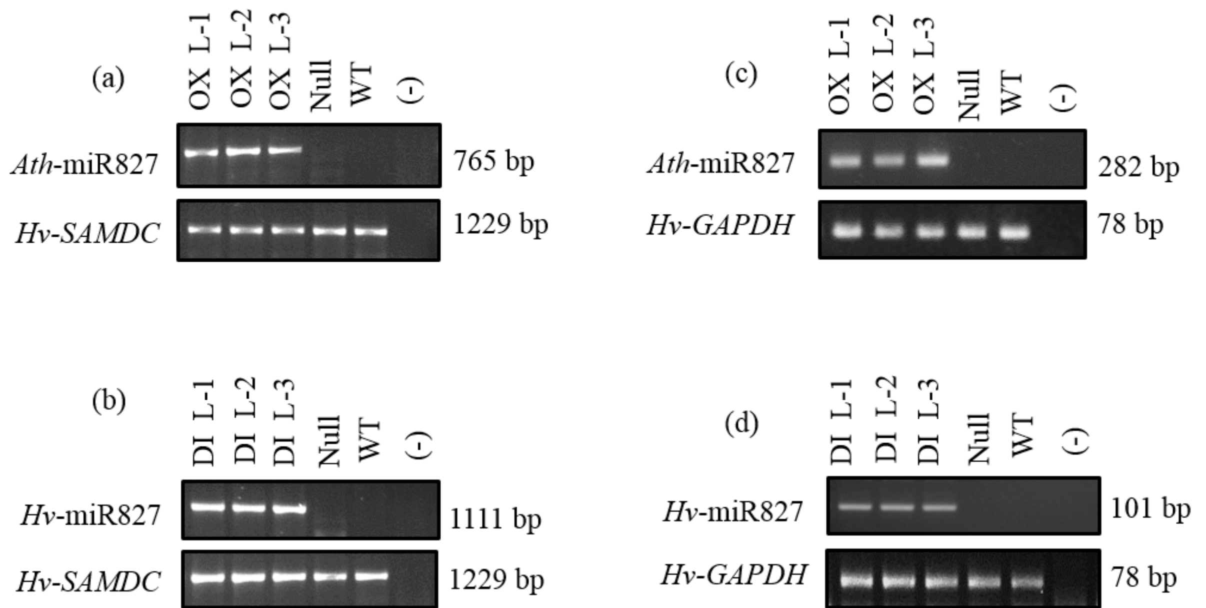
SPX domain encoding transcript (JLOC1_37332)



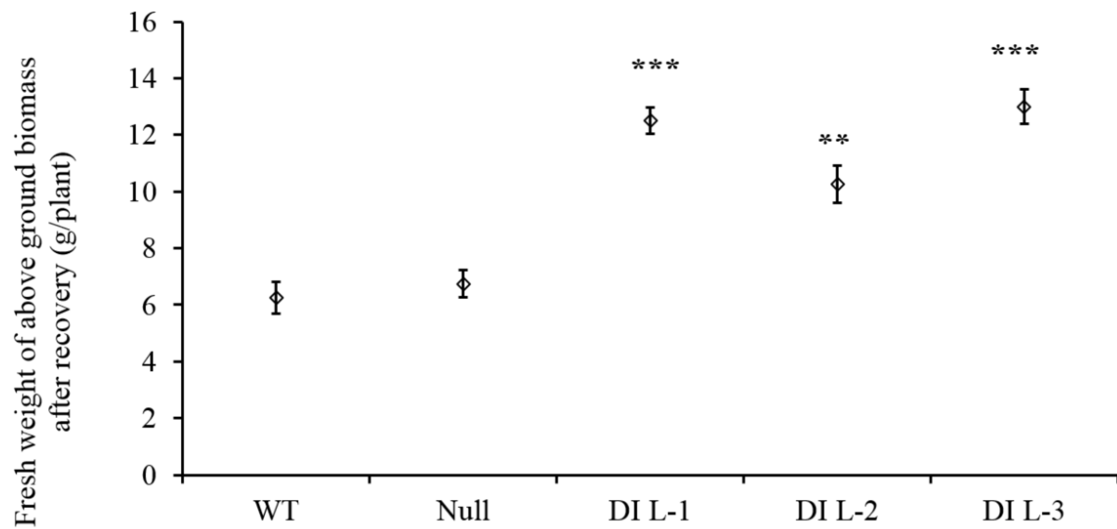
Supplementary Figure 2. Melt curve plots for miRNA and mRNA qRT-PCR.



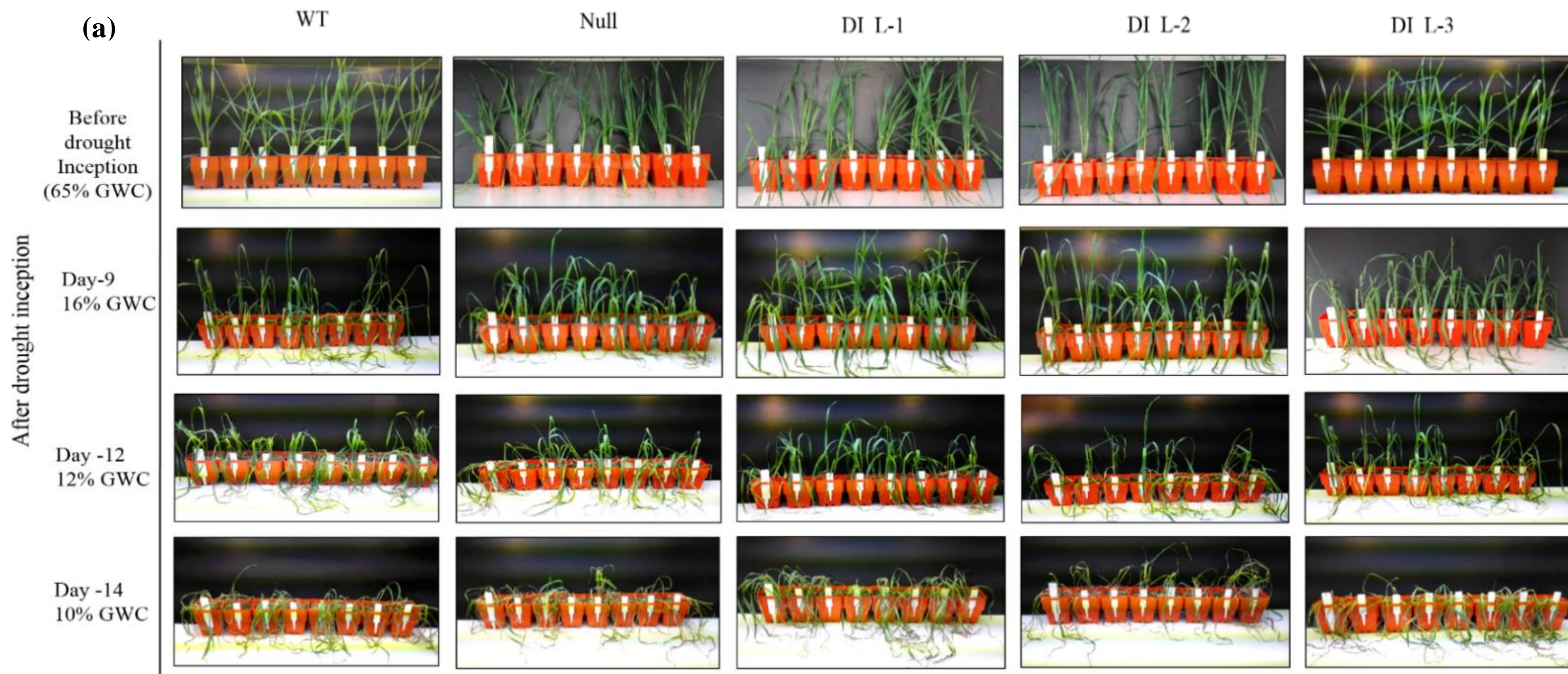
Supplementary Figure 3. Southern blot of the transgenic plants for selecting the copy number of the transgene. (a) *CaMV-35S*::*Ath*-miR827 (OX L-1, OX L-2 and OX L-3) and (b) *Zm-Rab17::Hv*-miR827 (DI L-1, DI L-2 and DI L-3) single copy plants (indicated in the red boxes) were selected.

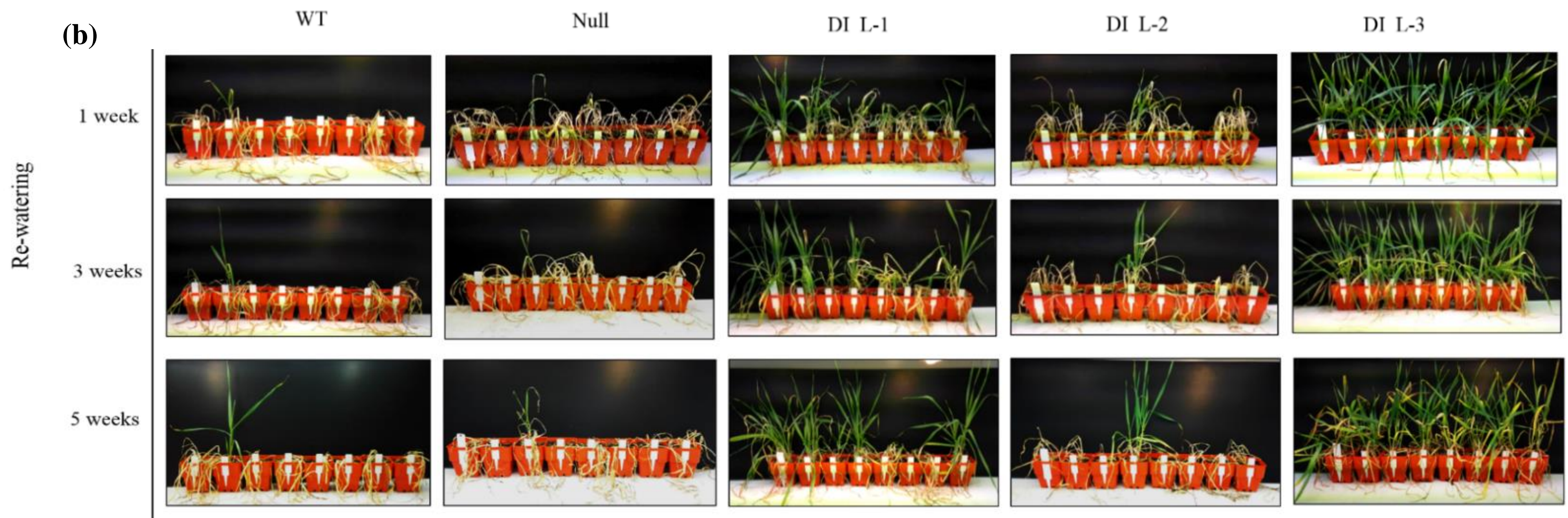


Supplementary Figure 4. Molecular characterization for transgenic barley expressing either *Ath*-miR827 under the control of *CaMV-35S* promoter or *Hv*-miR827 under the control of *Zm-Rab17* promoter. Polymerase Chain Reaction (PCR) detection for the presence of (a) *CaMV-35S*::*Ath*-miR827 transgene using *CaMV-35S* specific forward and *Nos*-terminator specific reverse primer, and (b) *Zm-Rab17*::*Hv*-miR827 transgene using *Zm-Rab17* specific forward and *Nos*-terminator specific reverse primer. The presence of a barley specific endogene *Hv-SAMDC* (internal control) was confirmed using *Hv-SAMDC* specific primers. RT-PCR used to detect expression of (c) *Ath*-miR827 with the precursor-*Ath*-miR827 and the *Nos*-terminator specific primers and of (d) *Hv*-miR827 with the precursor-*Hv*-miR827 and the Nos-terminator specific primers. *Hv-GAPDH* expression was used as an internal control. (-) = negative (water) control, e WT = wild type, nulls = plants regenerated from tissue culture and did not receive the transgene. For *CaMV-35S*::*Ath*-miR827 over-expressers, three independent events are shown in lane OX L-1, 2 and 3, whilst for *Zm-Rab17*::*Hv*-miR827 drought inducible expressers, three independent events are shown in lane DI L 1, 2 and 3.



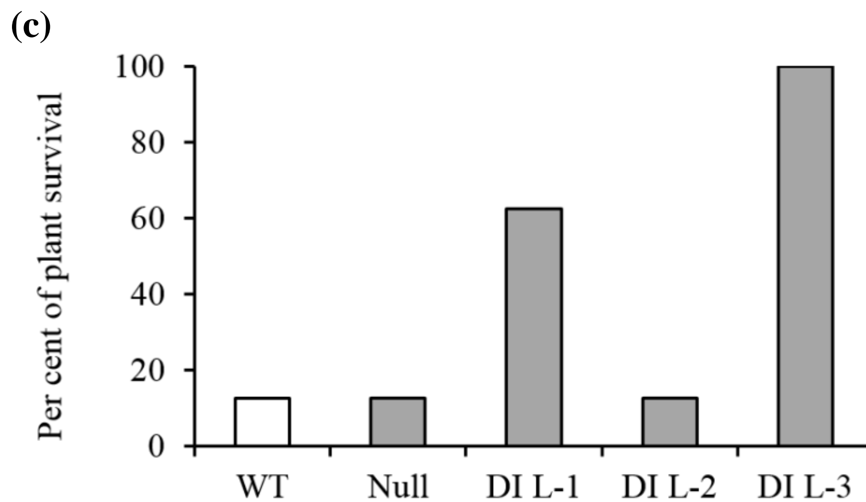
Supplementary Figure 5. Fresh weight of above ground biomass of the non-transgenic and transgenic (T4) plants (n= 8) with drought-inducible expression of *Hv*-miR827 (*Zm-Rab17*::*Hv*-miR827) lines after recovery from mild drought stress. Values are the mean ± s.e.m at $P < 0.05$ by t test. Significant difference compared to the wild type is depicted by asterisk (*).


(a)
WT
Null
DI L-1
DI L-2
DI L-3
After drought inception
Before drought Inception (65% GWC)
Day-9 16% GWC
Day -12 12% GWC
Day -14 10% GWC

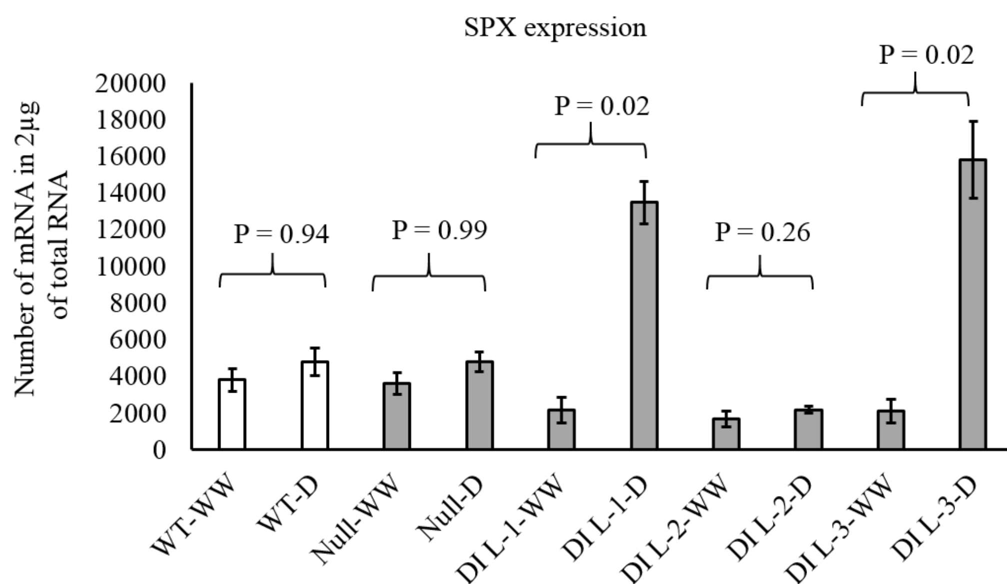


(b)
WT
Null
DI L-1
DI L-2
DI L-3
Re-watering
1 week
3 weeks
5 weeks

(c)



Supplementary Figure 6. Severe drought stress survival of non-transgenic and transgenic (T4 generation) plants. (a) Survival *Zm-Rab17*::*Hv*-miR827 barley plants from a severe drought stress. At 14 days post water deprivation, at which Gravimetric water content (GWC) declined to 10%, plants were re-watered. (b) DI lines showed difference in plant mortality and in speed of recovery post re-watering. DI L-1 and DI L-3 displayed increased drought stress survival relative to DI L-2 and non-transgenic controls. (c) Percentage survival of non-transgenic and transgenic DI lines upon re-watering post severe drought stress.

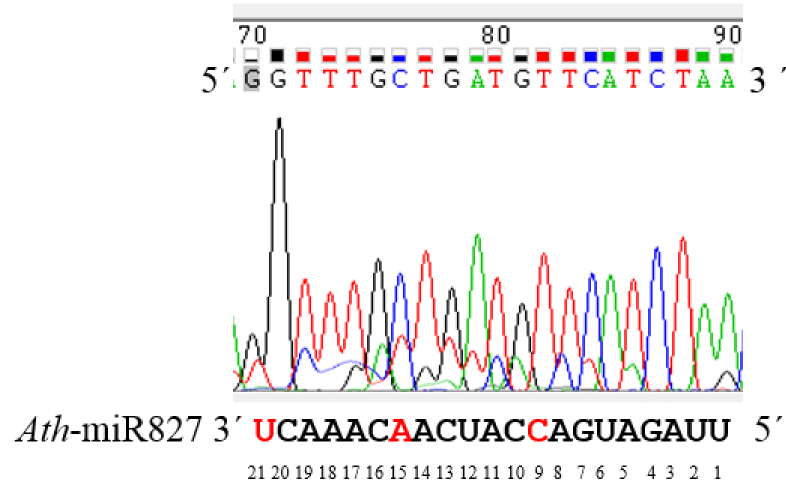


ANOVA

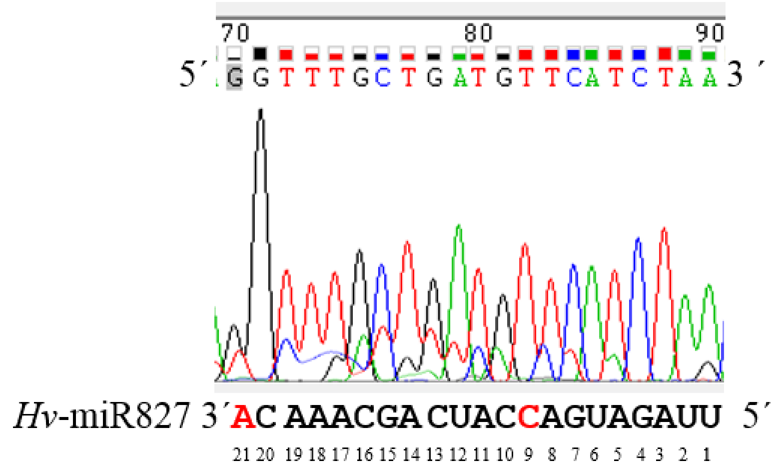
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Genotype	199089322.2	4	49772331	13.14	0.00002	2.87
Treatments	230631610.3	1	2.31E+08	60.87	1.71382E-07	4.35
Interaction	247630244.5	4	61907561	16.34	4.31943E-06	2.87
Within	75781375.91	20	3789069			
Total	753132552.9	29				

Supplementary Figure 7. Absolute transcript quantification of the putative *Hv*-miR827 target *SPX* in transgenic (*Zm-Rab17*::*Hv*-miR827, T4 generation) versus non-transgenic leaves. Expression quantification for *SPX* transcript (JLOC1_37332/ MLOC_57566) as detected by qRT-PCR on 2µg of total RNA from both well-watered and drought treated (at 12% GWC of soil) mature leaf samples. Three replicate mature leaf blades were collected from both non-transgenic (WT, Null) and transgenic lines (DI 1, 2 and 3). Samples from well-watered plants are depicted as 'WW' and those exposed to drought are depicted as 'D'. Values are the mean ± s.e.m at $P < 0.05$ by t test. ANOVA table from two-way ANOVA is shown below the graph.

(a) *Ath*-miR827: SPX transcript target site in 'Golden Promise'



(b) *Hv*-miR827: SPX transcript target site in 'Golden Promise'



Supplementary Figure 8. Sequence analysis of the target site within putative target transcript. Mismatches at (a) position 9nt, 15 nt and a G:U wobble pair at position 21nt (from 5'-end of the miRNA) between *Ath*-miR827 and target site sequence and (b) position 9nt and 21 nt (from 5'-end of the miRNA) between *Hv*-miR827 and target site sequence from barley cv. Golden Promise.

Chapter-6

General Discussion

This PhD project was designed to:

(i) investigate the natural variation in miRNAs among barley genotypes under drought stress to evaluate their promise for selecting drought tolerant genotypes based on miRNA expression profiles and

(ii) examine the potential value of miRNA over-expression as a tool to enhance drought tolerance using a sample miRNA in transgenic plants.

The main empirical findings are chapter specific and are presented in each chapter/paper. This chapter discusses the significance of outcomes generated in this PhD project and concludes with a consideration of future research directions.

Significance of the work

Barley is an economically important crop in much of the temperate world (Newton *et al*., 2011). Studies in plant molecular biology for improving barley drought tolerance have focussed on the analysis of candidate genes for pathways and processes associated with physiological responses to drought. However, the extant literature on upstream gene regulatory molecules such as miRNAs and their regulatory relationship to drought responsive target genes in barley is limited. In this PhD project, we detected significant changes in expression for several miRNAs when comparing expression under drought and well-watered conditions in barley, and showed that the differential expression of these miRNAs was genotype specific. This finding is important, because it demonstrates that even conserved miRNAs show natural variation in expression between genotypes of the same plant species. It is interesting to note that under drought, up or down regulation for several miRNAs observed in a previous study in drought sensitive genotypes (Hackenberg *et al*., 2015), showed opposite responses or no change in expression under drought in our study (Ferdous *et al*., 2016). This emphasised the high degree of expression variation between genotypes and the importance of using suitable controls for estimating miRNA

expression. The variation in miRNA expression between drought susceptible and tolerant genotypes might reflect important physiological differences in drought responses for these barley genotypes. Expression of miRNAs was also found to vary between tolerant and susceptible genotypes of other plant species including cowpea (Barrera-Figueroa *et al*., 2011), soybean (Kulcheski *et al*., 2011), wheat (Ma *et al*., 2015), sorghum (Katiyar *et al*., 2015) and rice (Cheah *et al*., 2015) under drought. To further investigate the relationship between the genotype dependent drought responsive expression of miRNAs and drought tolerance, drought susceptible and tolerant genotypes could be examined. Contrasting miRNA: target expression that show the strongest association with drought treatment could be used to evaluate the link between miRNA expression and drought tolerance. Promising miRNA: mRNA interactions could then be used as selection tools for breeders.

Target genes of miRNAs have vital roles in plant drought signalling and metabolism (Yin *et al*., 2014). Previous studies suggest that stress-induced miRNAs control genes that are negative regulators of stress responses or positive regulators of metabolic processes that are inhibited by stresses (reviewed in Khraiwesh *et al*., 2012). It is proposed that under drought, down-regulation of miR168, miR528, and miR167 might allow their respective targets, mitogen-activated protein kinase (MAPK), peroxidase (POD) and phospholipase D (PLD) to initiate the regulation of ABA-induced stomatal movement and antioxidant defense in maize (Wei *et al*., 2009). Further exploration of the function of target genes that play a role in the signalling networks of plants under drought stress will help our understanding of transcriptome homeostasis in drought tolerance.

Our study suggested that there is genetic variation in target response to miRNAs. That is, the regulation of miRNAs involved in controlling target gene expression appeared to depend on sequence differences within the miRNA binding site among the genotypes. This implies that miRNA-mediated target gene regulation varies between genotypes. This

also indicates the possibility that target could be modified within the sequences of miRNA binding site to avoid or suppress the mRNA cleavage by the miRNA.

The availability of deep sequencing information and bioinformatic analysis has resulted in extensive resources to support the identification and characterization of miRNAs in barley. Functional analysis of miRNAs and their target genes is difficult and generally relies on using transgenic plants to up or down regulate expression of the miRNA or target. However, the potential value of over-expression of promising miRNAs through stable genetic modification (GM) in barley has not been reported. We explored the feasibility of this approach through phenotyping and functional analysis of transgenic/cisgenic miRNAs in barley for performance under drought. The functional analysis of *Ath*/*Hv*-miR827 in the genetically modified barley indicated that this miRNA plays a regulatory role that can lead to improved plant performance under drought.

In developing transgenic germplasm there are two important issues to consider; the sources of the miRNA DNA sequence, 'transgenic' or 'cisgenic', and the nature of the promoter used to drive expression. From the findings of this study, it appeared that the use of *Hv*-miR827 under the control of drought-inducible promoter was the most promising option since this minimised the negative phenotypic effects of constitutive over-expression. Plants derived from the host species 'cisgenics' have greater consumer acceptance than 'transgenics' althought they are subject to the same regulatory conditions in most countries (Poltronieri and Reca, 2015). Nevertheless, genetically modified plants will require proper field trials to demonstrate if they really show improved yield under drought stress. There is active public debate about the potential complications associated with development of genetically modified plants and consumer acceptance. Field evaluation of genetically modified plants is also difficult due to stringent regulatory requirements. However since miRNAs are non-protein coding, miRNA-based genetic modification technology (miRNA-based GM tech) may overcome some of these concerns

for developing transgenic crops. In this context it is important to note that apple varieties where polyphenol oxidase has been inactivated through the use of miRNA have been accepted for commercial production in the USA (http://www.arcticapples.com/).

The results from this PhD offer opportunities for the utilization of miRNAs:mRNA as biomarkers for genotypic selection of drought tolerance, for the investigation of target gene functions and for the application of potential miRNAs to generate drought tolerant transgenic barley.

Future research directions

The objective of this PhD project was to examine the natural variation of miRNA expression among barley genotypes and to investigate the function of miRNA in the drought regulatory pathway. The results are encouraging but further research will be needed to define the regulatory network of the miRNAs identified in this project. Studies should include the following:

1. Upon drought stress, up or down regulation of the same miRNAs in different plant species varies even for conserved miRNAs. This implies that the function may also vary in a species-specific manner (Zhou and Luo, 2013). Future work should aim to assess the expression of potential miRNAs in different genotypes of barley and other crops adapted to environments differing in the frequency and severity of drought stress. Differences of miRNA expression in tissues, growth stages and extent of drought treatments will indicate greater sensitivity of certain miRNAs to the perception of drought in these genotypes. Only a small number of different barley genotypes were studied here. Therefore an investigation of miRNAs' expression in other genotypes will help to define the extent of miRNAs' expression variation and the relationship of expression to drought response. Genotype specific miRNA expression may be one of the effective selection criteria for recognizing drought tolerant genotypes in the future.

2. Some of the target genes of miRNAs revealed in our study have been previously reported to be associated with drought stress in model plants including *Arabidopsis* and *Brachypodium*. For example, NFY-A, a CCAAT-binding transcription factor where over-expression was reported to reduced leaf water loss in transgenic *Arabidopsis* compared with wild type plants (Li *et al*., 2008) and also reported to improved drought tolerance in transgenic *Arabidopsis* through an ABA signalling dependent pathway (Ni *et al*., 2013). The results seen in model species suggest that these miRNAs and their targets will be good candidates for further study.

3. There has been little research dedicated to functional characterization of miRNAs for their involvement in plant responses to drought stresses. In this project we found two potential miRNAs; *Ath*-miR169b and *Osa*-miR1432 that were implicated in drought responses in barley genotypes. Further work should aim to study the function of these miRNAs. The development of transgenic lines over or under-expressing these miRNAs is a promising strategy for functional analysis. Strategies including constitutive over-expression of these miRNAs or artificial miRNA (amiRNA) mediated gain of function and over-expression of miRNA inhibitors to create loss of function could be considered. Undesired side effects of using the constitutive promoter can be avoided by cutting down the duration of over-expression or utilizing stress-inducible promoters. In this project, though we observed a positive regulatory role of *Hv*-miR827 in transgenic barley plants, but we do not know if the plants will show better performance under field conditions. Therefore, it will be important to evaluate the transgenics at multiple field sites, and to elucidate the characteristics of the miRNA in the transgenics that results in the positive response to drought stress in barley. miRNA-based genetically modified plants could provide an approach to developing stress tolerant crop varieties.

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Appendices

Chapter 4: Supporting Information Table S1 - Predicted targets of miRNAs (JLOC numbers)

miRNA_Acc.	Target_Acc.	Degradome product search	WGS Morex Assembly v	annotation
Osa -miR1432	JLOC1_46633.1	Degradome product found	contig_57713	Calmodulin- related (EF hand containing) calcium sensor protein (PF13499)
Osa -miR1432	JLOC1_46105.1	No degradome product		None
Osa -miR1432	JLOC1_11545.1	No degradome product		None
Osa -miR1432	JLOC1_24180.1	No degradome product		None
Osa -miR1432	JLOC1_24180.2	No degradome product		protein|thioesterase family protein, putative, expressed
Osa -miR1432	JLOC1_3025.1	No degradome product		protein|rRNA 2-O-methyltransferase fibrillarin 2, putative, expressed
Osa -miR1432	JLOC1_12174.1	No degradome product		protein|zinc finger A20 and AN1 domain-containing stress-associated protein, putative, expressed
Osa -miR1432	JLOC1_35131.1	No degradome product		protein|thioesterase family protein, putative, expressed
Osa -miR1432	JLOC1_35131.2	No degradome product		protein|thioesterase family protein, putative, expressed
Hv -miR166b/c	JLOC1_7081.1	No degradome product		None
Hv -miR166b/c	JLOC1_38121.1	Degradome product found	contig_42852	START domain containing protein
Hv -miR166b/c	JLOC1_37511.8	No degradome product		None
Hv -miR166b/c	JLOC1_37511.4	No degradome product		None
Hv -miR166b/c	JLOC1_37511.6	No degradome product		None
Hv -miR166b/c	JLOC1_37511.5	No degradome product		None
Hv -miR166b/c	JLOC1_52684.1	Degradome product found	contig_8318	Homeobox domain, START domain, MEKHLA domain containing protein
Hv -miR166b/c	JLOC1_21944.1	Degradome product found	contig_241849	Homeobox domain, START domain, MEKHLA domain containing protein
Hv -miR166b/c	JLOC1_40258.1	Degradome product found	contig_45665	Homeobox domain, START domain, MEKHLA domain containing protein
Hv -miR166b/c	JLOC1_10154.1	No degradome product		None
Hv -miR166b/c	JLOC1_36120.1	No degradome product		None
Hv -miR166b/c	JLOC1_32943.1	No degradome product		None
Hv -miR166b/c	JLOC1_8624.1	No degradome product		protein|vacuolar protein sorting 55 family protein, putative, expressed
Hv -miR166b/c	JLOC1_47615.4	No degradome product		protein|wound responsive protein, putative, expressed
Hv -miR166b/c	JLOC1_47615.1	No degradome product		protein|wound responsive protein, putative, expressed
Hv -miR166b/c	JLOC1_47615.3	No degradome product		protein|wound responsive protein, putative, expressed
Hv -miR166b/c	JLOC1_47615.2	No degradome product		protein|wound responsive protein, putative, expressed
Hv -miR166b/c	JLOC1_21440.1	No degradome product		protein|mitochondrial ATP synthase g subunit family protein, putative, expressed

Ath -miR169b	JLOC1_18457.2	No degradome product		protein|SRPK4, putative, expressed
Ath -miR169b	JLOC1_18457.1	No degradome product		protein|SRPK4, putative, expressed
Ath -miR169b	JLOC1_18457.3	No degradome product		protein|SRPK4, putative, expressed
Ath -miR169b	JLOC1_49683.1	No degradome product		None
Ath -miR169b	JLOC1_44789.4	No degradome product		None
Ath -miR169b	JLOC1_44789.3	No degradome product		None
Ath -miR169b	JLOC1_44789.2	No degradome product		None
Ath -miR169b	JLOC1_44789.1	No degradome product		None
Ath -miR169b	JLOC1_23550.1	Degradome product found	contig_2546965	protein|nuclear transcription factor Y subunit, putative, expressed/ CCAAT-binding transcription factor (CBF-B/NF-YA) subunit
Ath -miR169b	JLOC1_47542.3	No degradome product		protein|expressed protein
Ath -miR169b	JLOC1_47542.1	No degradome product		protein|expressed protein
Ath -miR169b	JLOC1_3515.1	No degradome product		protein|expressed protein
Ath -miR169b	JLOC1_47542.4	No degradome product		protein|expressed protein
Ath -miR169b	JLOC1_44720.1	No degradome product		None
Ath -miR169b	JLOC1_23817.1	No degradome product		None

Osa -miR393a	JLOC1_36251.2	Degradome product found	contig_40541	Leucine-rich repeat (LRR) domain containing F-box protein
Osa -miR393a	JLOC1_6406.1	No degradome product		None
Osa -miR393a	JLOC1_36251.1	Degradome product found	contig_40541	Leucine-rich repeat (LRR) domain containing F-box protein
Osa -miR393a	JLOC1_32763.1	No degradome product		None
Osa -miR393a	JLOC1_41104.1	No degradome product		None
Osa -miR393a	JLOC1_34954.1	No degradome product		None
Osa -miR393a	JLOC1_46467.1	No degradome product		protein protein kinase, putative, expressed
Osa -miR393a	JLOC1_10553.4	No degradome product		None
Osa -miR393a	JLOC1_10553.2	No degradome product		None
Osa -miR393a	JLOC1_10553.3	No degradome product		None
Osa -miR393a	JLOC1_10553.1	No degradome product		None
Osa -miR393a	JLOC1_46467.2	No degradome product		protein protein kinase, putative, expressed
Osa -miR393a	JLOC1_10553.5	No degradome product		None
Osa -miR393a	JLOC1_45991.1	No degradome product		protein NB-ARC domain containing protein, expressed
Osa -miR393a	JLOC1_45448.2	No degradome product		protein retrotransposon protein, putative, unclassified
Osa -miR393a	JLOC1_45448.1	No degradome product		protein retrotransposon protein, putative, unclassified
Osa -miR393a	JLOC1_45448.3	No degradome product		protein retrotransposon protein, putative, unclassified
Osa -miR393a	JLOC1_13933.1	No degradome product		protein DEAD-box ATP-dependent RNA helicase, putative, expressed
Osa -miR393a	JLOC1_53528.2	No degradome product		None
Osa -miR393a	JLOC1_2445.8	No degradome product		None
Ata -miR9863a	JLOC1_16566.1	degradome product found	contig_163538	NB-ARC domain (PF00931)
Ata -miR9863a	JLOC1_39342.1	No degradome product		None
Ata -miR9863a	JLOC1_37368.2	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	JLOC1_37368.5	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	JLOC1_37368.1	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	JLOC1_37368.3	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	JLOC1_37368.4	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	JLOC1_15001.1	degradome product found	contig_1596863	NB-ARC domain (PF00931)
Ata -miR9863a	JLOC1_7300.1	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	JLOC1_39448.1	No degradome product		protein RGH1A, putative, expressed
Ata -miR9863a	JLOC1_20460.1	No degradome product		None
Ata -miR9863a	JLOC1_15074.1	No degradome product		protein RGH1A, putative, expressed
Ata -miR9863a	JLOC1_34903.1	No degradome product		protein RGH2B, putative, expressed
Ata -miR9863a	JLOC1_38367.1	No degradome product		protein mla1, putative, expressed
Ata -miR9863a	JLOC1_46239.1	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	JLOC1_42315.2	No degradome product		protein RGH1A, putative, expressed
Ata -miR9863a	JLOC1_41094.1	No degradome product		protein RGH1A, putative, expressed
Ata -miR9863a	JLOC1_42315.1	No degradome product		protein RGH1A, putative, expressed
Ata -miR9863a	JLOC1_4515.2	No degradome product		None
Ata -miR9863a	JLOC1_4515.1	No degradome product		None
Ata -miR9863a	JLOC1_37247.1	No degradome product		protein pentatricopeptide, putative, expressed

Hv -miR444b	JLOC1_39846.1	No degradome product		MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_39846.2	No degradome product		MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_729.1	No degradome product		None
Hv -miR444b	JLOC1_729.3	No degradome product		None
Hv -miR444b	JLOC1_11127.1	No degradome product		protein zinc finger, C3HC4 type domain containing protein, expressed
Hv -miR444b	JLOC1_44933.2	No degradome product		MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_4106.3	No degradome product		None
Hv -miR444b	JLOC1_51807.1	No degradome product		None
Hv -miR444b	JLOC1_2737.1	No degradome product		MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_6689.1	No degradome product		MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_6689.2	No degradome product		MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_39845.2	Degradome product found	contig_45023	MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_39845.1	same gene: Degradome product fo	contig_45023	MADS-box family gene with MIKCc type-box, expressed
Hv -miR444b	JLOC1_47577.2	No degradome product		None
Hv -miR444b	JLOC1_47577.1	No degradome product		None
Hv -miR444b	JLOC1_39845.3	same gene: Degradome product fo	contig_45023	MADS-box family gene with MIKCc type-box, expressed
Osa -miR169n	JLOC1_51189.1	No degradome product		None
Osa -miR169n	JLOC1_51189.4	No degradome product		None
Osa -miR169n	JLOC1_51189.2	No degradome product		None
Osa -miR169n	JLOC1_51189.3	No degradome product		None
Osa -miR169n	JLOC1_31769.1	No degradome product		None
Osa -miR169n	JLOC1_43532.2	No degradome product		None
Osa -miR169n	JLOC1_43532.1	No degradome product		None
Osa -miR169n	JLOC1_43532.3	No degradome product		None
Osa -miR169n	JLOC1_48661.1	No degradome product		None
Osa -miR169n	JLOC1_48661.2	No degradome product		None
Osa -miR169n	JLOC1_48661.3	No degradome product		None
Osa -miR169n	JLOC1_49683.1	No degradome product		protein cytochrome P450, putative, expressed
Osa -miR169n	JLOC1_18457.2	No degradome product		protein flavoprotein wrbA, putative, expressed
Osa -miR169n	JLOC1_18457.1	No degradome product		protein flavoprotein wrbA, putative, expressed
Osa -miR169n	JLOC1_23550.1	Degradome product found	contig_2546965	protein|nuclear transcription factor Y subunit A, putative, expressed
Osa -miR169n	JLOC1_10952.1	No degradome product		None
Osa -miR169n	JLOC1_18457.3	No degradome product		protein flavoprotein wrbA, putative, expressed
Osa -miR169n	JLOC1_48621.1	No degradome product		protein retrotransposon protein, putative, unclassified, expressed
Osa -miR169n	JLOC1_22366.1	No degradome product		protein ribosome biogenesis protein NEP1, putative, expressed
Osa -miR169n	JLOC1_325.1	No degradome product		protein retrotransposon protein, putative, unclassified
Osa -miR169n	JLOC1_46748.1	No degradome product		None

Bdi-miR396b-5p	JLOC1_21350.1	No degradome product		None
Bdi-miR396b-5p	JLOC1_43565.2	Degradome product found	contig_51136	WRC domain (PF08879)
Bdi-miR396b-5p	JLOC1_43565.1	same gene: Degradome product fo	contig_51136	WRC domain (PF08879)
Bdi-miR396b-5p	JLOC1_42015.1	No degradome product		protein growth regulating factor protein, putative, expressed
Bdi-miR396b-5p	JLOC1_4921.1	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	JLOC1_4921.4	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	JLOC1_4921.2	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	JLOC1_4921.3	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	JLOC1_22440.2	No degradome product		protein growth regulating factor protein, putative, expressed
Bdi-miR396b-5p	JLOC1_22440.1	No degradome product		protein growth regulating factor protein, putative, expressed
Bdi-miR396b-5p	JLOC1_22440.3	No degradome product		protein growth regulating factor protein, putative, expressed
Bdi-miR396b-5p	JLOC1_44340.1	Degradome product found	contig_52709	QLQ domain (PF08880) and WRC domain (PF08879) close to to each other
Bdi-miR396b-5p	JLOC1_44340.2	same gene: Degradome product fo	contig_52709	QLQ domain (PF08880) and WRC domain (PF08879) close to to each other
Bdi-miR396b-5p	JLOC1_125.1	No degradome product		None
Bdi-miR396b-5p	JLOC1_125.2	No degradome product		None
Bdi-miR396b-5p	JLOC1_42715.1	No degradome product		protein OsRhmbd12 - Putative Rhomboid homologue, expressed
Bdi-miR396b-5p	JLOC1_42715.2	No degradome product		protein OsRhmbd12 - Putative Rhomboid homologue, expressed
Bdi-miR396b-5p	JLOC1_42715.3	No degradome product		protein OsRhmbd12 - Putative Rhomboid homologue, expressed
Bdi-miR396b-5p	JLOC1_45054.2	No degradome product		None
Bdi-miR396b-5p	JLOC1_42715.4	No degradome product		protein OsRhmbd12 - Putative Rhomboid homologue, expressed
Hv-miR171-5p	JLOC1_12271.1	No degradome product		protein Leucine Rich Repeat family protein, expressed
Hv-miR171-5p	JLOC1_7081.1	No degradome product		None
Hv-miR171-5p	JLOC1_35526.1	No degradome product		protein xylose isomerase, putative, expressed
Hv-miR171-5p	JLOC1_43203.1	No degradome product		protein transmembrane 9 superfamily member, putative, expressed
Hv-miR171-5p	JLOC1_43203.2	No degradome product		protein transmembrane 9 superfamily member, putative, expressed
Hv-miR171-5p	JLOC1_24842.1	No degradome product		protein kinase domain containing protein, expressed
Hv-miR171-5p	JLOC1_50604.1	No degradome product		None
Hv-miR171-5p	JLOC1_33239.1	No degradome product		None
Hv-miR171-5p	JLOC1_41238.1	No degradome product		protein expressed protein
Hv-miR171-5p	JLOC1_33239.2	No degradome product		None
Hv-miR171-5p	JLOC1_33239.3	No degradome product		None
Hv-miR171-5p	JLOC1_20960.1	No degradome product		protein fringe-related protein, putative, expressed
Hv-miR171-5p	JLOC1_37932.2	No degradome product		None
Hv-miR5048	JLOC1_24612.6	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_24612.3	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_24612.1	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_46752.1	Degradome product found	contig_57988	Serine/threonine-protein kinase receptor precursor
Hv-miR5048	JLOC1_42422.1	No degradome product		protein jacalin-like lectin domain containing protein, expressed
Hv-miR5048	JLOC1_24674.3	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_35867.1	No degradome product		protein autophagy-related protein 12, putative, expressed
Hv-miR5048	JLOC1_4988.1	No degradome product		protein flavin monooxygenase, putative, expressed
Hv-miR5048	JLOC1_19953.1	No degradome product		None
Hv-miR5048	JLOC1_20683.1	No degradome product		None
Hv-miR5048	JLOC1_27081.1	No degradome product		None

Hv-miR5048	JLOC1_28251.1	No degradome product		protein cysteine-rich receptor-like protein kinase 7 precursor, putative, expressed
Hv-miR5048	JLOC1_51669.1	No degradome product		protein OsSigP5 - Putative Type I Signal Peptidase homologue; employs a putative Ser/His catalytic dyad, expressed
Hv-miR5048	JLOC1_24712.1	No degradome product		None
Hv-miR5048	JLOC1_25009.1	No degradome product		protein histidine acid phosphatase, putative, expressed
Hv-miR5048	JLOC1_24712.2	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_25009.3	No degradome product		protein histidine acid phosphatase, putative, expressed
Hv-miR5048	JLOC1_25009.2	No degradome product		protein histidine acid phosphatase, putative, expressed
Hv-miR5048	JLOC1_24712.3	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_3037.1	No degradome product		protein disease resistance protein RPS2, putative, expressed
Hv-miR5048	JLOC1_1395.1	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_1395.2	No degradome product		protein protein kinase domain containing protein, expressed
Hv-miR5048	JLOC1_3037.2	No degradome product		protein disease resistance protein RPS2, putative, expressed
Hv-miR5048	JLOC1_3037.3	No degradome product		protein disease resistance protein RPS2, putative, expressed
Hv-miR5048	JLOC1_14470.1	No degradome product		None
Hv-miRx5	JLOC1_37420.1	No degradome product		None
Hv-miRx5	JLOC1_24381.1	No degradome product		None
Hv-miRx5	JLOC1_41757.1	No degradome product		None
Hv-miRx5	JLOC1_45992.3	No degradome product		None
Hv-miRx5	JLOC1_45992.2	No degradome product		None
Hv-miRx5	JLOC1_45992.10	No degradome product		None
Hv-miRx5	JLOC1_45992.6	No degradome product		None
Hv-miRx5	JLOC1_45992.8	No degradome product		None
Hv-miRx5	JLOC1_45992.13	No degradome product		None
Hv-miRx5	JLOC1_45992.7	No degradome product		None
Hv-miRx5	JLOC1_45992.11	No degradome product		None
Hv-miRx5	JLOC1_45992.5	No degradome product		None
Hv-miRx5	JLOC1_45992.9	No degradome product		None
Hv-miRx5	JLOC1_45992.1	No degradome product		None
Hv-miRx5	JLOC1_45992.12	No degradome product		None
Hv-miRx5	JLOC1_45992.4	No degradome product		None
Hv-miRx5	JLOC1_8325.2	No degradome product		None
Hv-miRx5	JLOC1_41756.1	No degradome product		None
Hv-miRx5	JLOC1_26727.2	No degradome product		None
Hv-miRx5	JLOC1_39449.3	No degradome product		Protein RGH1A, putative, expressed, contains NB-ARC domain
Hv-miRx5	JLOC1_26727.1	No degradome product		None
Hv-miRx5	JLOC1_39449.2	No degradome product		Protein RGH1A, putative, expressed, contains NB-ARC domain
Hv-miRx5	JLOC1_44557.1	No degradome product		None
Hv-miRx5	JLOC1_6608.1	No degradome product		None
Hv-miRx5	JLOC1_39449.1	No degradome product		Protein RGH1A, putative, expressed, contains NB-ARC domain

Chapter 4: Supporting Information Table S1- Predicted targets of miRNAs (MLOC numbers)

miRNA_Acc.	Target_Acc.	Degradome product search	WGS Morex Assembly v3	annotation
Osa -miR1432	MLOC_70272.1	Degradome product found	contig_57713	Calmodulin- related (EF hand containing) calcium sensor protein (PF13499)
Osa -miR1432	AK361118	No degradome product		protein|alliin lyase precursor, putative, expressed
Osa -miR1432	MLOC_81070.1	No degradome product		protein|caffeoyl-CoA O-methyltransferase, putative, expressed
Osa -miR1432	MLOC_50020.1	No degradome product		protein transposon protein, putative, Ac/Ds sub-class
Osa -miR1432	MLOC_37349.2	No degradome product		None
Osa -miR1432	MLOC_37349.3	No degradome product		None
Osa -miR1432	MLOC_4782.1	No degradome product		protein rRNA 2-O-methyltransferase fibrillarin 2, putative, expressed
Osa -miR1432	MLOC_37349.4	No degradome product		None
Osa -miR1432	AK372087	No degradome product		protein|phosphatidylserine synthase, putative, expressed
Osa -miR1432	MLOC_7789.1	No degradome product		None
Hv -miR166b/c	MLOC_10786.1	No degradome product		None
Hv -miR166b/c	AK363495	No degradome product		protein START domain containing protein, expressed
Hv -miR166b/c	AK365312	Degradome product found	contig_8318	protein Homeobox domain (PF00046), START domain(PF01852), MEKHLA domain (PF08670)
Hv -miR166b/c	AK366024	No degradome product		None
Hv -miR166b/c	AK364215	Degradome product found	contig_241849	A target sequence (AK364215) contains Homeobox domain (PF00046), START domain(PF01852), MEKHLA domain (PF08670)
Hv -miR166b/c	AK362009	Degradome product found	contig_45665	protein|class III HD-Zip protein 8, putative, expressed
Hv -miR166b/c	AK354023	Degradome product found	contig_45665	MEKHLA Domain (PF08670)
Hv -miR166b/c	MLOC_39082.2	No degradome product		protein retrotransposon protein, putative, unclassified
Hv -miR166b/c	MLOC_39082.1	No degradome product		protein retrotransposon protein, putative, unclassified
Hv -miR166b/c	MLOC_14862.1	No degradome product		protein retrotransposon protein, putative, LINE subclass
Hv -miR166b/c	MLOC_55934.2	No degradome product		protein phosphatidylinositol 3- and 4-kinase family protein, putative, expressed
Hv -miR166b/c	MLOC_55934.7	No degradome product		protein phosphatidylinositol 3- and 4-kinase family protein, putative, expressed
Hv -miR166b/c	MLOC_55934.3	No degradome product		protein phosphatidylinositol 3- and 4-kinase family protein, putative, expressed
Hv -miR166b/c	MLOC_14896.1	No degradome product		protein MATE domain containing protein, expressed
Hv -miR166b/c	MLOC_36985.4	No degradome product		protein expressed protein
Hv -miR166b/c	MLOC_36985.1	No degradome product		protein expressed protein
Hv -miR166b/c	MLOC_81061.2	No degradome product		None
Hv -miR166b/c	MLOC_81061.1	No degradome product		None
Ath -miR169b	MLOC_22210.2	No degradome product		None
Ath -miR169b	MLOC_22210.1	No degradome product		None
Ath -miR169b	MLOC_30027.1	No degradome product		None
Ath -miR169b	MLOC_27317.1	No degradome product		protein SRPK4, putative, expressed
Ath -miR169b	MLOC_27317.3	No degradome product		protein SRPK4, putative, expressed
Ath -miR169b	MLOC_74547.1	No degradome product		protein|cytochrome P450, putative, expressed
Ath -miR169b	MLOC_27317.2	No degradome product		protein|NADPH-dependent FMN reductase domain containing protein, expressed
Ath -miR169b	MLOC_27318.1	No degradome product		protein flavoprotein wrbA, putative, expressed
Ath -miR169b	MLOC_67781.5	No degradome product		None
Ath -miR169b	MLOC_56054.3	No degradome product		protein serine palmitoyltransferase 1, putative, expressed

Ath -miR169b	MLOC_81951.4	No degradome product		None
Ath -miR169b	MLOC_81951.2	No degradome product		None
Ath -miR169b	MLOC_81951.3	No degradome product		None
Ath -miR169b	MLOC_81951.5	No degradome product		None
Osa -miR393a	AK355927	Degradome product found	contig_1557974	protein|OsFBL4 - F-box domain and LRR containing protein, expressed
Osa -miR393a	AK374984	Degradome product found	contig_1557974	protein|glycine-rich protein 2, putative
Osa -miR393a	MLOC_61582.3	No degradome product		protein expressed protein
Osa -miR393a	MLOC_61582.2	No degradome product		protein expressed protein
Osa -miR393a	MLOC_61582.1	No degradome product		protein expressed protein
Osa -miR393a	MLOC_12284.2	No degradome product		protein mitochondrial ATP synthase g subunit family protein, putative, expressed
Osa -miR393a	AK369673	No degradome product		None
Osa -miR393a	MLOC_59844.3	No degradome product		None
Osa -miR393a	MLOC_55936.3	No degradome product		protein nucleoside-triphosphatase, putative, expressed
Osa -miR393a	MLOC_55936.1	No degradome product		protein nucleoside-triphosphatase, putative, expressed
Osa -miR393a	MLOC_62777.1	No degradome product		None
Osa -miR393a	MLOC_70904.2	No degradome product		protein inactive receptor kinase At2g26730 precursor, putative, expressed
Osa -miR393a	MLOC_70904.1	No degradome product		protein inactive receptor kinase At2g26730 precursor, putative, expressed
Osa -miR393a	AK364310	No degradome product		None
Osa -miR393a	AK366571	No degradome product		None
Osa -miR393a	MLOC_15388.1	No degradome product		None
Osa -miR393a	MLOC_74475.1	No degradome product		None
Osa -miR393a	MLOC_74652.1	No degradome product		None
Osa -miR393a	AK374638	No degradome product		None
Osa -miR393a	MLOC_14579.1	No degradome product		None
Osa -miR393a	MLOC_54313.1	No degradome product		None
Ata -miR9863a	AK249764.1	No degradome product		None
Ata -miR9863a	MLOC_57619.4	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	MLOC_57619.1	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	MLOC_57619.2	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	MLOC_57619.3	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	MLOC_57619.6	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	MLOC_57619.5	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Ata -miR9863a	MLOC_31061.2	No degradome product		None
Ata -miR9863a	MLOC_31061.1	No degradome product		None
Ata -miR9863a	AK368780	No degradome product		None
Ata -miR9863a	AK356684	No degradome product		None
Ata -miR9863a	AK369908	No degradome product		None
Ata -miR9863a	AK372887	No degradome product		None
Ata -miR9863a	MLOC_54234.2	No degradome product		protein RGH2B, putative, expressed
Ata -miR9863a	MLOC_54234.3	No degradome product		protein RGH2B, putative, expressed
Ata -miR9863a	MLOC_54234.1	No degradome product		protein RGH2B, putative, expressed
Ata -miR9863a	MLOC_10425.3	No degradome product		protein RGH1A, putative, expressed
Ata -miR9863a	MLOC_56046.1	No degradome product		None
Ata -miR9863a	MLOC_36856.1	No degradome product		None

Ata-miR9863a	MLOC_56046.2	No degradome product		None
Ata-miR9863a	MLOC_10425.2	No degradome product		protein RGH1A, putative, expressed
Ata-miR9863a	AK365485	No degradome product		None
Ata-miR9863a	MLOC_64444.1	No degradome product		protein RGH1A, putative, expressed
Ata-miR9863a	AK364674	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Hv-miR444b	AK363243	No degradome product		None
Hv-miR444b	AK370732	No degradome product		None
Hv-miR444b	MLOC_20816.1	No degradome product		protein expressed protein
Hv-miR444b	MLOC_77702.1	No degradome product		None
Hv-miR444b	AK374170	No degradome product		None
Hv-miR444b	MLOC_61033.3	Degradome product found	contig_45023	MADS-box family gene with MIKCc type-box, expressed
Hv-miR444b	MLOC_71566.5	No degradome product		None
Hv-miR444b	MLOC_61032.1	No degradome product		None
Hv-miR444b	MLOC_61033.2	Degradome product found	contig_45023	MADS-box family gene with MIKCc type-box, expressed
Hv-miR444b	MLOC_61033.1	Degradome product found	contig_45023	MADS-box family gene with MIKCc type-box, expressed
Hv-miR444b	MLOC_71566.4	No degradome product		None
Hv-miR444b	MLOC_71566.3	No degradome product		None
Hv-miR444b	MLOC_71566.1	No degradome product		None
Hv-miR444b	MLOC_71566.2	No degradome product		None
Osa-miR169n	MLOC_22210.2	No degradome product		None
Osa-miR169n	MLOC_22210.1	No degradome product		None
Osa-miR169n	MLOC_76757.6	No degradome product		None
Osa-miR169n	MLOC_76757.2	No degradome product		None
Osa-miR169n	MLOC_76757.5	No degradome product		None
Osa-miR169n	MLOC_76757.1	No degradome product		None
Osa-miR169n	MLOC_76757.3	No degradome product		None
Osa-miR169n	MLOC_76757.4	No degradome product		None
Osa-miR169n	MLOC_19117.1	No degradome product		None
Osa-miR169n	MLOC_36685.2	No degradome product		protein resistance protein, putative, expressed
Osa-miR169n	MLOC_69031.1	No degradome product		protein flavin monooxygenase, putative, expressed
Osa-miR169n	MLOC_36685.1	No degradome product		protein resistance protein, putative, expressed
Osa-miR169n	AK370251	No degradome product		None
Osa-miR169n	MLOC_73118.3	No degradome product		None
Osa-miR169n	MLOC_73118.2	No degradome product		None
Bdi-miR396b-5p	MLOC_7461.1	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	MLOC_7461.2	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	AK375827	No degradome product		None
Bdi-miR396b-5p	AK250947.1	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	AK376404	No degradome product		None
Bdi-miR396b-5p	AK353813	No degradome product		None
Bdi-miR396b-5p	AK365188	No degradome product		None
Bdi-miR396b-5p	MLOC_7461.3	No degradome product		protein growth-regulating factor, putative, expressed
Bdi-miR396b-5p	MLOC_67201.4	Degradome product found	contig_52709	QLQ domain (PF08880) and WRC domain (PF08879) close to to each other
Bdi-miR396b-5p	AK373512	No degradome product		None
Bdi-miR396b-5p	MLOC_55035.2	No degradome product		protein inosine-uridine preferring nucleoside hydrolase family protein, putative, expressed
Bdi-miR396b-5p	MLOC_55035.1	No degradome product		protein inosine-uridine preferring nucleoside hydrolase family protein, putative, expressed

Hv -miR171-5p	MLOC_17874.2	No degradome product		None
Hv -miR171-5p	MLOC_17874.1	No degradome product		None
Hv -miR171-5p	MLOC_17769.1	No degradome product		protein Leucine Rich Repeat family protein, expressed
Hv -miR171-5p	MLOC_10786.1	No degradome product		None
Hv -miR171-5p	MLOC_30484.1	No degradome product		None
Hv -miR171-5p	MLOC_27199.1	No degradome product		protein hAT dimerisation domain-containing protein, putative, expressed
Hv -miR171-5p	MLOC_60565.1	No degradome product		protein retrotransposon protein, putative, unclassified
Hv -miR171-5p	MLOC_66038.1	No degradome product		protein protein binding protein, putative, expressed
Hv -miR5048	MLOC_1919.1	No degradome product		None
Hv -miR5048	MLOC_1919.2	No degradome product		None
Hv -miR5048	MLOC_1919.4	No degradome product		None
Hv -miR5048	MLOC_1919.3	No degradome product		None
Hv -miR5048	MLOC_1921.1	No degradome product		None
Hv -miR5048	MLOC_37930.3	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	MLOC_37930.2	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	MLOC_37930.5	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	MLOC_37930.6	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	MLOC_37930.7	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	MLOC_37930.1	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	MLOC_70446.6	Degradome product found	contig_57988	Pkinase (PF00069)
Hv -miR5048	MLOC_70446.1	Same sequence	contig_57988	Pkinase (PF00069)
Hv -miR5048	MLOC_70446.8	Same sequence	contig_57988	Pkinase (PF00069)
Hv -miR5048	MLOC_70446.5	Same sequence	contig_57988	Pkinase (PF00069)
Hv -miR5048	MLOC_70446.3	Same sequence	contig_57988	Pkinase (PF00069)
Hv -miR5048	MLOC_70446.7	Same sequence	contig_57988	Pkinase (PF00069)
Hv -miR5048	MLOC_70446.2	Same sequence	contig_57988	Pkinase (PF00069)
Hv -miR5048	MLOC_70446.4	Same sequence	contig_57988	Pkinase (PF00069)
Hv -miR5048	AK370751	Same sequence	contig_57988	None
Hv -miR5048	MLOC_64580.6	No degradome product		protein NBS-LRR disease resistance protein, putative, expressed
Hv -miR5048	MLOC_64580.3	No degradome product		protein NBS-LRR disease resistance protein, putative, expressed
Hv -miR5048	MLOC_38009.4	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	MLOC_38009.1	No degradome product		protein protein kinase domain containing protein, expressed
Hv -miR5048	AK369220	No degradome product		None

Hv -miRx5	MLOC_60487.1	No degradome product		None
Hv -miRx5	MLOC_60487.2	No degradome product		None
Hv -miRx5	MLOC_60487.3	No degradome product		None
Hv -miRx5	MLOC_60488.5	No degradome product		protein RGH1A, putative, expressed
Hv -miRx5	MLOC_60488.7	No degradome product		protein RGH1A, putative, expressed
Hv -miRx5	MLOC_60488.1	No degradome product		protein RGH1A, putative, expressed
Hv -miRx5	MLOC_60488.2	No degradome product		protein RGH1A, putative, expressed
Hv -miRx5	MLOC_60487.4	No degradome product		None
Hv -miRx5	MLOC_37634.3	No degradome product		protein MLA1, putative, expressed
Hv -miRx5	MLOC_37634.2	No degradome product		protein MLA1, putative, expressed
Hv -miRx5	MLOC_37634.4	No degradome product		protein MLA1, putative, expressed
Hv -miRx5	MLOC_16158.1	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Hv -miRx5	MLOC_16158.2	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Hv -miRx5	MLOC_16158.3	No degradome product		protein stripe rust resistance protein Yr10, putative, expressed
Hv -miRx5	AK252755.1	No degradome product		protein|NBS-LRR type disease resistance protein, putative, expressed
Hv -miRx5	MLOC_63704.2	No degradome product		None
Hv -miRx5	MLOC_63704.1	No degradome product		None
Hv -miRx5	MLOC_40934.1	No degradome product		protein RGH1A, putative, expressed
Hv -miRx5	MLOC_67477.2	No degradome product		protein MLA6 protein, putative, expressed
Hv -miRx5	MLOC_63704.4	No degradome product		None
Hv -miRx5	MLOC_63704.3	No degradome product		None
Hv -miRx5	MLOC_67477.1	No degradome product		protein MLA6 protein, putative, expressed
Hv -miRx5	MLOC_10137.1	No degradome product		None

Parameters used for target prediction:

Maximum expectation	4.0 (range: 0-5.0)
Length for complementarity scoring (hspsize):	20 (range: 15-30bp)
Target accessibility - allowed maximum energy to unpair the target site (UPE):	25 (range: 0-100, less is better)
Flanking length around target site for target accessibility analysis:	17 bp in upstream / 13 bp in downstream
Range of central mismatch leading to translational inhibition:	9 - 11 nt

Chapter 5: Supporting Information Table S1- Predicted targets of miRNAs (JLOC numbers)

miRNA_Acc.	Target_Acc.	Degradome product search	annotation
Hv- miR827	JLOC1_37332.4	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	JLOC1_37332.3	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	JLOC1_37332.1	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	JLOC1_37332.2	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	JLOC1_41660.2	No degradome product	uncharacterized membrane protein, putative, expressed
Hv- miR827	JLOC1_41660.1	No degradome product	uncharacterized membrane protein, putative, expressed
Hv- miR827	JLOC1_41660.3	No degradome product	uncharacterized membrane protein, putative, expressed
Hv- miR827	JLOC1_6921.1	No degradome product	PCRF domain and RF-1 domain
Hv- miR827	JLOC1_17996.1	No degradome product	U5 small nuclear ribonucleoprotein 200 kDa helicase, putative
Hv- miR827	JLOC1_28932.1	No degradome product	protein ribosomal protein L6, putative, expressed
Hv- miR827	JLOC1_7976.1	No degradome product	protein expressed protein
Hv- miR827	JLOC1_7610.1	No degradome product	protein CC-NBS-LRR protein, putative, expressed
Hv- miR827	JLOC1_36600.1	No degradome product	protein proline-rich family protein, putative, expressed
Hv- miR827	JLOC1_42994.5	No degradome product	Clp_N (Clp amino terminal domain, pathogenicity island component)
Hv- miR827	JLOC1_42994.4	No degradome product	Clp_N (Clp amino terminal domain, pathogenicity island component)
Hv- miR827	JLOC1_42994.1	No degradome product	Clp_N (Clp amino terminal domain, pathogenicity island component)
Hv- miR827	JLOC1_42994.3	No degradome product	Clp_N (Clp amino terminal domain, pathogenicity island component)
Hv- miR827	JLOC1_42994.2	No degradome product	Clp_N (Clp amino terminal domain, pathogenicity island component)
Hv- miR827	JLOC1_39118.1	No degradome product	protein aberrant pollen transmission 1, putative, expressed
Hv- miR827	JLOC1_39118.2	No degradome product	protein aberrant pollen transmission 1, putative, expressed
Hv- miR827	JLOC1_41751.1	No degradome product	protein TCP family transcription factor, putative, expressed
Hv- miR827	JLOC1_41812.1	No degradome product	protein T-complex protein, putative, expressed
Hv- miR827	JLOC1_22607.3	No degradome product	protein retrotransposon protein, putative, unclassified
Hv- miR827	JLOC1_37537.1	No degradome product	protein GDSL-like lipase/acylhydrolase, putative, expressed
Hv- miR827	JLOC1_33461.4	No degradome product	None

Ath -miR827	JLOC1_41660.2	No degradome product	uncharacterized membrane protein, putative, expressed
Ath -miR827	JLOC1_41660.1	No degradome product	uncharacterized membrane protein, putative, expressed
Ath -miR827	JLOC1_41660.3	No degradome product	uncharacterized membrane protein, putative, expressed
Ath -miR827	JLOC1_7610.1	No degradome product	protein CC-NBS-LRR protein, putative, expressed
Ath -miR827	JLOC1_39118.1	No degradome product	protein aberrant pollen transmission 1, putative, expressed
Ath -miR827	JLOC1_39118.2	No degradome product	protein aberrant pollen transmission 1, putative, expressed
Ath -miR827	JLOC1_37332.4	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	JLOC1_37332.3	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	JLOC1_37332.1	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	JLOC1_37332.2	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	JLOC1_8746.3	No degradome product	protein transposon protein, putative, unclassified
Ath -miR827	JLOC1_53950.1	No degradome product	protein csAtPR5, putative, expressed
Ath -miR827	JLOC1_34182.1	No degradome product	None
Ath -miR827	JLOC1_8746.1	No degradome product	protein transposon protein, putative, unclassified
Ath -miR827	JLOC1_8746.2	No degradome product	protein transposon protein, putative, unclassified
Ath -miR827	JLOC1_28932.1	No degradome product	protein ribosomal protein L6, putative, expressed
Ath -miR827	JLOC1_40467.2	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	JLOC1_40467.1	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	JLOC1_1693.1	No degradome product	None
Ath -miR827	JLOC1_40949.3	No degradome product	protein|F-box/LRR-repeat protein 2, putative, expressed
Ath -miR827	JLOC1_48390.1	No degradome product	protein|seven in absentia protein family protein, expressed
Ath -miR827	JLOC1_17996.1	No degradome product	U5 small nuclear ribonucleoprotein 200 kDa helicase, putative
Ath -miR827	JLOC1_43347.1	No degradome product	protein hypothetical protein
Ath -miR827	JLOC1_52865.1	No degradome product	None
Ath -miR827	JLOC1_46592.2	No degradome product	None

Chapter 5: Supporting Information Table S1- Predicted targets of miRNAs (MLOC numbers)

miRNA_Acc.	Target_Acc.	Degradome product search	annotation
Hv- miR827	MLOC_57566.1	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	MLOC_57566.3	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	MLOC_57566.2	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	MLOC_57566.4	No degradome product	protein membrane protein contains SPX doamin
Hv- miR827	MLOC_63586.1	No degradome product	protein uncharacterized membrane protein, putative, expressed
Hv- miR827	MLOC_63586.5	No degradome product	protein uncharacterized membrane protein, putative, expressed
Hv- miR827	MLOC_63586.4	No degradome product	protein uncharacterized membrane protein, putative, expressed
Hv- miR827	MLOC_63586.2	No degradome product	protein uncharacterized membrane protein, putative, expressed
Hv- miR827	MLOC_63586.3	No degradome product	protein uncharacterized membrane protein, putative, expressed
Hv- miR827	AK249938.1	No degradome product	None
Hv- miR827	AK361500	No degradome product	None
Hv- miR827	AK365126	No degradome product	None
Hv- miR827	AK357685	No degradome product	None
Hv- miR827	AK363697	No degradome product	None
Hv- miR827	MLOC_60035.1	No degradome product	protein aberrant pollen transmission 1, putative, expressed
Hv- miR827	MLOC_63773.1	No degradome product	protein T-complex protein, putative, expressed
Hv- miR827	MLOC_63874.1	No degradome product	None
Hv- miR827	MLOC_75608.1	No degradome product	None
Hv- miR827	MLOC_57817.3	No degradome product	protein GDSL-like lipase/acylhydrolase, putative, expressed
Hv- miR827	MLOC_52252.4	No degradome product	None
Hv- miR827	MLOC_57817.1	No degradome product	protein GDSL-like lipase/acylhydrolase, putative, expressed
Hv- miR827	MLOC_57817.2	No degradome product	protein GDSL-like lipase/acylhydrolase, putative, expressed
Hv- miR827	MLOC_52252.3	No degradome product	None
Hv- miR827	MLOC_52252.2	No degradome product	None
Hv- miR827	MLOC_52252.1	No degradome product	None

Ath -miR827	MLOC_63586.1	No degradome product	protein uncharacterized membrane protein, putative, expressed
Ath -miR827	MLOC_63586.5	No degradome product	protein uncharacterized membrane protein, putative, expressed
Ath -miR827	MLOC_63586.4	No degradome product	protein uncharacterized membrane protein, putative, expressed
Ath -miR827	MLOC_63586.2	No degradome product	protein uncharacterized membrane protein, putative, expressed
Ath -miR827	MLOC_63586.3	No degradome product	protein uncharacterized membrane protein, putative, expressed
Ath -miR827	MLOC_60035.1	No degradome product	protein aberrant pollen transmission 1, putative, expressed
Ath -miR827	MLOC_57566.1	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	MLOC_57566.3	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	MLOC_57566.2	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	MLOC_57566.4	No degradome product	protein membrane protein contains SPX doamin
Ath -miR827	MLOC_53238.2	No degradome product	None
Ath -miR827	AK358898	No degradome product	None
Ath -miR827	MLOC_53238.1	No degradome product	None
Ath -miR827	MLOC_61891.6	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	MLOC_61891.1	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	MLOC_61891.4	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	MLOC_61891.2	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	MLOC_61891.3	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	MLOC_61891.5	No degradome product	protein protein kinase domain containing protein, expressed
Ath -miR827	MLOC_73772.3	No degradome product	protein serine/threonine-protein kinase receptor precursor, putative, expressed
Ath -miR827	MLOC_73772.5	No degradome product	protein serine/threonine-protein kinase receptor precursor, putative, expressed
Ath -miR827	MLOC_73772.2	No degradome product	protein serine/threonine-protein kinase receptor precursor, putative, expressed
Ath -miR827	MLOC_7178.1	No degradome product	None
Ath -miR827	MLOC_75657.4	No degradome product	None
Ath -miR827	MLOC_75657.1	No degradome product	None