Final Scientific Report

Cover Page

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Project Title: Analysis of small RNAs associated with plant senescence

Investigators

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Keywords *not* appearing in the title and in order of importance.

miRNA, leaf senescence, silique senescence, nutrient responsiveness, tubulin

Abbreviations commonly used in the report, in alphabetical order:

MicroRNAs (miRNAs); Parallel Analysis of RNA Ends (PARE);

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Signature
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Signature
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Final Scientific Report

Publication Summary (numbers)

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Refereed (published, in press, accepted) BARD support acknowledged				
Submitted, in review, in preparation	1			1
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses		1		1
Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	2	1		3
Longer Visits (Sabbaticals)				

Description Cooperation:

The initial stages of the research including, growth of plants, sampling of leaves at specific senescence stages and RNA extraction were done in the Israeli laboratory by Dr. Shaul Burd. The RNA was shipped frozen to be used in the US laboratory for library construction and sequencing. The bioinformatics and Northern blot analyses which led to identification of the senescence associated miRNAs were also performed in the US laboratory by the Ph.D. student Shawn Thatcher. Based on the results from these experiments, specific miRNAs which levels are regulated in senescence were analyzed in detail. These miRNAs, as well as their target genes, are being subjected to functional analysis already initiated in the Israeli laboratory which will be continued as a collaborative research. The US and Israeli PIs agreed to make efforts for submitting to BARD a continuation research proposal on this topic.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
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Abstract

Senescence is an agriculturally significant process due to its negative impact to crop yield and postharvest quality. The genetic regulatory systems controlling senescence induction and progress respond to both developmental and environmental stress signals and involve numerous gene expression changes. Knowledge about the key molecular factors which control senescence is very limited. MicroRNAs (miRNAs) are a class of small RNAs which typically function by guiding cleavage of target messenger RNAs. They have been shown to play major roles in a variety of plant processes including development, responses to environmental stresses, and senescence. The long-term goal of this work is to elucidate roles of small RNAs associated with plant senescence. The hypothesis underlying this research is that miRNA-mediated regulation makes important contributions to the senescence process in plants. Specific, original research objectives included: 1) Profiling of small RNAs from senescing plants; 2) Data Analysis and public access via a user-friendly web interface; 3) Validation of senescence-associated miRNAs and target RNAs; 4) Development of transgenic plants for functional analysis of miRNAs in Arabidopsis. Major revisions made in the research compared to the original work plan included 1) Exclusion of the planned work with tomato as recommended by the BARD review panel; 2) Performing miRNA study also in senescing Arabidopsis siliques, in addition to senescing leaves. To identify senescence-regulation of miRNAs in Arabidopsis thaliana, eight small RNA libraries were constructed and sequenced at four different stages of development and senescence from both leaves and siliques, resulting in more than 200 million genome-matched sequences. Parallel Analysis of RNA Ends (PARE) libraries, which enable the large-scale examination of miRNA-guided cleavage products, were also constructed and sequenced, resulting in over 750 million genome-matched sequences. These massive datasets lead to the identification of new miRNAs, as well as new regulation of known miRNAs and their target genes during senescence, many of which have established roles in nutrient responsiveness and cell structural integrity. In keeping with remobilization of nutrients thought to occur during senescence, many miRNAs and targets had opposite expression pattern changes between leaf and silique tissues during the progression of senescence. Taken together, these findings highlight the integral role that miRNAs may play in the remobilization of resources and alteration of cellular structure that is known to occur in senescence. Experiments were initiated for functional analysis of specific senescence-associated miRNAs and respective target genes. Transgenic Arabidopsis plants were generated in which miR408, found in this study to be significantly induced in leaf senescence, was over-expressed either constitutively or under a senescence-specific promoter. These plants are currently being characterized for any altered phenotypes. In addition T-DNA knock out mutants for various target genes identified in this research are being analyzed. This work provides insights about specific miRNAs that contribute to leaf and silique senescence. The knowledge generated may suggest new strategies to monitor and alter the progression of senescence in crops for agricultural improvement.

RESEARCH ACHIEVEMENTS

In this study, we have utilized high-throughput sequencing of small RNA libraries constructed from both senescing leaf and silique tissue to discover new miRNAs and differential miRNA expression in *Arabidopsis*. Expression levels of target genes for regulated miRNAs were examined in detail, and often had tissue-specific regulation as senescence progressed. This analysis was complemented through the construction and sequencing of PARE libraries, which enabled examination of miRNA-guided cleavage events during senescence. The combination of small RNA, target mRNA and degradome data lead to a much more complete picture of the roles that miRNAs may play in *Arabidopsis* senescence.

Small RNA libraries from senescing *Arabidopsis* leaves and siliques

Small RNA libraries were constructed from leaves and siliques at four different developmental time points. Leaves were selected based on age (Fig. 1A, *figures are located in the manuscript attached in the appendix*) young leaves, mature leaves, early senescing leaves, and late senescing leaves. Siliques were selected based on visual morphology indicative of developmental and senescence stage (Fig. 1B). These eight small RNA libraries yielded more than 200 million genome-matched sequences (Table 1), and represent the first massive small RNA sequencing from senescing plant tissues.

Differential expression of known miRNAs during senescence

miRNA expression levels from young, early senescence and late senescence leaf and silique libraries were compared to libraries of their respective mature tissue to determine if any miRNAs were differentially expressed. In general, the expression of miRNAs across libraries was highly similar; with mature versus young showing the strongest miRNA expression correlation in both leaves and siliques libraries and mature versus late senescence leaves and siliques libraries showing the weakest correlation. Despite mostly similar expression patterns, certain miRNAs did show significant increases or decreases in early and late senescing tissue. miRNAs showing expression changes greater than fivefold from mature to early or late senescence were confirmed. miR164, which targets the positive regulator of senescence *ORE1*, has been previously reported to decline in leaves as senescence progresses (Kim et al., 2009). Both small RNA sequencing data and small RNA northern blot validation reproduced this regulation (Fig. 3).

In addition, differential expression of seven other *Arabidopsis* miRNAs during the course of senescence was observed (Fig. 3). Interestingly, five out of these seven miRNAs have been reported to be nutrient responsive. miR408 targets a host of genes involved in pollen tube guiding, copper metabolism and cell wall integrity (Sunkar and Zhu, 2004) and has been shown to be responsive to low copper levels (Abdel-Ghany and Pilon, 2008). Both small RNA sequencing data and northern blot validation showed that this miRNA increased approximately seven fold during senescence in leaf tissue (Fig. 3). However, miR408's expression pattern in siliques differed from leaves, only increasing very slightly (Fig. 3).

Several other nutrient responsive miRNAs also showed different expression trends between leaf and silique during senescence. Both miR399, which targets the negative repressor of phosphate transport, *PHOSPHATE2*, and miR827 which targets the nitrogen/phosphate balance regulator

NITROGEN LIMITATION ADAPTATION are well known to be strongly induced by low phosphate conditions (Chiou et al., 2006; Kant et al., 2011). Both of these miRNAs increased as senescence progressed in leaves (Fig. 3) but had different expression patterns in siliques. In both cases, a reduction in expression occurred in early senescing siliques compared to mature.

miR447's precursor has been demonstrated to increase under both phosphate and nitrate limiting conditions and the mature miRNA targets *2-PHOSPHOGLYCERATE KINASE*, a gene involved in phytic acid metabolism (Allen et al., 2005; Pant et al., 2009; Kim and Tai, 2011). Our results indicated that the mature miRNA increases slightly in leaves during senescence (Fig. 3). In siliques, however, miR447 is reduced at the outset of senescence and then increased in late senescence (Fig. 3). miR398 has been reported to respond to a host of nutrients including phosphate, nitrate and sucrose and targets genes involved in glucose metabolism and oxidative stress response (Sunkar et al., 2006; Dugas and Bartel, 2008; Pant et al., 2009). miR398 showed a mild induction as senescence progressed in leaves and a much larger reduction in siliques (Fig. 3).

Two other regulated miRNA families without clear roles in nutrient responsiveness also showed senescence regulation (Fig. 4). The miR156/157 family is a highly conserved miRNA family which targets *SQUAMOSA PROMOTER LIKE BINDING (SPL)* genes that are crucial for development. Intriguingly, miR157 showed a slight decrease during senescence in leaves while its close relative, miR156 was unchanged (Fig. 4A). Both miR156 and miR157 had different expression patterns in siliques compared to leaves, where they both increased strongly in late senescence. miR396a increased very slightly in both leaf and silique senescence (Fig. 4B). miR396a-3p had a larger increase in expression in leaves and a steep decline in siliques during late senescence (Fig. 4B), indicating that this newly annotated miRNA may play a previously unknown role during senescence.

New senescence-inducible miRNA locus targets the alpha tubulin family

One new miRNA locus was discovered, and named "miRsen1" (Fig. 5A, Table 2). miRsen1 generates five abundant mature miRNAs, miRsen1.1-5 which were confirmed to be senescence-regulated by northern blotting. All miRNAs generated from the miRsen1 locus increased to their highest level in late senescing leaves (Fig. 5B). In siliques, miRsen1's mature miRNAs also showed their highest expression levels in late senescence (Fig. 5B).

Targets were also predicted for all mature miRNAs arising from the *miRsen1* locus using psiRNA target prediction software (Dai and Zhao, 2011). miRsen1.3 was predicted to target a conserved region of the alpha tubulin family, pairing to *TUA2*, *TUA4* and *TUA6* with scores of 4.0, 3.5 and 4.0 respectively (Fig. 5C). Abundant cleavage products generated by miRsen1.3 and a variant which differs by one nucleotide were both found in a previously constructed PARE library (German et al., 2008).

Analysis of PARE data from senescing leaves

Six PARE libraries were constructed from *Arabidopsis* leaves to examine targets of regulated miRNAs. These libraries yielded more than 750 million genome matched sequences and represent the first large scale analysis of the RNA degradome during *Arabidopsis* senescence (Table 3). Early and late senescing leaf libraries had significantly reduced genome-matched distinct sequences,

despite reconstruction and resequencing from biological replicates (Table 3). Initial analysis of these PARE libraries focused on examining targets of regulated miRNAs. Due to the reduction or absence of most miRNA target decay products in senescing leaves, it is not possible to draw conclusions about lower levels of decay intermediates for targets whose miRNAs were shown to be reduced as senescence progresses. However, miR408's strong induction in leaves was accompanied by an increase in the cleavage products generated for all four of its validated targets (Fig. 6).

Differential expression of senescence regulated miRNA targets

Expression changes of the genes targeted by senescence regulated miRNAs were analyzed. Although the majority of senescence regulated miRNAs have previously been shown to be nutrient responsive, their targets possessed a wide variety of functions ranging from nutrient mobilization to cell wall integrity. Like many senescence regulated miRNAs, these targets often showed different expression changes between leaf and silique tissue and in many cases showed incoherent regulation with the miRNAs targeting them (Fig. 7).

Functional analysis

Experiments were initiated for functional analysis of specific senescence-associated miRNAs and respective target genes. Transgenic Arabidopsis plants were generated in which the miR408 gene was over-expressed either constitutively or under a senescence-specific *BFN1* promoter. Transgenic lines were confirmed for over expression and are currently being characterized for any altered phenotypes, with emphasis of senescence progress. In addition, T-DNA knock out mutants for the different miRNA genes which expression was found to be altered during senescence, as well as for the different target genes identified, were already received from the stock center and are being subjected to analysis for evaluation of phenotypic consequences.

Details of cooperation – This research was based on close and synergistic collaboration between the US and Israeli laboratories. The initial stages of the research including, growth of plants, sampling of leaves at specific senescence stages and RNA extraction were done in the Israeli laboratory by Dr. Shaul Burd. The RNA was shipped frozen to be used in the US laboratory for library construction and sequencing. The bioinformatics and Northern blot analyses which led to identification of the senescence associated miRNAs were also performed in the US laboratory by the Ph.D. student Shawn Thatcher. Based on the results from these experiments specific miRNAs which levels are regulated in senescence were chosen for further research. These miRNAs, as well as their target genes, are being subjected to functional analysis already initiated in the Israeli laboratory which will be continued as collaborative research. The US and Israeli PIs agreed to make efforts for submitting to BARD a continuation research proposal on this topic.

Appendix for BARD Project Number: IS-4180-08

Manuscript to be submitted to Plant Physiology

Title:

Differential expression of miRNAs and their target genes in senescing leaf and silique

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One-Sentence Summary

The first massive sequencing of small RNAs and RNA decay intermediates from senescing tissue lead to
the discovery of a new miRNA locus as well as senescence regulation of known miRNAs and their target
genes.

FOOTNOTES

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ABSTRACT

MicroRNAs (miRNAs) are a class of small RNAs which typically function by guiding cleavage of target messenger RNAs. They have been shown to play major roles in a variety of plant processes including development, responses to environmental stresses, and senescence. To identify senescence-regulation of miRNAs in *Arabidopsis thaliana*, eight small RNA libraries were constructed and sequenced at four different stages of development and senescence from both leaves and siliques, resulting in more than 200 million genome-matched sequences. Parallel Analysis of RNA Ends (PARE) libraries, which enable the large-scale examination of miRNA-guided cleavage products, were also constructed and sequenced, resulting in over 750 million genome-matched sequences. These massive datasets lead to the identification of new miRNAs, as well as new regulation of known miRNAs and their target genes during senescence, many of which have established roles in nutrient responsiveness and cell structural integrity. In keeping with remobilization of nutrients thought to occur during senescence, many miRNAs and targets had opposite expression pattern changes between leaf and silique tissues during the progression of senescence. Taken together, these findings highlight the integral role that miRNAs may play in the remobilization of resources and alteration of cellular structure that is known to occur in senescence.

1 INTRODUCTION

2 In plants, senescence is a broad term that describes whole-plant as well as tissue-specific changes that
3 take place over the course of aging. During senescence, the leaf's cellular structure, metabolic
4 activities, and physiological role are greatly altered. Chloroplasts degenerate and the
5 photosynthetic apparatus is disassembled (Hörtensteiner, 2006; Thomas, 2013). Senescence is
6 characterized by a wide and significant change in the pattern of gene expression (Wagstaff et al.,
7 2009; Breeze et al., 2011): the expression of many genes, such as those associated with photosynthesis, is
8 repressed, while that of many other genes termed senescence-associated genes (SAGs) is induced. The
9 molecular mechanisms governing senescence regulation have been suggested to form a complex network
10 responsible for activation of the different SAGs (Guo and Gan, 2005). Various SAGs exhibit differential
11 expression in different tissues and in response to different senescence-promoting factors, including plant
12 endogenous factors such as hormones, and environmental factors such as abiotic stress (Park et al., 1998;
13 Weaver et al., 1998; Lim et al., 2007; Fischer, 2012). Senescence regulation involves the action of a large
14 number of transcription factors (TFs), and in particular the NAC and WRKY families are enriched for
15 senescence-regulated TFs in many plant species (Gregersen and Holm, 2007; Balazadeh et al., 2008;
16 Breeze et al., 2011). The involvement of epigenetic programming in senescence via histone
17 modifications was also demonstrated (Ay et al., 2009; Brusslan et al., 2012).

18 Due to the tight regulatory control exerted during the progression of senescence, it can be seen as a final
19 developmental stage (Gan and Amasino, 1997). In annual plants, including *Arabidopsis*, leaf senescence
20 is thought to be a response to dwindling nutrient availability as each growing season comes to a close.
21 Faced with a limited supply of micro and macronutrients, plants direct senescence in leaves in order to
22 mobilize resources, such as copper, phosphate and nitrate, into reproductive tissue to enhance seed yield
23 (Amasino and Himmelblau, 2001; Hörtensteiner and Feller, 2002; Diaz et al., 2008; Gregersen et al., 2008).
24 This idea is known as the nutrient relocation hypothesis (Guiboileau et al., 2010). Given their
25 established role in responding to nutrient availability, it is likely that microRNAs (miRNAs) may play a
26 crucial and currently underappreciated part in guiding this process.

27 miRNAs are a class of small RNAs which typically function by guiding ARGONAUTE-mediated
28 cleavage of target messenger RNAs. They have been shown to regulate a host of processes ranging from
29 development to responses to nutrient levels, such as phosphate and nitrate (Chiou et al., 2006; Kant et al.,
30 2011). miRNAs were directly implicated in regulating *Arabidopsis* senescence when it was shown that
31 miR164 is a key player in the senescence regulatory pathway. Natural reduction of miR164 level during
32 senescence results in increased cell death due to its control on its target, the senescence transcriptional

1 activator gene *ORE1* (Kim et al., 2009). However, no large scale sequencing of small RNAs or analysis of
2 their target genes over the course of senescence has been presented thus far.

3 In addition to changes in transcript abundance, senescence is also known to result in broad changes in
4 RNA decay through increased expression of RNAses (Taylor et al., 1993; Bariola et al., 1999). It has been
5 proposed that this increase in RNA turnover may be an adaptive response to senescence-related changes
6 in phosphate levels (Bariola et al., 1994; Green, 1994). Construction and sequencing of PARE (Parallel
7 Analysis of RNA Ends) libraries enables the precise examination of not only decay intermediates
8 generated by miRNA-guided cleavages, but also other RNA which possess a 5' monophosphate (German
9 et al., 2009). By utilizing this technique, a clearer picture of the RNA degradome during the course of
10 senescence could be obtained.

11 In this study, we utilize high-throughput sequencing of small RNA libraries constructed from both
12 senescing leaf and silique tissue to discover new miRNAs and differential miRNA expression in
13 *Arabidopsis*. Expression levels of target genes for many regulated miRNAs were also examined in detail,
14 and often had tissue-specific regulation as senescence progressed. This analysis was complemented
15 through the construction and sequencing of PARE libraries, which enabled examination of miRNA-
16 guided cleavage events during senescence. The combination of small RNA, target mRNA and degradome
17 data lead to a much more complete picture of the roles that miRNAs may play in *Arabidopsis* senescence.

RESULTS

Small RNA libraries from senescing *Arabidopsis* leaves and siliques

In order to examine the expression patterns of miRNAs during *Arabidopsis* senescence, small RNA libraries were constructed from leaves and siliques at four different developmental time points. Leaves five and six were selected based on age after germination (Fig. 1A): young leaves (35 days), mature leaves (45 days), early senescing leaves (50 days), and late senescing leaves (55 days). Siliques were selected based on visual morphology indicative of developmental and senescence stage (Fig. 1B). After trimming of adaptors and removal of rRNA, tRNA and snoRNAs, these eight small RNA libraries yielded more than 200 million genome-matched sequences (Table 1), and represent the first massive small RNA sequencing from senescing plant tissues.

Differential expression of known miRNAs during senescence

miRNA expression levels from young, early senescence and late senescence leaf and silique libraries were compared to libraries of their respective mature tissue to determine if any miRNAs were differentially expressed. As an initial screen for regulated candidates, the abundance of annotated miRNAs in mature leaf and silique libraries with abundance greater than 50 TP2M in any library were plotted against their abundance in young, early senescence or late senescence libraries (Fig. 2). In general, the expression of miRNAs across libraries was highly similar; with mature versus young showing the strongest miRNA expression correlation in both leaves and siliques libraries (R^2 of 0.95 and 0.94, respectively) and mature versus late senescence leaves and siliques libraries showing the weakest correlation (R^2 of 0.79 and 0.81, respectively). Despite mostly similar expression patterns, certain miRNAs did show significant increases or decreases in early and late senescing tissue.

miRNAs showing expression changes greater than fivefold from mature to early or late senescence were confirmed in two or more independent biological replicates via northern blotting or splinted ligation. miR164, which targets the positive regulator of senescence-induced cell death *ORE1* (*AT5G39610*), has been previously reported to decline in leaves as senescence progresses (Kim et al., 2009). Both small RNA sequencing data and small RNA northern blot validation reproduced this regulation, showing a steady decline from young to late senescence time points in leaves (Fig. 3). Interestingly, miR164 was reduced from young to mature siliques but did not continue to decline as senescence progressed in this tissue (Fig. 3); the first indication that senescence-responsive miRNAs may change in a tissue-dependent manner.

1 In addition to reproducing miR164's regulation, differential expression of seven other *Arabidopsis*
2 miRNAs during the course of senescence was observed (Fig. 3). Interestingly, five out of these seven
3 miRNAs have been reported to be nutrient responsive. miR408 targets a host of genes involved in pollen
4 tube guiding, copper metabolism and cell wall integrity (Sunkar and Zhu, 2004) and has been shown to be
5 responsive to low copper levels (Abdel-Ghany and Pilon, 2008). Both small RNA sequencing data and
6 northern blot validation showed that this miRNA increased approximately seven fold during senescence
7 in leaf tissue (Fig. 3). However, miR408's expression pattern in siliques differed from leaves, only
8 increasing very slightly (Fig. 3).

9 Several other nutrient responsive miRNAs also showed different expression trends between leaf and
10 silique during senescence. Both miR399, which targets the negative repressor of phosphate transport,
11 *PHOSPHATE2* (*PHO2*, *AT2G33770*), and miR827 which targets the nitrogen/phosphate balance
12 regulator *NITROGEN LIMITATION ADAPTATION* (*NLA*, *AT1G02860*) are well known to be strongly
13 induced by low phosphate conditions (Chiou et al., 2006; Kant et al., 2011). Both of these miRNAs
14 increased as senescence progressed in leaves (Fig. 3) but had different expression patterns in siliques. In
15 both cases, a reduction in expression occurred in early senescing siliques compared to mature. In late
16 senescence, miR827 continued to decline, dropping to nearly undetectable levels. miR399, on the other
17 hand, was strongly increased at the end of senescence, indicating that the regulation of these two miRNAs
18 may not be entirely dependent on phosphate levels during senescence.

19 miR447's precursor has been demonstrated to increase under both phosphate and nitrate limiting
20 conditions and the mature miRNA targets *2-PHOSPHOGLYCERATE KINASE* (*2PGK*, *AT5G60760*), a
21 gene involved in phytic acid metabolism (Allen et al., 2005; Pant et al., 2009; Kim and Tai, 2011). Our
22 results indicated that the mature miRNA increases slightly in leaves during senescence (Fig. 3). In
23 siliques, however, miR447 is reduced at the outset of senescence and then increased in late senescence
24 (Fig. 3). miR398 has been reported to respond to a host of nutrients including phosphate, nitrate and
25 sucrose and targets genes involved in glucose metabolism and oxidative stress response (Sunkar et al.,
26 2006; Dugas and Bartel, 2008; Pant et al., 2009). miR398 showed a mild induction as senescence
27 progressed in leaves and a much larger reduction in siliques (Fig. 3).

28 Two other regulated miRNA families without clear roles in nutrient responsiveness also showed
29 senescence regulation (Fig. 4). The miR156/157 family is a highly conserved miRNA family which
30 targets *SQUAMOSA PROMOTER LIKE BINDING* (*SPL*) genes that are crucial for development.
31 Intriguingly, miR157 showed a slight decrease during senescence in leaves while its close relative,
32 miR156 was unchanged (Fig. 4A). Both miR156 and miR157 had different expression patterns in siliques

1 compared to leaves, where they both increased strongly in late senescence. miR396a, which increased
2 very slightly in both leaf and silique senescence (Fig. 4B), was recently found to produce an abundant
3 miRNA*, miR396a-3p in certain tissues (Jeong and Thatcher *et al.*, unpublished). miR396a-3p had a
4 larger increase in expression in leaves and a steep decline in siliques during late senescence (Fig. 4B),
5 indicating that this newly annotated miRNA may play a previously unknown role during senescence.

6 **New senescence-inducible miRNA locus targets the alpha tubulin family**

7 Given that no large scale sequencing of small RNAs has been carried out in senescencing tissue, an
8 analysis of senescence libraries could reveal miRNAs which are only significantly expressed at this stage.
9 To find new senescence-induced miRNAs, small RNA data was first filtered to remove tRNA, rRNA,
10 snoRNA and previously annotated miRNAs. Regions surrounding the remaining small RNAs which were
11 elevated threefold or more in senescing leaf libraries compared to mature were evaluated using criteria
12 described by earlier (Meyers et al., 2008), including strand bias of greater than 90% and abundance bias
13 of 50%. Remaining regions were then subjected to manual folding analysis using Centroidfold (Sato et al.,
14 2009) to determine their ability to form stem-loop structures.

15 After application of these filters, one new miRNA locus was discovered, and named “miRsen1” (Fig. 5A,
16 Table 2). miRsen1 generates five abundant mature miRNAs, miRsen1.1-5 which were confirmed to be
17 senescence-regulated by northern blotting. All miRNAs generated from the miRsen1 locus increased to
18 their highest level in late senescing leaves (Fig. 5B). In siliques, miRsen1’s mature miRNAs also showed
19 their highest expression levels in late senescence (Fig. 5B). It is important to note that miRsen1 fails the
20 abundance bias cutoff for annotated miRNAs in young and mature leaf libraries but passes it in
21 senescencing leaf libraries, highlighting the importance of using a variety of tissues and conditions in the
22 search for bona fide miRNAs.

23 Targets were also predicted for all mature miRNAs arising from the *miRsen1* locus using psiRNA target
24 prediction software (Dai and Zhao, 2011). Published PARE (Parallel Analysis of RNA Ends) data from
25 *xrn4* flowers were examined to search for evidence of cleavage for each potential target (German et al.,
26 2008). miRsen1.3 was predicted to target a conserved region of the alpha tubulin family, pairing to *TUA2*,
27 *TUA4* and *TUA6* with scores of 4.0, 3.5 and 4.0 respectively (Fig. 5C). Abundant cleavage products
28 generated by miRsen1.3 and a variant which differs by one nucleotide were both found in this library,
29 although the conserved nature of this region makes it impossible to distinguish whether this decay
30 intermediate is arising from cleavage of one, two or all three alpha tubulin family members.

31 **Analysis of PARE data from senescing leaves**

1 In addition to utilizing small RNA libraries, six PARE libraries were constructed from *Arabidopsis* leaves
2 to examine targets of regulated miRNAs. After trimming of adaptors, these libraries yielded more than
3 750 million genome matched sequences and represent the first large scale analysis of the RNA degradome
4 during *Arabidopsis* senescence (Table 3). Early and late senescing leaf libraries had significantly reduced
5 genome-matched distinct sequences, despite reconstruction and resequencing from biological replicates
6 (Table 3). In keeping with this reduction in distincts, the majority of miRNA target decay products were
7 either missing or significantly reduced.

8 Initial analysis of these PARE libraries focused on examining targets of regulated miRNAs. Due to the
9 reduction or absence of most miRNA target decay products in senescing leaves, it is not possible to draw
10 conclusions about lower levels of decay intermediates for targets whose miRNAs were shown to be
11 reduced as senescence progresses. However, miR408's strong induction in leaves was accompanied by an
12 increase in the cleavage products generated for all four of its validated targets (Fig. 6). Similarly NLA had
13 a significant increase in the cleavage product generated by miR827-guided cleavage (data not shown).

14 **Differential expression of senescence regulated miRNA targets**

15 Expression changes of the genes targeted by senescence regulated miRNAs were also analyzed. Although
16 the majority of senescence regulated miRNAs have previously been shown to be nutrient responsive, their
17 targets possessed a wide variety of functions ranging from nutrient mobilization to cell wall integrity.
18 Like many senescence regulated miRNAs, these targets often showed different expression changes
19 between leaf and silique tissue and in many cases showed incoherent regulation with the miRNAs
20 targeting them (Fig. 7).

21 Four of miR408's targets which had increased cleavage products in senescence PARE data (Fig. 6) were
22 analyzed via northern blotting. *AT1G72230*, a member of the *CUPREDOXIN* superfamily which binds
23 copper and functions in the synthesis of xylan, a polysaccharide component of cell walls (Borner et al.,
24 2002; Mutwil et al., 2009), decreased as aging progressed in both leaves and siliques (Fig. 7A).
25 *UCLACYANIN 2 (UCC2, AT2G44790)* also contains a copper binding domain but its function remains
26 uncertain (Borner et al., 2002; Mutwil et al., 2009). Unlike *AT1G72230*, *UCC2* was mostly unchanged
27 during leaf senescence and actually increased in senescing siliques (Fig. 7A). *LAC3 (AT2G30210)* is a
28 member of the laccase family that has been proposed to be involved in maintaining cell wall integrity
29 (Turlapati et al., 2011). *LAC3* expression was not detected via northern blotting in leaves, but it was
30 highly expressed in young and mature siliques and decreased rapidly during senescence (Fig. 7A).
31 *PLANTACYANIN (AT2G02850)*, which is thought to function in pollen tube guiding, has only been
32 reported to be expressed significantly in the pistil and root (Dong et al., 2005; Abdel-Ghany and Pilon,

2008). Despite a highly abundant cleavage product present in leaf PARE libraries, no expression was detected in this tissue through northern blotting (Fig. 7A). However, *PLANTACYANIN* was detectable in early senescing siliques and increased further during late senescence, indicating incoherent regulation with miR408.

Both miR399, which targets *PHO2*, and miR827, which targets *NLA*, were strongly induced during senescence in leaves (Fig. 3). Interestingly, *PHO2* and *NLA* were themselves increased in senescing leaves, showing incoherent regulation with the miRNAs which target them (Figs. 7B and C). While full length *NLA* increased during leaf senescence, its miR827 guided cleavage product increased even more strongly (Fig. 7C). This result is consistent with an increase of miR827 and an accumulation of miR827 directed decay product in PARE results and implies that miR827 may be functioning to moderate *NLA*'s expression increase during senescence instead of acting as an on/off switch. In siliques, *PHO2* and *NLA* showed mostly coherent regulation, increasing to their highest levels in early senescence as miR399 and miR827 decreased.

miR447's target *2PGK* has previously been reported to be flower and silique preferential (Kim and Tai, 2011). In keeping with published work, *2PGK* was undetectable in leaves at any point during senescence (Fig. 7D). In siliques, *2PGK* increased from young to early senescence time points, reproducing earlier work demonstrating its role in increased phytic acid levels during siliques maturation (Kim and Tai, 2011). However, *2PGK* decreased as siliques progressed to late senescence, again showing coherent regulation with miR447's increase at this time.

miR398 targets a host of genes involved in a variety of different processes ranging from glucose metabolism to oxidative stress response. *COX5B-1* (*AT3G15640*), which is part of the glucose catabolic process (Comelli et al., 2009), was selected for northern blot analysis based known macro-nutrient changes during senescence (Wingler and Roitsch, 2008). Surprisingly, *COX5B-1*'s expression was relatively unchanged in leaves or siliques during senescence despite regulation of miR398 itself (Fig. 7E). miR398's other targets, *SUPEROXIDE DISMUTASE 1* (*CSD1*) and *SUPEROXIDE DISMUTASE 2* (*CSD2*), which are responsible for reducing reactive oxygen species (ROS) during oxidative stress, were unchanged in leaves but declined in senescing siliques (Fig. 7E).

miRsen1.3's targets include three members of the alpha tubulin family, *TUA2*, *TUA4* and *TUA6* (Fig. 5C), which were examined via northern blotting. All three of these targets declined to approximately half of their normal expression level in late senescing leaves (Fig. 8). In siliques, this decline was substantially stronger, with all of miRsen1.3's targets declining to nearly undetectable levels by late senescence (Fig. 8).

DISCUSSION

In this study, we report the first small RNA and PARE libraries from senescing leaves and siliques, which enabled large scale examination of miRNA expression and RNA decay in these tissues. Seven previously annotated miRNAs were demonstrated to change as senescence progressed, in a tissue specific manner (Figs. 3 and 4). Deep sequencing data also enabled identification of a new miRNA locus, miRsen1, which is most strongly expressed in senescing leaves and siliques (Fig. 5). Examination of miRNA targets' expression level and decay intermediates revealed that they also undergo tissue-specific changes during the course of senescence, often showing incoherent regulation with the miRNAs which target them (Fig. 7).

Differential expression of miRNAs and their target mRNAs in leaves and siliques

It has been reported that a significant change in the levels of both micro and macro nutrients occurs in senescing leaves (Bleecker and Patterson, 1997; Gan and Amasino, 1997). These changes are thought to be to be the result of a mobilization of resources away from vegetative tissue and towards developing reproductive tissue and lead to a host of expression changes in both tissues (Leopold, 1961; Guiboileau et al., 2010). Our analysis of miRNA expression during the course of senescence revealed different, and often completely opposite, expression patterns between leaves and siliques. In keeping with the nutrient remobilization hypothesis, five out of the seven miRNAs (miR408, miR399, miR827, miR447 and miR398) which were senescence regulated have been previously reported to be nutrient responsive (Fujii et al., 2005; Abdel-Ghany and Pilon, 2008; Dugas and Bartel, 2008; Pant et al., 2009; Kant et al., 2011), indicating that they may play a currently underappreciated role in controlling the flow of nutrients from vegetative to reproductive tissue.

miR399 and miR827 were both strongly induced during senescence in leaves but showed more complex pattern of regulation in siliques, where their expression was initially lower in early senescence (Fig. 3). The correlation between these two miRNAs in both leaves and siliques is consistent with their reported response to phosphate levels (Kant et al., 2011). However, their target genes *PHO2* and *NLA*, which are both negative repressors of phosphate transport, responded differently to senescence than they do to phosphate deficiency (Figs. 7 B and C) (Fujii et al., 2005). In leaves, *PHO2* gradually increased during senescence, showing incoherent regulation with miR399 and an opposite expression change than has been reported in non-senescence induced phosphate deficiency. However, it has been shown that while miR399 and *PHO2* are coexpressed in vascular tissue, miR399 alone is expressed in mesophyll cells where it may have a currently unidentified target (Aung et al., 2006), which could be repressed during senescence. Additionally, *Arabidopsis* expresses target mimics that sequester miR399, which could explain how both

miR399 and *PHO2* levels could increase under the same conditions (Franco-Zorrilla et al., 2007). miR827's target *NLA* also increased in senescing leaves, showing incoherent regulation and an opposite pattern than has been reported in phosphate deficient *Arabidopsis*. However, both PARE (data not shown) and northern blot (Fig. 7C) showed an even stronger accumulation of the miR827 directed cleavage product, which is consistent with elevated target and miRNA levels. Given *NLA*'s role in regulating phosphate transport in a nitrate dependent manner, it is likely that miR827 may be functioning to fine tune this relationship during senescence instead of acting as an on/off switch. Additionally, *NLA* has been shown to positively affect anthocyanin production, which is a well-known senescence phenotype (Peng et al., 2007).

miR447, which has been shown to be induced under nitrate deficient conditions (Pant et al., 2009), was mildly increased during senescence in leaves (Fig. 3), where it is known that nitrate levels decrease with age (Diaz et al., 2008). Its target *2PGK*, however, has been reported to be strongly silique and flower preferential and was undetectable in leaves at any time point. In siliques, miR447 had the lowest level of expression in early senescence where its target *2PGK* showed its highest expression (Fig. 7). The increase of *2PGK* as siliques transitioned from young to early senescence is consistent with earlier findings which implicate increases of this gene in the silique maturation-induced accumulation of phytic acid, a phosphate storage compound found in seeds which is inaccessible to human absorption and considered an anti-nutrient (Stevenson-Paulik et al., 2005). Knockouts of *2PGK* have been shown to have reduced phytic acid, a desirable agricultural trait, but also have reduced seed germination under stress (Kim and Tai, 2011). The natural repression of *2PGK* by miR447, which we show is reduced in late senescence, may be amenable to manipulation in future work.

Of the five nutrient responsive miRNAs which changed during the course of aging, only miR398 responded in an opposite manner in senescence compared to its demonstrated response to nutrient deficiency. While sucrose, phosphate and nitrate deficiencies have been reported to repress miR398 (Dugas and Bartel, 2008; Pant et al., 2009), its levels were actually increased during aging in leaves. Surprisingly miR398's target, *COX5b-1* was unchanged during senescence (Fig. 7E) despite its reported responses to sucrose levels (Comelli et al., 2009). miR398's other targets, *CSD1* and *CSD2*, however were down-regulated during senescence in siliques. This is surprising, given miR398's reduction during senescence in siliques (Fig. 2.3) and the known increase in reactive oxygen species during senescence (Woo et al., 2004) and indicates that miR398's role in senescence warrants further investigation.

The majority of senescence regulated miRNAs have previously been reported to respond to changes in nutrient levels. However, members of the miR156/157 family and miR396a-3p have not been shown to change significantly under nutrient starvation. miR396a itself has been reported to target the *GROWTH REGULATING TRANSCRIPTION FACTOR (GRF)* family (Jones-Rhoades and Bartel, 2004), but only increased very slightly in senescing leaves and siliques. Interestingly, miR396a's precursor was recently reported to produce an abundant miR star, miR396a-5p (Jeong and Thatcher *et al.*, unpublished) which was induced during senescence in leaves and repressed in siliques (Fig. 4B). This miRNA is currently without any validated targets, making it difficult to speculate on its potential role in senescence. All members of the miR156/157 family are predicted to target members of the *SPL* transcription factors which control development. Despite overlapping predicted targets, it has recently been reported that miR156 and miR157 family members do exhibit differential cleavages of specific *SPL* genes (Meng *et al.*, 2012). The senescence repression of miR157 in leaves without any change in miR156 therefore may be a fine tuning mechanism to control expression of specific *SPL* members as senescence progresses in leaves. In siliques, both miRNAs were strongly induced in late senescence which could lead to repression of all *SPL* family members in this tissue.

miR408 targets genes involved in many different biological processes and its senescence-regulation could potentially have a variety of effects. Although both *UCC2* and *PLANTACYANIN* are both validated targets of miR408 they showed incoherent regulation by increasing as senescence progressed (Fig. 7), and are involved in processes which have no known involvement in the senescence process (Dong *et al.*, 2005). *CUPREDOXIN* and *LAC3*, however, both showed the expected reduction during miR408's senescence induction (Fig. 7). *CUPREDOXIN* has been proposed to be involved in xylan biosynthesis (Mutwil *et al.*, 2009) and its down-regulation by miR408 could therefore affect cell wall integrity during senescence. Similarly *LAC3*, which was also reduced during senescence, is thought to act on the cell wall. Transgenic knockdowns of *LAC3* have been reported to have altered cell wall integrity and more readily detachable xylem fiber cell walls in poplar (Ranocha *et al.*, 1999). Senescence is known to bring about a host of cell wall changes (Bleecker and Patterson, 1997) and the regulation of *CUPREDOXIN* and *LAC3* by miR408 imply that this miRNA may be playing an important role in the process.

miRsen1 is induced by senescence and targets the alpha tubulin gene family

Arabidopsis has been studied extensively and is thought to be mostly saturated for miRNA discovery. However, the discovery of senescence-inducible *miRsen1* through deep sequencing of small RNAs in senescing tissues highlights the importance of continued investigation of the small RNA transcriptome under diverse sets of conditions. Although the mature miRNAs produced from this locus are detectable in

1 non-senescent tissues (Fig. 5B), the locus which generates them just barely fails the abundance bias cutoff
2 for annotating bona-fid miRNAs (Meyers et al., 2008) due to the amount of low abundance small RNAs
3 coming off of the predicted precursor in non-senescent small RNA libraries. The lack of precise
4 processing of this precursor indicates that *miRsen1* may be a young miRNA but its tight regulation under
5 senescence conditions and miRsen1.3's validated cleavage of alpha tubulin family members imply its
6 potential functional importance (Fig. 5C). Intriguingly, nearly all alpha tubulin members have been
7 reported to decline during senescence (Wagstaff et al., 2009; Keech et al., 2010; Breeze et al., 2011). We
8 show that all alpha tubulin members that miRsen1.3 targets decline slightly in leaves and drastically in
9 siliques (Fig. 8), which has the potential to lead to massive changes in cellular integrity. miRsen1.3 may
10 therefore be playing an important role in this structural shift by targeting alpha tubulins and represents an
11 interesting candidate for future studies investigating senescence-based changes in cell structure.

12 Taken together, these findings highlight the integral, and currently underappreciated, role that miRNAs
13 play during senescence. The high percentage senescence regulated miRNAs which have previously been
14 noted as nutrient-responsive, and their predominantly antagonistic regulation between leaves and siliques,
15 implies they may function in the regulatory pathways which govern mobilization of nutrients away from
16 vegetative tissue and toward reproductive tissue during the course of senescence. Beyond the mobilization
17 of nutrients, several miRNAs have target genes which could be of strong interest agriculturally, such as
18 miR447 which controls phytic acid accumulation, or miR408 and miRsen1.3 which have the potential to
19 alter cell structure and integrity. This study greatly expands upon the existing knowledge of miRNAs'
20 roles in senescence and provides a number of new avenues to explore in studying natural as well as stress-
21 induced senescence.

22

MATERIALS AND METHODS

Plant Growth and Stress Treatments

Plant material was harvested from *Arabidopsis* Col-0 wild type plants. Plants were grown in short days (8 hours) for two weeks on 1/2 x MS 0.8% agar plates. Seedlings were transferred to artificial soil in 7x7x8 cm containers and grown with a light/dark cycle of 16/8 hours (long days). For leaf tissue, leaves five and six were selected at four different stages: young (20 days after transfer), mature (30 days after transfer), early senescence (35 days after transfer) and late senescence (40 days after transfer). Silique tissue was staged based on visual morphology indicative of senescence. All tissues were harvested, immediately frozen in liquid nitrogen and stored at -80°C. RNA was extracted using Trizol reagent (Molecular Research center)

Small RNA Library Construction and Sequencing

Small RNA libraries were constructed as described previously (Lu et al., 2007). Briefly, small RNA was isolated from total RNAs using PEG8000/NaCl –mediated low molecular weight RNA precipitation and then run on a 15% denaturing PAGE gel to select and extract 20-30 nt RNAs. 5' and 3' adaptors were ligated, followed by additional PAGE purification after each reaction. RNA containing adaptors was reverse transcribed and amplified via 18 cycles of PCR followed by PAGE purification to select the final product. Libraries were sequenced using an Illumina IIGX platform.

Analysis of Small RNA Sequencing Data

Sequencing data from small RNA libraries were trimmed to remove adaptor sequences, and then mapped to the *Arabidopsis* genome (The Arabidopsis Information Resource [TAIR] version 10; <http://www.arabidopsis.org>). Reads matching perfectly to this genome and not matching tRNA, rRNA and snoRNA were retained for further analysis. Remaining small RNAs which did not match to known miRNAs which were elevated three fold or more in early or late senescence leaf libraries compared to mature leaf libraries were considered as possible new senescence-inducible miRNAs. Regions surrounding putative miRNAs were subjected to manual folding using Centroidfold (Sato et al., 2009).

RNA Blot Analysis

Small RNA analyses were carried out by running low molecular weight RNA on a 15% denaturing PAGE gel. RNA was transferred onto Hybond N+ membrane (Amersham) and fixed using UV crosslinking. Membranes were then prehybridized for at least two hours in Ultrahyb-Oligo hybridization buffer (Ambion). DNA oligos complementary to the miRNA of interest were labeled with [³²P]ATP using

Optikinas (Amersham Biosciences), filtered using QIAquick nucleotide removal kit (Qiagen). Labeled probes were added to the hybridization solution and allowed to hybridize for at least 12 hours. mRNA blots were carried out by running 20 µg of total RNA onto 1.5% formaldehyde agarose gels, followed by transfer onto Hybond N⁺ membranes. Membranes were then prehybridized overnight using hybridization buffer (Church and Gilbert, 1984) and probed with ³²P labeled probes. The probes were generated by PCR using primers described in Supplemental Table XXX. mRNAs were visualized using a Typhoon phosphorimager (GE Healthcare Life Sciences).

Splinted-Ligation-mediated miRNA Detection

miRNAs were examined by miRtec-IT miRNA labeling and detection kit (Affymetrix) as described previously (Maroney et al., 2008; Jeong and Green, 2012). In brief, miRNAs were captured from two µg of total RNA using a bridge oligonucleotide, that is designed to specifically detect one specific miRNA and ligated to a ³²P labeled detection oligo using T4 DNA ligase. The resulting products were separated via a 15% PAGE gel and visualized using a Typhoon phosphorimager (GE Healthcare Life Sciences).

Prediction and validation of miRNA targets

Targets were predicted for all new miRNAs using psRNATarget (Dai and Zhao, 2011). Targets with scores < 4.0 were considered potential new miRNA targets. To validate predicted cleavage, senescence PARE sequencing data as well as PARE sequencing data from *xrn4* flowers obtained from NCBI's Gene Expression Omnibus with accession number GSM280227 were utilized. Sequences matching to cleavage products starting between bases 10 and 11 from the 5' end of the predicted miRNA pairings were considered as evidence for miRNA-guided cleavage and were obtained through use of a custom perl script.

Accession Numbers

Small RNA and PARE data have been added to the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) (XXX-XXX). These data are also available at <http://mpss.udel.edu/XXX>.

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Figure 1. Leaves and siliques over the course of development and senescence

The course of development and senescence is represent by four time points.

(A) Leaves at four time points: Young (35 days), Mature (45 days), Early Senescence (50 days), Late Senescence (55 days). **(B)** Siliques at four time points: stage determined and selected based on visual inspection.

Figure 2. Comparison of miRNA abundance between mature and other time points

miRNA abundance comparison between mature and other time points. MiRNAs which were subjected to northern blot validation are highlighted in red. Correlation coefficients for each comparison are also shown.

(A) Mature leaves compared to young leaves, early senescing leaves and late senescing leaves. **(B)** Mature siliques compared to young siliques, early senescing siliques and late senescing siliques.

Figure 3. Expression of nutrient-responsive miRNAs in Arabidopsis leaf and silique over the course of senescence and development

miRNA expression level was examined via northern blotting. Time points **Y** (Young), **M** (Mature), **ES** (Early Senescence) and **LS** (Late Senescence) were selected as described in Figure 1. U6-normalized quantification values are displayed below each time point. One representative U6 is shown.

Figure 4. Expression of miRNAs in Arabidopsis leaf and silique

Time point selection and abbreviation as described in Figure 1.

(A) miR156/157 family members' expression level was examined via northern blotting. U6-normalized quantification values are displayed below each time point.

(B) miR396a and miR396a-3p expression was examined via splint-ligation-mediated miRNA detection. Quantification numbers are displayed below.

Figure 5. miRsen1's expression and target cleavage

(A) Small RNA plot of miRsen1. The abundance of small RNA sequences is plotted across their position within the miRNA locus's predicted precursor. Mature miRNAs are designated in red.

(B) miRsen1's expression level was examined via northern blotting. U6-normalized quantification values are displayed below each time point. Time points were selected as described in Figure 1.

(C) Predicted cleavage sites of miRsen1.3 are indicated by arrows and bold 5' nucleotides on target mRNAs. Target scores for each miRNA are also shown. Target cleavage data were obtained from PARE data of *xrn4* flower (German et al., 2008). PARE abundance is shown in histograms. Gray bars indicates cleavage by annotated miRNAs while white bars indicate cleavage by variants and black bars indicate other degradations

Figure 6. PARE sequences corresponding to precise miR408 target sites

PARE libraries data showing the level of decay intermediates generated by miR408's cleavage of *PLANTACYANIN*, *AT1G72230*, *LAC3* and *UCC2*. Cleavage data were obtained from PARE libraries constructed at 4 time points during development and senescence. Bars represent the abundance of decay intermediates generated by miR408 guided cleavage.

Figure 7. Senescence regulation of nutrient-responsive miRNA targets in leaf and silique

Northern blots showing differential expression of nutrient regulated miRNA targets in senescing leaf and silique. Time points were selected as described in Figure 1. *UBC* (*At5g25760*) normalized quantification values are displayed below each time point. **(A)** miR408's targets *UCC2*, *PLANTACYANIN*, *AT1G72230* and *LAC3*. **(B)** miR399's target *PHO2* **(C)** miR827's target *NLA* **(D)** miR447's target *2PGK* **(E)** miR398's targets *COX5B-1*, *CSD1* and *CSD2* **(F)** Representative *UBC*.

Figure 8. Senescence regulation of miRsen1.3's targets

Northern blots showing differential expression of miRsen1's targets in senescing leaf and silique. Time points were selected as described in Figure 1. *UBC* (*At5g25760*) normalized quantification values. One representative *UBC* is shown.