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MICROARRAY ANALYSIS OF RICE GRAIN ABSCISSION REGULATED BY *SH4*

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Major Professor's Signature

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MICROARRAY ANALYSIS OF RICE GRAIN ABSCISSION REGULATED BY *SH4*

By

Ailing Zhou

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ABSTRACT

MICROARRAY ANALYSIS OF RICE GRAIN ABSCISSION REGULATED BY *SH4*

By

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Abscission is the programmed organ separation from the main body of the mother plant. Separation takes place at predetermined positions called abscission zones. Although abscission is essential for the plant life cycle, the molecular regulation of abscission remains poorly understood. The rice grain shattering gene *sh4*, which encodes a putative MYB3 transcription factor, is involved in the development and function of the abscission zone between a rice grain and its pedicel. To investigate genes potentially regulated by *sh4* during the abscission process, microarray analysis was conducted using *sh4* transgenic plants. The study identified several categories of genes that were up-regulated by *sh4*. These include cell wall hydrolytic enzymes, expansins, pathogenesis-related genes, and abscisic acid (ABA) and stress responsive genes. Further studies indicated that exogenous ABA was capable of promoting flower abscission in wild rice species and the *sh4* transgenic plants. ABA from the developing embryo may have served as the signal for the initiation of rice grain abscission. The study suggests that ABA is the hormone that signals rice grain abscission and *sh4* is a regulator in the signaling pathway.

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INTRODUCTION

Abscission refers to a developmental process that leads to the detachment of a plant organ. Abscission is a widespread phenomenon and is very important for the plant life cycle. Most deciduous plants abscise their leaves before winter, while evergreen plants continuously shed older leaves. A plant may drop its unwanted organs such as petals, sepals or filaments when they are no longer functionally essential. Fruits and seeds may be shed for seed dispersal. A plant may also abscise immature fruits to conserve resources needed to bring the remaining fruits to maturity. In response to disease or pathogen attack, a plant may shed its infected organs to protect the rest of the plant.

Abscission also impacts agricultural productivity. Easy shedding of fruits causes considerable yield loss of crops. One of the essential steps of plant domestication was the reduction in fruit or seed shattering to allow effective field harvest. Important examples include the reduction of grain shattering in cereals and pod shattering of legumes.

Abscission Zone

Abscission consists of two developmental stages. The first is to form an abscission zone (AZ) and the second involves the response to environmental and

hormonal signals that trigger cell wall hydrolysis in the abscission zone (Roberts et. al., 2002). The formation of the AZ at the location of organ separation may be completed months before organ detachment actually occurs. (Sexton and Roberts 1982; Gonzalez-Carranza et al., 1997).

In dicotyledonous plants such as *Arabidopsis*, tomato, and bean, an abscission zone usually encompasses several layers of cells. In comparison to adjacent cells, AZ cells are smaller, contain denser protoplasm and larger deposits of starch, and have smaller intercellular spaces. Cell expansion in AZ often occurs during the abscission process (reviewed by Sexton and Roberts, 1982).

In monocotyledonous plants such as in rice and oat, the AZ is located between the flower (or grain) and pedicel, and mostly consists of one layer of small, thin-walled cells. It is thus also called an abscission layer (Sargent 1984; Jin 1986). In rice, the abscission layer forms 16-20 days before flowers open for pollination (Jin 1986).

Positive Hormonal Regulation of Abscission

In 1955, Osborne published the first evidence that a diffusible substance in senescent leaves in beans and several other species accelerated abscission when applied to bean leaflet explants (Osborne 1955). Subsequently, several studies reported physiologically similar substances from leaves and fruits. Carns and his colleagues found a diffusible substance collected from the base of the cotton fruit

(*Gossypium hirsutum*) promoted abscission (Carns 1958). This work led to the purification and partial identification of abscisin I, a weak abscission-accelerating crystallized substance isolated from mature fruit walls of cotton (Liu and Carns, 1961).

Two years later, Ohkuma et al. identified and purified a strong abscission-accelerating acid from young cotton fruits and named it abscisin II (Ohkuma et al., 1963). The structure of abscisin II was then determined by Ohkuma et al. (1965). Shortly after, an abscission accelerator from the fruits of yellow lupin (*lupinus luteus*) was identified and found to be identical to abscisin II (Porter and van Steveninck, 1966). However, the name abscisin II was called into question by some chemists because they concerned that a name such as abscisin II could raise difficulties for further naming analogues and derivatives. They favored a name that ends in "-ic acid". At the Sixth international Conference of Plant Growth Substances held in Ottawa in 1967, a group of 12 scientists agreed to change the name to abscisic acid (ABA). This concluded the discovery and naming of the plant hormone, ABA, whose function, as indicated by its name, was thought to positively regulate plant organ abscission.

ABA-accelerated abscission was observed in buds, leaves, pedals, flowers, and fruits (Addicott and Carns, 1983). ABA can also increase the rate of leaf senescence (Jackson and Osborne, 1972), promote ethylene synthesis (Riov et al., 1990), and induce cell wall hydrolytic enzymes such as cellulase (Craker and

Abeles, 1969). However, shortly after its discovery, studies began to show that ABA is not particularly effective in promoting abscission. For example, the leaf abscission responses to ABA in *Citrus* were found to be season-dependent. Leaves sprayed with ABA in summer fell off, but those sprayed in winter did not (Cooper et al., 1968). Work by Jackson and Osborne suggests that ABA probably stimulates the abscission process through its ability to promote tissue senescence and ethylene climacteric (Jackson and Osborne, 1972). This is further supported by the result that ABA is not capable of speeding up abscission in *Citrus* if the tissue's ability to produce ethylene is inhibited by aminoethoxyvinylglycine (AVG) (Sagee et al., 1980), suggesting that ABA functions through ethylene to accelerate abscission. These observations raised the question of whether ABA is the major abscission accelerator.

The ABA's role in regulating abscission was further obscured after the discovery of its essential roles in regulating dormancy and stomatal closure. When the role of ethylene in accelerating abscission was increasingly demonstrated in dicots, the role of ABA in regulating abscission was considered to be minor (Patterson 2001).

Numerous studies have shown that ethylene can promote abscission of leaves, floral organs and fruits. Evidence mainly falls into four categories. First of all, there is a correlation between elevated ethylene production and the onset of abscission. Many fruits produce considerable amounts of ethylene in correlation with ripening and abscission (Pratt 1974; Walsh 1977). Natural ethylene or ethylene precursor

level is often higher during fruit abscission (Brady et al., 1991; Burdon and Sexton, 1993). Further study of β-glucuronidase (GUS) expression driven by the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (the enzyme that synthesizes the ethylene precursor ACC) promoter exhibits localized expression within the abscission zone (Ecker and Theologis, 1994).

Second, abscission can be delayed by ethylene inhibitors. When ethylene production was inhibited by the antisense of ACC oxidase, the enzyme that converts ACC to ethylene, the activation of fruit abscission in melon was not activated and the fruit did not drop (Ayub et al., 1996). Likewise, the ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG), could reduce apple fruit drop (Kondo and Hayata, 1995).

Third, abscission is delayed in ethylene insensitive mutants. Arabidopsis ethylene insensitive mutants *etr1-1* (Bleecker et al., 1988) and *ein2* (Guzman and Ecker, 1990) show significantly delayed shedding of floral parts (Bleecker and Patterson, 1997). Finally, ethylene can up-regulate the expression of cell wall degrading enzymes in the abscission zone (Boller et al., 1983; Taylor et al., 1990). Genes encoding these enzymes contain promoter elements that are subject to regulation by ethylene (Bleecker and Patterson, 1997).

In the applied side, ethylene has been used as a chemical abscission agent for fruit harvest (reviewed by Addicott, 1982). Fruits can be shaken off from trees using mechanical devices when the abscission processes are well advanced by

ethylene. This is particularly helpful for cultivars whose fruits are not yet dropping at the optimum harvest time and cannot be removed by the limited degree of shaking that a tree can tolerate. The best examples include the use of ethylene to help harvest of walnuts and pecans. Ethephon, a chemical that is easily converted to ethylene when absorbed by plants, has been effectively used for help harvest apples, cherries, plums, prunes, olives, oranges, and coffee berries.

While ethylene has become the leading plant hormone in the basic research and agricultural applications related to abscission, researchers recently began to question how widespread the role ethylene plays as the primary hormone regulator of abscission. van Doorn and Stead surveyed more than 300 flowering plant species and found that although in most dicots flower abscission was regulated by ethylene, most monocots showed ethylene-insensitive petal abscission (van Doorn and Stead, 1997; van Doorn 2002). Work by Sexton et al. indicates that tulip tepal abscission does not respond to ethylene or ethylene antagonists (Sexton et al., 2000). Aneja et al. reported that during cocoa flower abscission, ABA levels increased dramatically prior to abscission, while ethylene production only increased slightly. Furthermore, the ABA synthesis inhibitor, fluridone, inhibited the formation of an abscission zone and consequently the abscission or senescence of flowers; whereas the ethylene biosynthesis inhibitor, AVG, only slightly delayed but did not prevent abscission (Aneja et al., 1999).

In addition, studies in grain abscission of wild oat (Sargent et. al., 1984)

indicated that neither ethylene nor ACC promoted the cell separation process, and AVG did not delay abscission. In contrast, it was ABA that accelerated abscission of grains of wild oat. These observations raised the questions of whether the abscission of monocotyledons and dicotyledons is controlled by different hormones (Sargent et al., 1981, 1984), whether the abscission of different organs, such as leaf and fruit or flower is regulated by the same hormone, and whether the key regulator has yet to be determined (Roberts et. al., 2002).

Genes Up-regulated in the Abscission Process

Abscission occurs in multiple organs, such as leaf, flower, seed and fruit. The model plant *Arabidopsis* does not have leaf or fruit abscission, but it does display floral organ abscission (Patterson 2001). Progress has been made recently in studying *Arabidopsis* floral organ abscission, which led the identification of several genes with novel functions (reviewed by Lewis et al., 2006). Genes involved in abscission of other organs are relatively poorly understood. In general, at least two categories of genes are up-regulated in almost all organ abscission systems studied to date.

The first group includes cell wall hydrolytic enzymes and expansins. During abscission process, cell wall degrading enzymes play vital roles to degrade middle lamella or even primary cell wall. The first enzyme observed to be involve in cell wall degradation at the site of abscission was β-1,4-glucanase, or cellulase

(Horton and Osborne, 1967; Lewis and Varner, 1970; Sexton and Roberts, 1982). This gene belongs to a large gene family, and seven different isozymes (Cel1 to Cel7) have been cloned in tomato (reviewed by Roberts et al. 2002). During tomato flower abscission, an increased expression of Cel1, Cel2, and Cel5 has been detected (del Campillo and Bennett, 1996; Gonzalez-Bosch et al., 1997; Kalaitzis et al. 1999). But which, if any, of these isoforms may contribute to abscission is still unclear.

Polygalacturonase (PG) is another abscission-related enzyme. The increased enzyme activity was reported during the abscission of leaves (Taylor et al., 1993), flowers (Tucker et al., 1984) and fruits (Bonghi et al., 1992; Henderson et al., 2001). It has been proved that in tomato three PG isoforms (TAPG1, TAPG2 and TAPG4) are associated with abscission. The GUS expression of *TAPG1* and *TAPG2* was localized specifically in the abscission zone of leaf petioles, flower and fruit pedicels, petal corolla, and stigma, and the expression was increased by ethylene and inhibited by IAA (Hong et al., 2000). In *Arabidopsis*, an abscission-related PG (*PGAZAT)* was shown to be specifically expressed at the base of the anther filaments (Gonzalez-Carranza et al., 2002). A null mutant of *PGAZAT* was found slightly delayed in ethylene-promoted flower abscission (Roberts et al., 2002). Expansins are associated with cell wall loosening. Two expansin genes have been cloned from the abscission zone region of ethylene-treated leaflets, and have been found to be specifically up-regulated during cell separation (Belfield et al. 2005).

The second group included pathogenesis-related genes. Abscission provides an ideal site for pathogen invasion, and can be recognized as a classical defense response in many plants (Eyal et al., 1993). Pathogenesis-related (PR) genes are shown to express at a higher level during abscission. del Campillo and Lewis (1992a) found that abscission of the primary leaves of bean was accompanied by the accumulation of a number of PR proteins. Such proteins were also accumulated in bean anthers and pistils during flower abscission (del Campillo and Lewis, 1992b). Coupe et al. (1997) isolated several types of PR cDNA clones from ethylene treated leaflet abscission zone in *Sambucus nigra*. When Bleecker and Patterson fused the chitinase promoter to GUS and transformed the construct into *Arabidopsis*, the expression was observed at floral abscission zone (Bleecker and Patterson, 1997; Patterson and Bleecker, 2004). Plant defense associated enzymes, β-1,3-glucanases (Volko et al., 1998) and jasmonic acid biosynthetic enzymes (Kubigsteltig et al., 1999) were up-regulated specifically in the floral abscission zone region in response to different stresses. Metallothionein-like proteins also accumulate during ethylene-promoted abscission. These proteins might act to scavenge the free radicals within the abscission zone tissues (Coupe et al., 1995).

Genes Involved in Grain Abscission During Rice Domestication

Rice (*Oryza Sativa*), the world's staple food, feeds more than half of the world's population. Wild rice species shed their mature grains rapidly to ensure efficient seed dispersal. However, to allow effective field harvest, human must select those plants that can hold on to their ripe grains. Reduction in grain shattering is one of the first and most important agricultural traits selected by humans (Harlan 1975). The genetic basis of rice grain shattering remained unknown until recently. Two grain shattering genes, *sh4* (Li et. al., 2006b) and *qSH1* (Konishi et. al., 2006) were identified in rice through quantitative trait loci (QTL) mapping. *qSH1* was cloned from a shattering- type cultivar and shown to encode a BEL1-type homeobox gene. A single-nucleotide polymorphism (SNP) in the 5' regulatory region was found responsible for the loss of grain shattering. A near isogenic line (NIL) that contained the *qSH1* region exhibited an improved abscission layer, suggesting *qSH1* is involved in the development of abscission layer.

s*h4* is a grain shattering QTL identified from crosses between a wild rice species *Oryza nivara* and a cultivar of *O. sativa ssp. indica* (Li et al., 2006a). It was positionally cloned and the mutation responsible for the derivation of nonshattering in cultivated rice was mapped to a 1.7 kb region of a gene with a previously unknown function. Sequence analyses of additional species of wild rice and

cultivars, along with gene transformation experiments confirmed that a single amino acid substitution from lysine to asparagine in the predicted MYB3-like DNA-binding domain caused the reduction of shattering in cultivated rice. The nuclear localization of sh4 protein, together with bioinformatic analysis, suggests the gene is a transcription factor. *sh4* is required for the development of the abscission layer between a grain and pedicel that controls programmed cell separation. The amino acid substitution weakened the function of *sh4* and caused the incomplete development of the abscission layer in cultivated rice. However, the mutation did not eliminate abscission layer function and allowed manual separation of the grains from the pedicel, a part of the rice harvest process known as threshing. The increased expression of *sh4* in the late stage of seed maturation suggests that the gene may also be involved in the activation of the abscission process (Li et al., 2006b), which occurs after the formation of abscission layer. To test this hypothesis, one of the appropriate approaches would be to examine what genes are regulated by *sh4*.

Microarray analysis has proved to be a powerful tool to identify gene transcription profiles at the genome-wide level (Brown and Botstein, 1999; Young 2000; Lockhart and Winzeler, 2000; Harmer et. al, 2000). This sensitive and productive method should generate more information about changes in transcription during abscission process, and opens a door to further explore functions of abscission related genes. To identify genes regulated by *sh4* during

the grain abscission process, microarray analysis was conducted using *sh4* transgenic plants and the controls.

MATERIALS AND METHODS

Rice Gene Transformation

Mature rice seeds of a *joponica* cultivar, T309, were dehusked, surface-sterilized with 70% ethanol for 1 min, rinsed with sterile water 3 times and sterilized again with 20% Chlorox bleach for 20 min. After being washed 3 times again with sterile water, ten seeds were placed on each callus induction medium plate (Table 1) with sterilized forcep. Plates were sealed with parafilm and set under light at 30 ^{0}C for 3 to 4 weeks. On the day of the transformation, the proliferated calli derived from the scutella were divided into 2 mm pieces and transferred onto co-cultivation medium plates and each plate had about 100 pieces.

One day before the transformation, *Agrobacterium* tumefacias strain EHA 101 carrying *sh4* construct 1, which harbored *O. sativa* promoter and lysine region of *O. nivara* allele (Li et al., 2006b) was grown in AB liquid medium (Chilton et al., 1974) containing 50 mg/L streptomycin overnight at 28^{0}C with vigorous shaking at 250 rpm. *Agrobacterium* cells were harvested by centrifugation at 3000 g for 10 min at 4^{0}C, resuspended in PIM2 medium (Kant et al., 2001) supplemented with 100 μM acetosyringone and 50 mg/L hygromycin (Table 1). The *Agrobacterium* cell cultures were diluted with PIM2 medium to OD_{600} < 0.1, measured with a spectrophotometer (Barnstead II Thermolyne Corporation, Dubuque, IA, USA).

Ten microliter of the diluted aliquot were pipetted onto a callus piece on co-cultivation medium (Table 1) and set in the dark at room temperature for 2-3 days. The cocultivated calli were washed with sterile water containing 250 mg/L cefotaxime (Sigma, St. Louis, MO, USA) to kill the *Agrobacterium*. The seeds were blotted dry on sterilized filter paper and transferred to selection medium (Table 1) containing 50 mg/L hygromycin. Plates were sealed with parafilm and put under continuous light at 30^0C for 4 weeks. Fast growing calli on selection medium were transferred to 20x150 mm shoot induction medium plates (Table1), and set under 16-h-light/8-h-dark cycles at 30^0C. Regenerated shoots were transferred to root induction medium (Toki 1997, Table 1) for 2-3 weeks. Regenerated plantlets were then transferred to sterilized soil in small pots with a plastic cover to maintain humidity. After one week the seedlings were transplanted to big pots and grown to maturity in a greenhouse with 16-h-light/8-h-dark cycles.

Table 1. Media used for rice transformation

Medium	Composition
YEP medium	10 g/L yeast extract, 10g/L peptone, 5 g/L NaCl 5g/L, 50 mg/L streptomycin, 15 g/L agar, PH 7.5
AB medium	1x AB buffer (3 g/L K_2HPO4, 1 g/L NaH_2PO4), 1x AB salts (1 g/L NH_4Cl, 0.15 g/L KCl, 0.01 g/L $CaCl_2 \cdot 2H2O$, 2.5 mg/L $FeSO4 \cdot 7H2O$), 5 g/L glucose, 50 mg/L streptomycin, PH 7.2
Calli induction medium	MS salts and vitamins [1], 30 g/L sucrose, 2 mg/L 2,4-D, 0.3 g/L casamino acids, 2.8 g/L proline, 6 g/L agarose, PH 5.8
Co-cultivation medium	MS salts and vitamins[1], 30 g/L sucrose, 10 g/L glucose, 2 mg/L 2,4-D, 1 g/L casamino acids, 100 μM acetosyringone, 6 g/L agarose, PH 5.2
PIM2 medium	1% glucose, 75 mM MES (PH 5.6), 2 mM NaPO4 buffer (PH 5.6), 1xAB salts (1 g/L NH_4Cl, 0.15 g/L KCl, 0.01 g/L $CaCl2 \cdot 2H2O$, 2.5 mg/L $FeSO4 \cdot 7H2O$)
Selection medium	MS salts and vitamins[1], 30 g/L sucrose, 0.3 g/l casamino acids, 2.8 g/L proline, 2 mg/L 2,4-D, 50 mg/L hygromycin, 250 mg/L cefotaxime, 6 g/L agarose, PH 5.8
Shoot induction medium	MS salts and vitamins[1], 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 2 mg/L benzylaminopurine, 0.05 mg/L naphthylacetic acid, 50 mg/L hygromycin, 4 g/L gelrite, PH 5.8
Root induction medium	MS salts and vitamins[1], 30 g/L sucrose, 50 mg/L hygromycin, 2 g/L gerite, PH 5.8

[1](Murashige and Skoog, 1962, purchased from SIGMA-ALDRICH, Inc., St. Louis, MO, USA.)

DNA Isolation and Screening of Transgenic Plants

DNA was isolated from leaf tissues of 10-day old seedlings as described previously (Li et al., 2006b). Transgenic plants were first screened by plasmid primers, Lac F (5'-TGGAGCTCCAGCTTTTGTTC-3') and Lac R (5'-AGTTAGCTCACTCATTAGGC- 3'). Plants that failed to amplify the Lac band

were further screened by coding region primers, ssh4-7819sF (5'-GAGAGCGCGTCGTAGACCTC-3') and ssh4-8201sR (5'-GCAAGGGGACTGGACGCTG-3') to rule out the possibility of the Lac negative band being caused by low DNA quality.

Segregation Analysis

Ten-day-old T0 and T1 seedlings were grown in small pots in a tray, and plants containing the transgene were identified by PCR with the above primers. Segregation ratios were calculated by counting the number of plants carrying the *sh4* transgene and those lacking the transgene.

Phenotypic Evaluation

Phenotype was evaluated by measuring the force required to pull away flowers or grains from the pedicel (Li et al., 2006b). Five panicles of each plant were evaluated.

Oligo Microarray

Expression profiling was conducted with the 45K NSF rice oligo nucleotide array (http://www.ricearray.org/index.shtml), which consisted of two slides and was printed with 43,482 oligos designed for 45,116 TIGR V3 rice gene models from the TIGR Rice Annotation Database that have EST and/or full-length cDNA support.

These oligos were cross referenced to the Kikuchi full-length cDNA dataset (Kikuchi et al., 2003) and were designed using PICKY-2 (Chou et al., 2004). In addition to the rice gene oligos, 456 hygromycin resistant gene oligos were randomly spotted as controls. These hygromycin oligos served as positive controls in our case.

RNA Isolation and Quality Check

Total RNA was isolated and purified from tissues at the junction of flower/grain and pedicel using the Qiagen Plant RNeasy Kit (Qiagen Sciences, Germantown, MD, USA). Four developmental stages, pollination (flowering stage), grain soft dough (grain is less than half full), grain hard dough (grain is full but still green), fully mature grain (grain is full and yellow) were assayed. Tissues from three to five plants were pooled for each sample. RNA was prepared from three biological replicates for each developmental stage and was examined by an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) for integrity and NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) for purity. To ensure data quality, only samples with well-defined 18S and 28S rRNA bands, and with A260/A280 ratios of 1.9-2.1 were included in the microarray analyses.

RNA Labeling

Because of the limited amount of tissues, fluorescent cRNA was generated using an Agilent Low Input Fluorescent Linear Amplification Kit (Agilent Technologies, Inc., Wilmington, DE, USA) with modifications. Briefly, both first and second strand cDNA were synthesized by incubating 450 ng total RNA with 1.2 µl of T7 promoter primer in nuclease-free water at 65 ^{0}C for 10 min followed by incubation with 4 µl of 5× first strand buffer, 2 µl of 0.1MDTT, 1 µl of 10mM dNTP, 1 µl of 200 U/µl MMLV RT, and 0.5 µl of 40U/ul RNaseOUT at 40 ^{0}C for 2 h. Incubate samples at 65 ^{0}C for 15 min to inactivate MMLV RT. Immediately following cDNA synthesis, the reaction mixture was incubated with 2.4 µl of 10 mM cyanine-3- or cyanine-5-CTP (Agilent), 15.3 µl of nuclease-free water, 20 µl of transcription buffer, 6 µl of 0.1M DTT, 8 µl of NTP mix, 6.4 µl of 50% PEG, 0.5 µl of RNaseOUT, 0.6 µl of inorganic pyrophosphatase, and 0.8 µl of T7 RNA polymerase at 40 ^{0}C for 4 h. Cyanine 3 (Cy3) or cyanine 5 (Cy5) labeled cRNA was purified with Qiagen's RNeasy mini spin columns. Amplified cRNA was quantified using a NanoDrop spectrophotometer. Only cRNA with a concentration higher than 1.5 µg/µl and the specificity higher than 8.0 pmol Cy3 or Cy5 per µg cRNA was used for hybridization.

Hybridization and Washing

Prior to hybridization, microarray slides were immersed in pre-hyb buffer (185 ml ddH_2O, 62.5 ml 20XSSC, 2.5 ml 10% SDS and 2.5 g BSA) at 42 ^{0}C for 45 min, and

washed in 0.1XSSC and ddH_2O. Mixed Cy3 and Cy5 labeled cRNA was dried and resuspended in 4 µl 10 mM EDTA. One microliter of 10 mg/ml yeast tRNA was added to the reaction mix as the block solution. The mixture was heated at 95 ^{0}C for 10 min, and 60 µl of pre-warmed SlideHyb buffer (Ambion, Austin, TX, USA) was added. The total of 64 µl mixture was applied to the array and hybridized at 48 ^{0}C for 16 h. After hybridization, the array was washed sequentially with solution I (1×SSC and 0.2% SDS in ddH2O), solution II (0.1× SSC and 0.2% SDS in ddH2O), and solution III (0.1×SSC in ddH2O).

Data Acquisition and Analysis

Dried arrays were immediately scanned with an Affymetrix 428^{TM} Array Scanner (Affymetrix, Inc., Santa Clara, CA, USA), and TIFF images were processed with GenePix pro 3.0 software. Spots with aberrant morphology were manually flagged. Raw data GPR files generated from GenePix were uploaded in R-based limmaGUI package (Wettenhall and Smyth, 2004). Hybridization quality of each slide was checked with image array plot before normalization was performed. Various background correction methods were compared, and no background correction option was finally adopted. Scale normalization method was used. Toptable for differentially expressed genes was generated with FDR adjusted method. For the three replicates, a spot was removed from further analysis if any one replicate has flagged value, or any two of the three replicates has 40 percent feature pixels with

intensities lower than two standard deviations above the background pixel intensity in both channels. With a cutoff of p value < 0.05 and two fold change, both up-regulated and down-regulated genes were identified for three developmental stages.

ABA Treatment

Flowering panicles from *O. nivara* and T309 transgenic plants were treated with 0.1 mM, 1.0 mM abscisic acid (A.G. Scientific, Inc., San Diego, CA, USA) and H_2O, respectively. Percentage of flower shattering was calculated every 24 hr by counting the number of flowers dropped when panicles were tapped by hand. Eight to ten panicles from at least three plants were examined for each treatment.

Stigmas of a portion of unopened flowers on *O. nivara* panicles were removed. The panicles were observed for 30 days on plants and then removed and treated with 1.0 mM ABA.

Seed ABA Extraction and Analysis

Forty grains of *O. nivara* were sampled from four different developmental stages, Milky endosperm (grain less than half full), soft dough (grain half to 3/4 full), green hard dough (grain full but still green) and yellow hard dough (grain fully full and yellow). Grains were dehusked and ground to power in a mortar under liquid nitrogen, and 5 ml ABA extraction buffer (100 mg/L 2,6-ditert-butyl-methyl phenol,

500 mg/L citric acid in 80% methanol) added, rotated at 4^0C in the dark for 16h. The suspension was centrifuged at 1000x g at 4^0C for 20 min and then transferred to four new microcentrifuge tubes and vacuum dried. The dried residue was dissolved with 100 µl of Tris saline buffer (TBS, 25mM Tris, 100mM sodium chloride, 1mM magnesium chloride hexahydrate) containing 0.02% sodium azide, and then the extracted ABA from the four tubes was combined together.

Quantitative analysis of ABA was performed by the indirect enzyme-linked immunosorbent assay (ELISA) method, using the Phytodetek-ABA-Kit as described by the manufacturer (Sigma-Aldrich, Inc., St. Louis, MO, USA), and measured with a Spectra Max M2 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

RESULTS

RNA Quality Check

RNA samples were assessed quantitatively and qualitatively by the Nanodrop spectrophotometer. If an A260/A280 ratio was below 1.9, the extraction procedure was repeated. RNA samples with accepted A260/A280 ratio were further analyzed using an Agilent bioanalyzer (Figure 1). The 18s and 28s peaks were clearly visible at 41 and 47 seconds respectively in stage I, II and III, whereas 28s and 18s peaks were absent in stage IV. The result indicates RNA samples from the Stage I, II and III were qualified for microarray. The total RNA from stage IV was almost degraded for the reason of seed maturation. Thus stage IV could not be included in the microarray experiment.

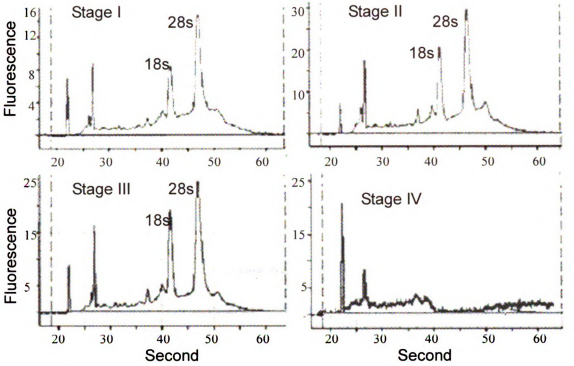

Figure 1. RNA integrity assessment. The purified total RNA samples from different developmental stages were measured by Agilent 2100 Bioanalyzer and the results were presented as Electropherograms. RNA of one biological replicate from each stage was presented.

Developing Transgenic Lines

Characterization of T0 Transgenic Plants

sh4 construct 1 which harbored *O. sativa* promoter and most of the coding region of *O. nivara* that contains the functional SNP was transformed into an *O. sativa ssp. Japonica* cultivar, T309. DNA was extracted from transgenic plants, and PCR analysis indicated that all of the transgenic plants carried the *sh4* transgene.

In comparison with the control, most of T309 transgenic lines (T0) had loosened flowers (Li et al., 2006b). Four transgenic lines with the clear phenotype, S11, S12, S14, and S15, were grown for advanced generations, T1, T2, and so on.

Segregation Analysis of T1 Transgenic Plants

DNA was extracted from 10-day-old T1 seedlings derived from T0 plants through self-fertilization. PCR analysis was performed to determine the presence of the *sh4* transgene. The transgene segregated in all four lines, and the Chi square (χ^2) test indicated that the transgene was introduced into each of the four T0 transgenic lnes at a single locus (P>0.05) (Table 2).

Table 2. χ^2 test of T1 transgenic plants

Line	Observed number		Expected number		χ^2	P value
	With transgene	without transgene	with transgene	without transgene		
S11	67	25	69	23	0.232	0.630
S12	65	23	66	22	0.061	0.805
S14	54	19	55	18	0.074	0.786
S15	68	15	62	21	2.295	0.130

Df=1

Phenotypic Evaluation of T1 Transgenic Plants

The phenotype of all T1 transgenic plants grown under the same conditions in the greenhouse was evaluated by measuring the force required to pull away the mature grains from the pedicels (Li et al., 2006a). Compared with plants without the trangene, the force required to break away the grains of plants carrying the transgene was greatly reduced (Figure 2-5). All four lines showed significant difference of grain shattering between transgenic plants with and without the trangene ($p = 0.004$, 0.002, 0.001 and 0.023 for S11, S12, S14 and S15, respectively; student's t-test). The variance of the measurement was relatively large due to the limited number of grains available from individual plants for the phenotypic evaluation.

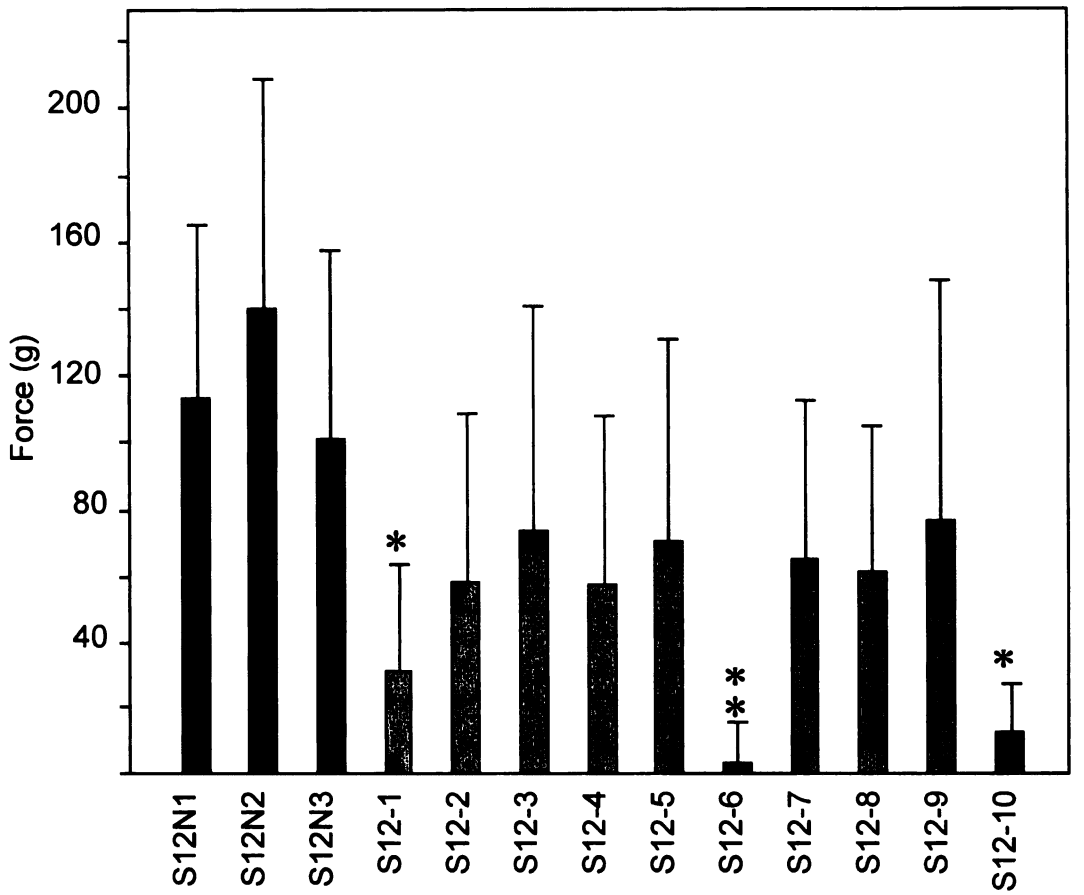

Figure 2. Grain shattering phenotype in line S12 T1 plants. Grey bars indicate plants carrying *sh4* transgene, black bars indicate plants that segregated from the same T0 parent and did not carry the transgene. The average force with standard deviation required to pull away a grain from the pedicel is illustrated. Plants marked with * were selected to grow for further segregation and phenotypic evaluation. Total number of grains evaluated was between 30 and 100, depending on the number of grains available at the time of measurement.

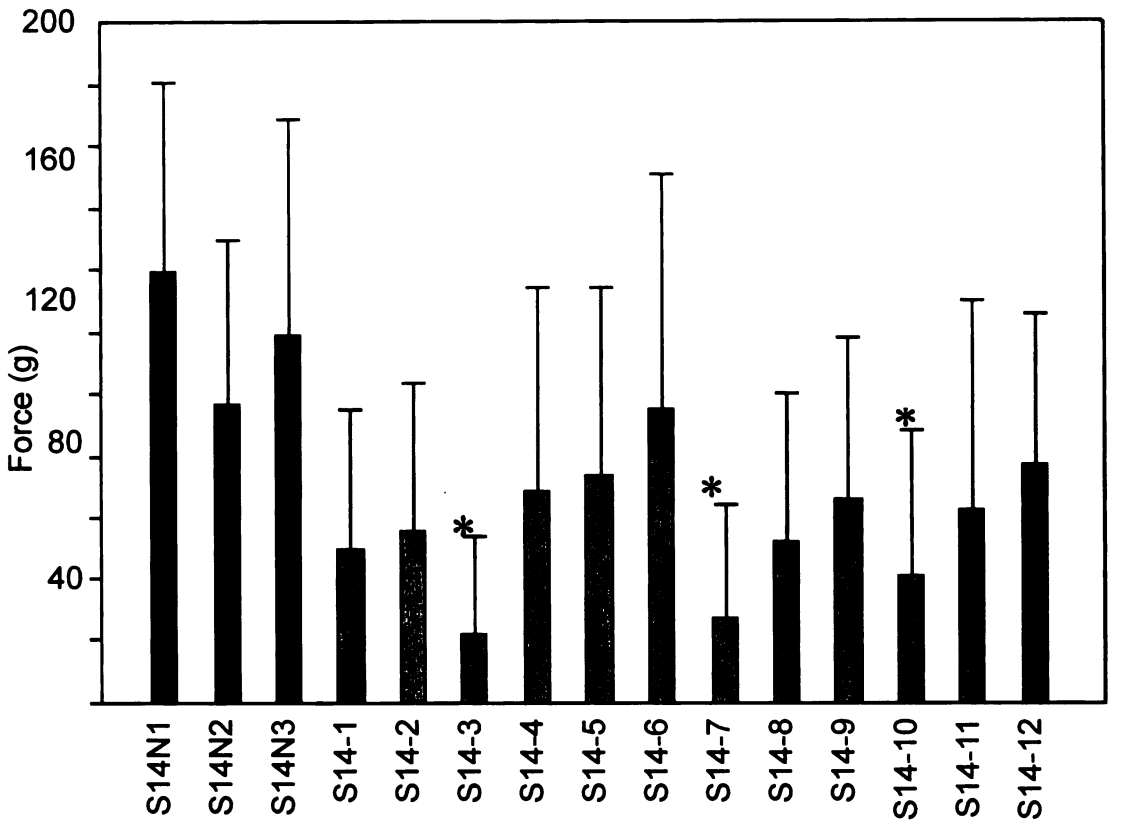

Figure 3. Grain shattering phenotype in line S14 T1 plants. Grey bars indicate plants carrying *sh4* transgene, black bars indicate plants that segregated from the same T0 parent and did not carry the transgene. The average force with standard deviation required to pull away a grain from the pedicel is illustrated. Total number of grains evaluated from each plant varied between 40 and 100, depending on the number of grains available at the time of measurement. Plants marked with * were grown for further phenotypic evaluation.

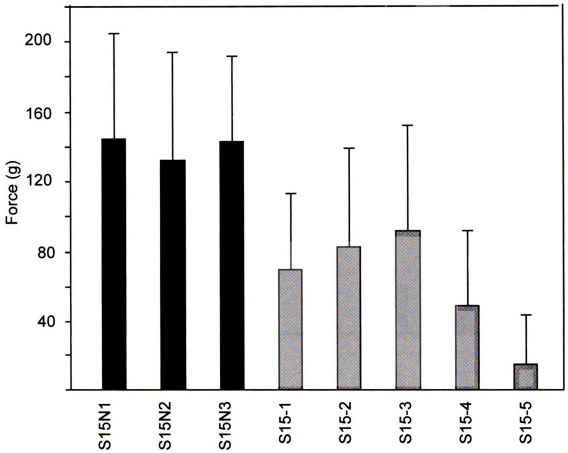

Figure 4. Grain shattering phenotype in line S15 T1 plants. Grey bars indicate plants carrying *sh4* transgene, black bars indicate plants that segregated from the same T0 parent and did not carry the transgene. The average force with standard deviation required to pull away a grain from the pedicel is illustrated. Total number of grains evaluated was between 20 and 50, depending on the number of grains available at the time of measurement.

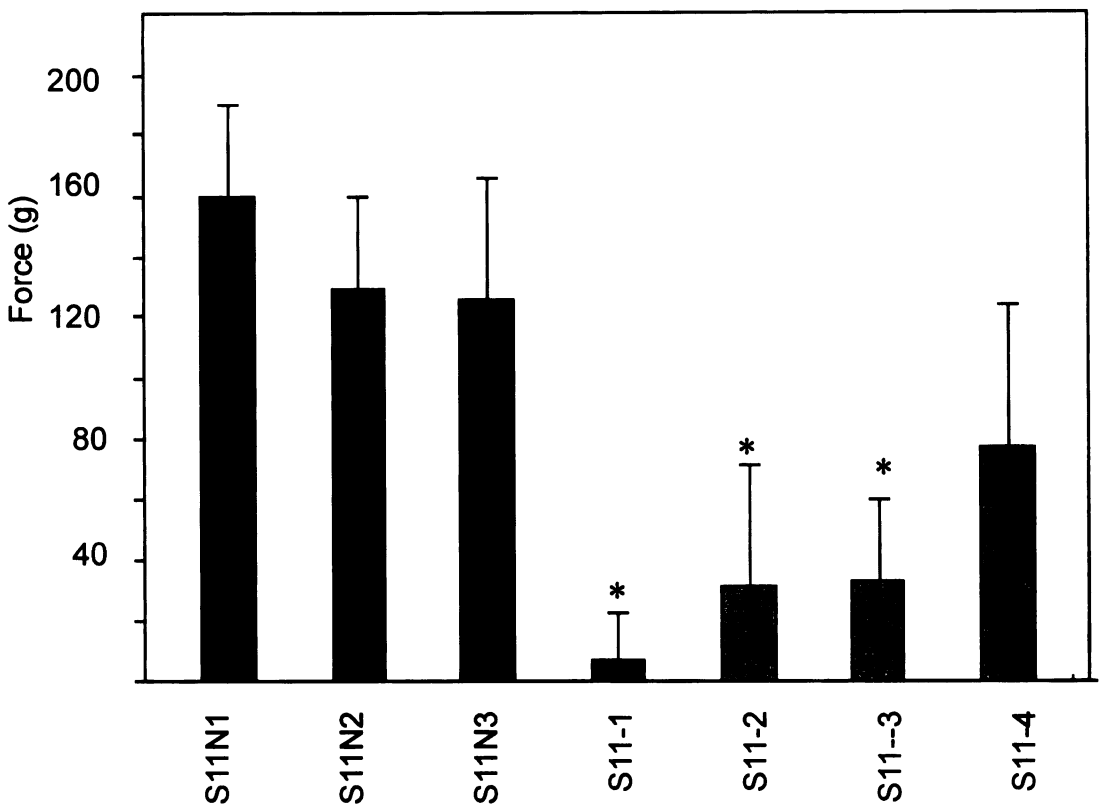

Figure 5. Grain shattering phenotype in line S11 T1 plants. Grey bars indicate plants carrying *sh4* transgene, black bars indicate plants segregated from the same T0 parent and did not carry the transgene. The average force with standard deviation required to pull away a grain from the pedicel is illustrated. Plants marked with * were grown for further phenotypic evaluation. Total number of grains evaluated was between 20 and 50, depending on the number of grains available at the time of measurement.

Among the T1 transgenic lines, plant S12-6 showed the best phenotype. Thus transgenic and the control seeds randomly selected from S12-6 were grown for

further analysis (double * in Figure 2). Meanwhile, two other plants from line S12 and three easiest-shattering plants from line S11 and S14 were chosen for further phenotypic evaluation. DNA was extracted from 10-day-old seedlings of T2 plants derived from the T1 line S12-6. PCR analysis from 32 T2 individuals demonstrated that the transgene was no longer segregating, indicating that this line was homozygous at the transgene locus.

Phenotypic Evaluation of S12-6 T2 Homozygous Transgenic Plants

The T2 plants derived from S12-6 showed an easy shattering phenotype (Figure 6). The plants segregated from the S12 T0 line that did not carry the transgene showed non-shattering phenotype. The Student's t-test indicated a highly significant difference between the phenotypes ($p<0.0001$). Therefore, the T2 plants derived from homozygous transgene line S12-6 and the line without trangene were used for the microarray analysis.

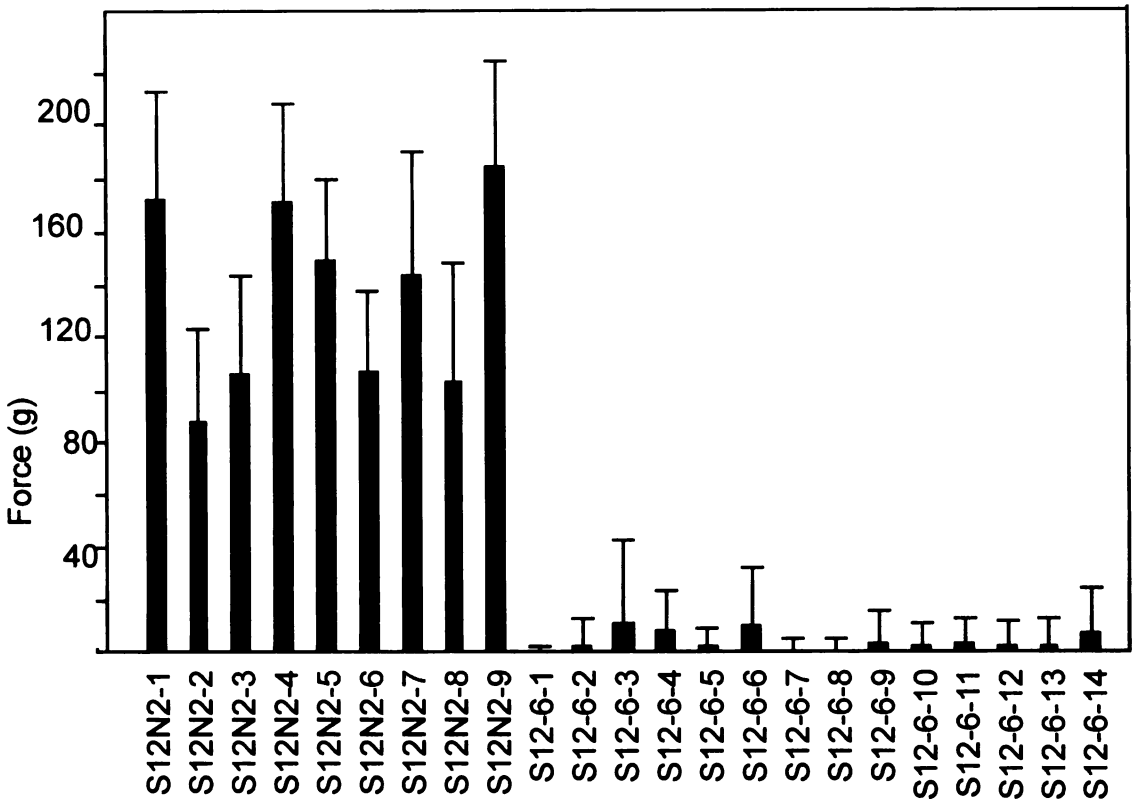

Figure 6. Grain shattering phenotype of the T2 plants derived from S12. Grey bars indicate plants carrying *sh4* transgene (from T1 line S12-6), black bars indicate control plants (from T1 line S12N2) that did not carry the transgene. The average force with standard deviation required to pull away a grain from the pedicel is illustrated. Fifty grains from each plant were evaluated.

Microarray Analyses

Comparison of Different Background Correction Methods

For the raw data generated from genepix, several background correction methods, including None, Subtract, Half, Minimum and RMA were compared in

R-based Limma package (Smyth 2004) (Figure 7).

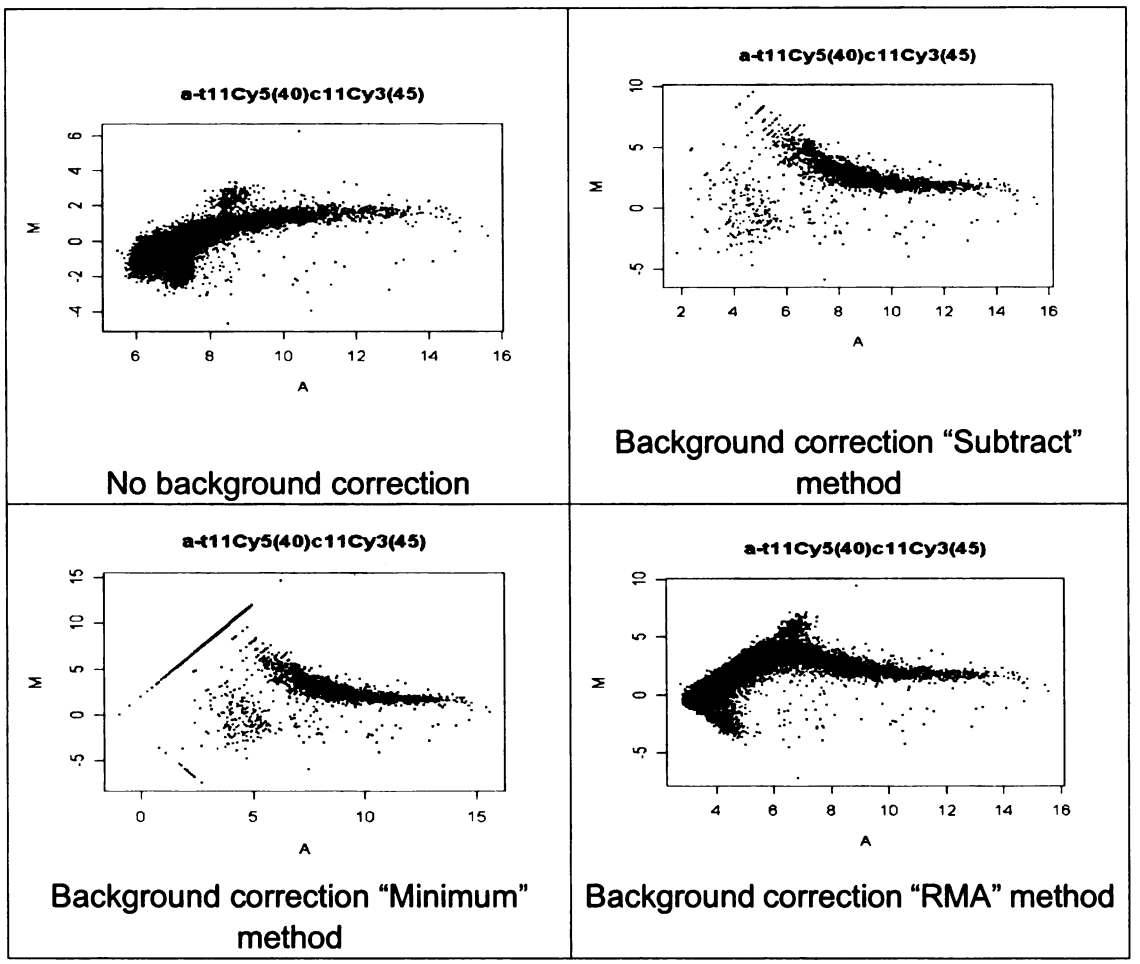

Figure 7. MA-plots for one slide of stage 1 after different methods of background correction without any normalization. MA-plots for other slides were similar for each of these background correction methods.

From the log-ratio M and log-intensity A plot (MA-plot), we can see that the "Subtract" method was not suitable for our dataset, which may be due to many negative values after background subtraction. These values cannot be used for

further analysis since Log cannot deal with negative values, so they were treated as missing observations. The "Minimum" and the "Half" (graph not shown) methods did not give better results as they assign some fixed positive values for the nonnegative intensities after subtracting the background intensities. The RMA method gave some normal plots. However, this method still showed biased distribution for our dataset. Based on the comparison of the MA-plot, no background correction seems to generate the least bias, thus it was adopted in our microarray data analysis.

Genes Up-regulated by sh4

With a cutoff of two fold change and a p value of 0.05, a total of 278 genes were identified as differentially expressed in *sh4* transgenic and control plants for three developmental stages. Two hundred and forty one genes were up-regulated by *sh4* (Table 3). They were grouped into the following functional categories (Table 4).

Table 3. Number of up-regulated genes in various functional categories identified in three developmental stages

Functional category	**Stage 1**	**Stage 2**	**Stage 3**
Cell wall hydrolytic enzymes and expansins	9	0	1
Pathogenesis- related genes	15	2	2
ABA/stress related genes	10	3	6
DNA binding/transcription factor	3	0	3
Signal transduction	3	0	2
Membrane protein	4	0	2
Transport	3	0	4
Protein biosynthesis/modification	16	0	5
Metabolism	11	3	9
Photosynthesis	23	3	4
Others	29	4	22
Unknown	38	7	12

Table 4. Genes up-regulated by *sh4*

Category	Annotation	ID	Stage
Cell wall hydrolytic enzymes and expansins			
	Alpha-expansin 11 precursor, putative	LOC_Os06g50400	1
	Expansin-related protein 2 precursor, putative	LOC_Os09g29710	1
	Mannan endo-1,4-beta-mannosidase, putative	LOC_Os01g47400	1
	Pectinesterase family protein	LOC_Os03g28090	1
	Endo-beta-mannanase, putative	LOC_Os03g61270	3
	Glycosyl hydrolases family 16 protein	LOC_Os04g53950	1
	Glycosyl hydrolase family 1 protein	LOC_Os03g11420	1
	Hydrolase, alpha/beta fold family protein, putative	LOC_Os03g27110	1
	Glycosyl hydrolase family 14 protein	LOC_Os10g32810	1
	Beta-fructofuranosidase 1 precursor, putative	LOC_Os02g01590	1
Pathogenesis-related			
	Glycosyl hydrolases family 18 protein	LOC_Os10g28080	1
	Endochitinase A precursor, putative	LOC_Os04g41620	1
	26 kDa endochitinase 1 precursor, putative	LOC_Os05g33150	1
	Chitinase 1 precursor, putative	LOC_Os10g28120	1

Table 4 cont'd

Endochitinase A precursor, putative	LOC_Os04g41680	1
Pectinesterase inhibitor domain containing protein	LOC_Os03g61530	1
Pectinesterase inhibitor domain containing protein	LOC_Os05g46530	1
Polygalacturonase inhibitor 1 precursor, putative	LOC_Os07g38130	1
Win1 precursor, putative	LOC_Os11g37970	1
Xylanase inhibitor protein 1 precursor, putative	LOC_Os07g43820	1
Xylanase inhibitor protein 2 precursor, putative	LOC_Os05g15770	1
Glucan endo-1,3-beta-glucosidase 5 precursor	LOC_Os06g39060	1
Glucan endo-1,3-beta-glucosidase GV, putative	LOC_Os01g71810	1
Lectin precursor, putative	LOC_Os04g09390	3
Metallothionein-like protein type 3, putative	LOC_Os05g11320	1,2
P21 protein, putative	LOC_Os03g46070	1
Pathogenesis-related protein Bet v I family protein	LOC_Os04g39150	2
Pathogenesis-related protein Bet v I family protein	LOC_Os04g39150	3
ABA/Stress response		
Hsp20/alpha crystallin family protein	LOC_Os01g04350	1
Salt stress-induced protein	LOC_Os01g24710	1
Wound/stress protein	LOC_Os02g51710	1
Wound induced protein	LOC_Os04g54240	1,2
Wound induced protein, putative	LOC_Os04g54300	1,2
Universal stress protein family protein	LOC_Os05g28740	1
Late embryogenesis abundant protein	LOC_Os05g29930	1
ABA/WDS induced protein	LOC_Os11g06720	1
Late embryogenesis abundant protein Lea14-A, putative	LOC_Os01g12580	3
ABA/WDS induced protein	LOC_Os04g34600	3
Stress responsive protein, putative	LOC_Os01g01450	3
Universal stress protein family protein	LOC_Os05g37970	3
BURP domain containing protein	LOC_Os05g12640	1,2
16.9 kDa class I heat shock protein, putative	LOC_Os01g04380	3
Zinc finger A20 and AN1 domains containing protein At2g36320	LOC_Os07g07350	3
Dehydrin family protein	LOC_Os11g26790	1
DNA binding/transcription factor		
Floral homeotic protein APETALA1, putative	LOC_Os03g54160	1
Homeobox domain containing protein	LOC_Os04g45810	1
LIM domain containing protein	LOC_Os10g35930	1

Table 4 cont'd

AP2 domain containing protein	LOC_Os09g35010	3
MYB6, putative	LOC_Os05g35500	3
DNA-binding protein S1FA2, putative	LOC_Os04g33440	3
Signal transduction		
EF hand family protein	LOC_Os06g47640	1
EF hand family protein	LOC_Os05g31620	3
EF hand family protein	LOC_Os02g39380	1
Ras-related protein Rab-2-B, putative	LOC_Os02g37420	1
Protein kinase domain containing protein	LOC_Os12g41090	3
Membrane protein		
Outer membrane lipoprotein blc precursor, putative	LOC_Os02g39930	1
Plant integral membrane protein TIGR01569 containing protein	LOC_Os04g21320	1
16kDa membrane protein, putative	LOC_Os04g33830	1
Plasma membrane ATPase 1, putative	LOC_Os07g09340	1
Secretory carrier membrane protein family protein	LOC_Os05g42330	3
Pyrophosphate-energized vacuolar membrane proton pump, putative	LOC_Os06g08080	3
Transport		
Aquaporin RWC3, putative	LOC_Os04g47220	1
Aquaporin TIP-type RB7-18C, putative	LOC_Os06g22960	1
Aquaporin TIP3.1, putative	LOC_Os05g14240	3
POT family protein	LOC_Os01g65100	3
Nonspecific lipid-transfer protein precursor, putative	LOC_Os01g60740	1
Adaptin N terminal region family protein	LOC_Os11g07280	3
Major intrinsic protein	LOC_Os01g13130	3
Protein biosynthesis/modification		
Eukaryotic aspartyl protease family protein	LOC_Os01g41550	1
Eukaryotic aspartyl protease family protein	LOC_Os03g08790	1
Eukaryotic aspartyl protease family protein	LOC_Os04g58840	1
Ubiquitin-conjugating enzyme E2-17 kDa, putative	LOC_Os03g57790	1
Subtilisin N-terminal Region family protein	LOC_Os07g39020	1
Ubiquitin-conjugating enzyme E2-17 kDa, putative	LOC_Os04g57220	3
Bowman-Birk serine protease inhibitor family protein	LOC_Os03g60840	1
5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, putative	LOC_Os12g42884	3
60S ribosomal protein L12, putative	LOC_Os02g47140	1

Table 4 cont'd

60S ribosomal protein L31, putative	LOC_Os02g48660	1
40S ribosomal protein SA, putative	LOC_Os03g08440	1
60S ribosomal protein L15, putative	LOC_Os03g40180	1
Ribosomal protein L36 containing protein	LOC_Os03g59720	1
40S ribosomal protein S8, putative	LOC_Os04g28180	1
40S ribosomal protein S10, putative	LOC_Os04g35090	1
60S ribosomal protein L6, putative	LOC_Os04g39700	1
60S ribosomal protein L4, putative	LOC_Os07g08330	1
60S ribosomal protein L22-2, putative	LOC_Os07g47710	1
40S ribosomal protein S3a, putative	LOC_Os03g10340	3
Ribosome associated membrane protein RAMP4 containing protein	LOC_Os07g39400	3
60S ribosomal protein L17, putative, expressed	LOC_Os08g41810	3
Metabolism		
Triosephosphate isomerase, cytosolic, putative	LOC_Os01g05490	1
Cytochrome P450 family protein	LOC_Os04g40470	1
Nucleoside diphosphate kinase 1, putative	LOC_Os07g30970	1
Fructose-bisphosphate aldolase, cytoplasmic isozyme	LOC_Os08g02700	1
ATP synthase subunit C family protein	LOC_Os12g34110	3
Enolase, C-terminal TIM barrel domain containing protein	LOC_Os03g15950	1
Geranylgeranyl hydrogenase, putative	LOC_Os02g51080	1
Glutathione S-transferase GSTU6, putative	LOC_Os10g38740	3
Oxidoreductase, 2OG-Fe oxygenase family protein	LOC_Os01g70930	3
Annexin-like protein RJ4, putative	LOC_Os02g51750	1
GDSL-like Lipase/Acylhydrolase family protein	LOC_Os10g32580	2
Farnesyl pyrophosphate synthetase, putative	LOC_Os01g50760	1,2
NAD dependent epimerase/dehydratase family protein	LOC_Os07g40690	3
NAD dependent epimerase/dehydratase family protein	LOC_Os06g44180	3
Heme oxygenase 1, putative	LOC_Os06g40080	1
2-Hydroxyisoflavanone dehydratase, putative	LOC_Os09g28630	1
Gibberellin 2-beta-dioxygenase, putative	LOC_Os01g55240	3
Tropinone reductase, putative	LOC_Os11g25700	3
Alcohol dehydrogenase class III, putative	LOC_Os02g57040	3
Cyanate hydratase, putative	LOC_Os10g33270	3
SAM dependent carboxyl methyltransferase family protein	LOC_Os02g48770	2

Table 4 cont'd

O-methyltransferase family protein	LOC_Os10g02880	1
Photosynthesis		
Chlorophyll a-b binding protein 2, chloroplast precursor, putative	LOC_Os01g41710	1,2,3
Chlorophyll a-b binding protein CP24 10B, chloroplast precursor, putative	LOC_Os04g38410	1
Chlorophyll a-b binding protein of LHCII type III, chloroplast precursor, putative	LOC_Os07g37550	1
Chlorophyll a-b binding protein 6A, chloroplast precursor, putative	LOC_Os06g21590	1
Chlorophyll a-b binding protein 8, chloroplast precursor, putative	LOC_Os02g10390	1,3
Chlorophyll a-b binding protein 7, chloroplast precursor, putative	LOC_Os07g38960	1
Chlorophyll a-b binding protein 8, chloroplast precursor, putative	LOC_Os02g10390	3
Thioredoxin F-type 2, chloroplast precursor, putative	LOC_Os01g68480	1
Ferredoxin-NADP reductase, leaf isozyme, chloroplast precursor, putative	LOC_Os02g01340	1
Elongation factor Tu, chloroplast precursor, putative	LOC_Os02g38210	1
Chloroplast 30S ribosomal protein S10, putative	LOC_Os03g10060	1
Acyl-desaturase, chloroplast precursor, putative	LOC_Os03g30950	1
Photosystem I reaction center subunit psaK, chloroplast precursor, putative	LOC_Os07g05480	1
Ferredoxin-1, chloroplast precursor, putative	LOC_Os08g01380	1,2
Photosystem I reaction center subunit V, chloroplast precursor, putative	LOC_Os09g30340	1
Photosystem I reaction center subunit VI, chloroplast precursor, putative	LOC_Os05g48630	1
Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor, putative	LOC_Os11g47970	1
Expressed protein;Photosystem I reaction center subunit III, chloroplast precursor, putative	LOC_Os03g56660	1
Oxygen-evolving enhancer protein 3-1, chloroplast precursor, putative	LOC_Os07g36080	1
Ferritin 1, chloroplast precursor, putative	LOC_Os12g01530	1,2,3
Possible Photosystem II reaction center Psb27 protein, putative	LOC_Os03g21560	1

Table 4 cont'd

Ribulose bisphosphate carboxylase large chain precursor, putative	LOC_Os10g21280	1
Ribulose bisphosphate carboxylase large chain, catalytic domain containing protein, putative	LOC_Os06g39730	1
50S ribosomal protein L12-2, chloroplast precursor, putative	LOC_Os01g47330	1
Others		
Histone H4, putative	LOC_Os04g49420	1
Histone H4, putative	LOC_Os02g45940	1
Histone H3, putative	LOC_Os06g06460	1
Histone H4, putative	LOC_Os09g38020	1
Histone H4, putative	LOC_Os09g26340	3
Histone H2A, putative	LOC_Os05g38640	3
Histone H4, putative	LOC_Os03g02780	3
Peroxidase 21 precursor, putative	LOC_Os07g49360	1
AhpC/TSA family protein	LOC_Os01g48420	1
Protein phosphatase 2C, putative	LOC_Os05g38290	3
Peptidyl-prolyl cis-trans isomerase 1, putative	LOC_Os06g49480	3
RNA recognition motif family protein	LOC_Os03g61990	3
Fasciclin domain containing protein	LOC_Os01g06580	3
Cytidine and deoxycytidylate deaminase zinc-binding region family protein	LOC_Os01g51540	1
ADP-ribosylation factor, putative	LOC_Os01g59790	3
GTP-binding nuclear protein Ran/TC4, putative	LOC_Os05g49890	3
RPT2, putative, expressed	LOC_Os11g02610	1
Cupin family protein, expressed	LOC_Os08g03410	1
Shrunken seed protein, putative	LOC_Os02g03070	2
Pollen-specific protein C13 precursor, putative	LOC_Os04g32680	1
B-type cyclin, putative	LOC_Os02g33330	1
Fasciclin-like arabinogalactan protein 1 precursor, putative	LOC_Os02g49420	1
Dof domain, zinc finger family protein	LOC_Os02g49440	1
CP12, putative	LOC_Os03g19380	1
ER6 protein, putative	LOC_Os02g52314	1,3
MTERF family protein	LOC_Os03g24590	1,2
Yippee, putative	LOC_Os03g49150	1
Enzyme of the cupin superfamily, putative	LOC_Os04g36760	1

Table 4 cont'd

MA3 domain-containing protein, putative	LOC_Os08g02690	1
Postsynaptic protein Cript, putative	LOC_Os08g09180	1
Phytosulfokines 2 precursor, putative	LOC_Os11g05190	1
Early nodulin 93, putative	LOC_Os06g04990	2
Dirigent-like protein	LOC_Os08g28790	3
Zinc finger, C3HC4 type family protein	LOC_Os06g34450	3
PPIC-type PPIASE domain containing protein	LOC_Os09g24540	3
NC domain-containing protein, putative	LOC_Os05g24560	3
Pi starvation-induced protein, putative	LOC_Os05g35740	3
Nuclear protein, putative	LOC_Os05g45450	3
CCT motif family protein	LOC_Os11g05930	3
Seed maturation protein	LOC_Os03g06360	3
:al Meristem protein	LOC_Os04g38720	3
ARI, RING finger protein, putative	LOC_Os08g42740	3
DNA-directed RNA polymerase alpha chain, putative	LOC_Os01g57944	1
Tubulin beta-2 chain, putative	LOC_Os03g01530	1
Actin-related protein 2/3 complex 34kDa subunit family, putative	LOC_Os04g43290	3
Retrotransposon protein, putative, Ty3-gypsy subclass	LOC_Os01g52690	1
Transposon protein, putative, CACTA, En/Spm sub-class	LOC_Os01g69020	1
:trotransposon protein, putative, Ty3-gypsy subclass	LOC_Os03g19600	1
Transposon protein, putative, CACTA, En/Spm sub-class	LOC_Os07g23640	1
Transposon protein, putative, CACTA, En/Spm sub-class	LOC_Os04g34170	1
Transposon protein, putative, CACTA, En/Spm sub-class	LOC_Os07g48910	1
Retrotransposon protein, putative, unclassified	LOC_Os08g21960	2
Retrotransposon protein, putative, Ty3-gypsy subclass	LOC_Os01g13650	3
Unknown		
Expressed protein	LOC_Os01g06620	1,2
Expressed protein	LOC_Os01g10400	1
Expressed protein	LOC_Os01g32770	1
Expressed protein	LOC_Os01g42520	1
Expressed protein	LOC_Os02g03510	1
Expressed protein	LOC_Os02g03520	1

Table 4 cont'd

Expressed protein	LOC_Os02g03710	1
Expressed protein	LOC_Os02g45930	1
Expressed protein	LOC_Os03g05520	1,2
Expressed protein	LOC_Os03g14570	1
Expressed protein	LOC_Os03g39830	1
Expressed protein	LOC_Os03g42600	1,3
Expressed protein	LOC_Os03g47270	1
Expressed protein	LOC_Os03g48626	1
Expressed protein	LOC_Os03g48626	1
Expressed protein	LOC_Os03g58850	1
Expressed protein	LOC_Os04g33310	1
Expressed protein	LOC_Os04g33710	1
Uncharacterized plant-specific domain TIGR01570 family protein	LOC_Os04g54600	1
Expressed protein	LOC_Os07g02340	1
Hypothetical protein	LOC_Os07g05500	1
Expressed protein	LOC_Os07g05810	1
Hypothetical protein	LOC_Os07g25320	1
Expressed protein	LOC_Os07g31490	1
Expressed protein	LOC_Os07g37240	1
Expressed protein	LOC_Os07g43020	1
Uncharacterized plant-specific domain TIGR01568 family protein	LOC_Os07g48150	1
Expressed protein	LOC_Os10g34270	1
Expressed protein	LOC_Os06g09900	1
Expressed protein	LOC_Os06g15400	1
Expressed protein	LOC_Os06g17450	1
Expressed protein	LOC_Os05g10800	1
Expressed protein	LOC_Os05g30400	1
Expressed protein	LOC_Os05g33280	1,2
Expressed protein	LOC_Os12g35470	1,2
Expressed protein	LOC_Os11g31400	1
Expressed protein	LOC_Os08g39330	1
Expressed protein	LOC_Os11g02330	1
Expressed protein	LOC_Os03g57220	2
Expressed protein	LOC_Os10g39150	2
Expressed protein	LOC_Os04g54230	2

Table 4 cont'd

Expressed protein	LOC_Os01g13660	3
Expressed protein	LOC_Os01g49210	3
Expressed protein	LOC_Os03g15920	3
Expressed protein	LOC_Os08g08190	3
Uncharacterised protein family containing protein	LOC_Os06g37070	3
Expressed protein	LOC_Os05g33620	3
Expressed protein	LOC_Os12g07550	3
Expressed protein	LOC_Os11g05490	3
Expressed protein	LOC_Os11g28420	3
Expressed protein	LOC_Os01g71540	3
Expressed protein	LOC_Os06g40520	3

Cell wall hydrolytic enzymes have been consistently found to be related to abscission. As we expected, four such enzymes including mannan endo-1,4-beta-mannosidase, pectinesterase family protein, endo-beta-mannanase and glycosyl hydrolases family 16 protein were up-regulated by *sh4*. These enzymes can degrade cell wall component during abscission process. Two expansin genes were induced by *sh4*. Expansins are cell wall associated enzymes involved in cell elongation.

A total of 19 pathogenesis-related genes showed increased expression in *sh4* transgenic plants. Five genes encode chitinases including four chitinases and one glycosyl hydrolase family 18 protein. These chitinases can degrade chitin, the major cell wall component of fungi. Two genes encoding pathogenesis-related protein (PR) Bet v I family protein. In addition, genes encoding cell wall hydrolytic enzyme inhibitors, such as pectinesterase inhibitor domain containing protein,

polygalacturonase inhibitor 1 precursor, xylanase inhibitor protein 1 precursor and xylanase inhibitor protein 2 precursor, were also up-regulated by *sh4*. They inhibit the activity of hydrolytic enzymes secreted by fungi to degrade plant cell wall during their infection.

Stress related genes were up-regulated by *sh4*. These stress responsive genes are usually up-regulated by ABA and abiotic stress such as drought, salt, cold, and wound. The up-regulation of these groups of genes was not previously reported associated with the abscission processes.

Genes encoding transcription factors, signal transduction, transport, protein biosynthesis or degradation, and metabolism were also induced by *sh4*, indicating abscission is an active process involving many aspects of molecular regulation. A relatively large number of genes involved in photosynthesis were up-regulated in *sh4* transgenic plants.

Genes Down-regulated by *sh4*

In comparison to the number of up-regulated genes, fewer genes were down-regulated by *sh4* (Table 5). It is noticeable that of 37 down-regulated genes, 11 encode DNA binding/transcription factors (Table 6).

Table 5. Number of genes down-regulated by *sh4*

Functional category	**Stage 1**	**Stage 2**	**Stage 3**
Cell wall hydrolysis	0	0	1
Stress responsive	0	1	0
Pathogenesis-related	0	1	0
DNA binding/transcription factor	2	9	1
Signal transduction	0	3	0
Metabolism	0	4	0
Transport	0	2	0
Others	0	11	0
Unknown	0	5	0

Table 6. Genes down-regulated by *sh4*

Category	Annotation	ID	Stage
DNA binding/transcription factor			
	Myb-like DNA-binding domain containing protein	LOC_Os01g65370	2
	Helix-turn-helix family protein	LOC_Os06g39240	3
	Helix-loop-helix DNA-binding domain containing protein	LOC_Os04g23550	1,2
	Helix-loop-helix DNA-binding domain containing protein	LOC_Os03g53020	2
	Transcription initiation factor IID, 31kD subunit family protein	LOC_Os07g42150	2
	AP2 domain containing protein	LOC_Os09g35010	1
	AP2 domain containing protein	LOC_Os02g45450	2
	AP2 domain containing protein	LOC_Os03g09170	2
	Zinc finger, C2H2 type family protein	LOC_Os03g60570	2
	Homeobox domain containing protein	LOC_Os04g45810	2
	JmjC domain containing protein	LOC_Os02g01940	2
Metabolism			
	Chemocyanin precursor, putative	LOC_Os03g50160	2
	Fatty acid elongase, putative	LOC_Os02g11070	2
	Phenylalanine ammonia-lyase, putative	LOC_Os02g41670	2
	Oxidoreductase, 2OG-Fe oxygenase family protein	LOC_Os04g49210	2

Table 6 cont'd

Cell wall hydrolysis		
Beta-fructofuranosidase 1 precursor, putative	LOC_Os02g01590	3
Signal transduction		
Protein kinase domain containing protein	LOC_Os01g50410	2
Caltractin, putative	LOC_Os07g42660	2
Serine/threonine-protein kinase SAPK2, putative	LOC_Os07g42940	2
Transport		
Sugar transporter family protein	LOC_Os07g39350	2
Amino acid transporter, putative	LOC_Os12g08130	2
Others		
Heavy metal-associated domain containing protein	LOC_Os04g39360	2
ATPase, AAA family protein	LOC_Os12g28137	2
Reticulon family protein	LOC_Os01g63240	2
Harpin-induced protein 1 containing protein	LOC_Os01g64470	2
VQ motif family protein	LOC_Os03g47280	2
ZIM motif family protein	LOC_Os07g42370	2
Las1-like family protein	LOC_Os12g08800	2
Senescence-associated protein	LOC_Os03g13840	2
Hevamine A precursor, putative	LOC_Os01g64110	2
Ankyrin-1, putative	LOC_Os08g42960	2
AN1-like Zinc finger family protein	LOC_Os09g21710	2
Unknown		
Expressed protein	LOC_Os01g03980	2
Expressed protein	LOC_Os01g72360	2
Expressed protein	LOC_Os03g13870	2
Expressed protein	LOC_Os03g32420	2
Expressed protein	LOC_Os03g32490	2

ABA Regulation of Abscission

Flower Shattering in Response of ABA Treatment

Our microarray data suggested ABA and stress related genes were up-regulated by *sh4*, which led to an investigation of the role of ABA in rice grain

abscission. Young panicles that just began to flower were treated with 0.1 mM and 1.0 mM ABA. Panicles treated with H_2O were used as the control. Percentage of flower shattering was scored every 24 hr. Both 0.1 mM and 1.0 mM ABA caused flower abscission. After 24 hours in 1.0 mM ABA, nearly 60% of flowers shattered, and at 48 hr almost all flowers dropped when tapped by hand. In response of 0.1 mM ABA treatment, the percentage of flower shattering increased gradually with the length of treatment, and about 60% flowers shattered after 96 hr (Figure 8).

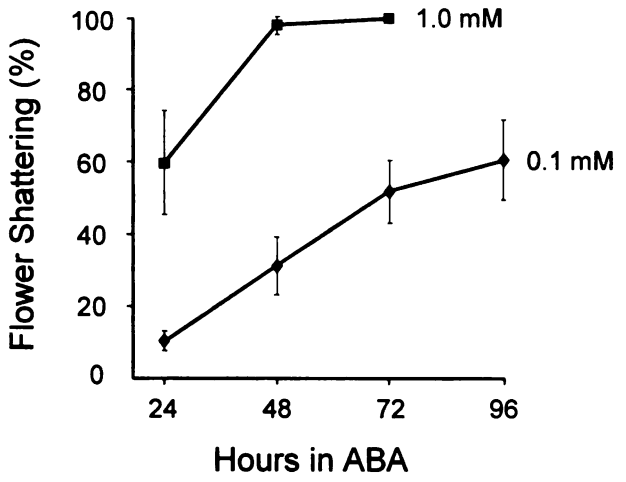

Figure 8. *O. nivara* flower shattering in response of ABA treatment. Same developmental stage young panicles were treated with 0.1 mM and 1.0 mM ABA. Percentage of flower shattering were scored every 24 hrs. Each dot represents the average plus standard deviation of three replicates.

Panicles from T2 transgenic plants with and without the trangene were treated with 1.0 mM ABA under the same conditions. For plants with the transgene, The

average force required to pull away flowers from the pedicels became substantially lower than that treated with H_2O after 48 hours of the treatment. Wherease plants that did not carry the *sh4* transgene showed no difference between the ABA and H_2O treatment (Figure 9).

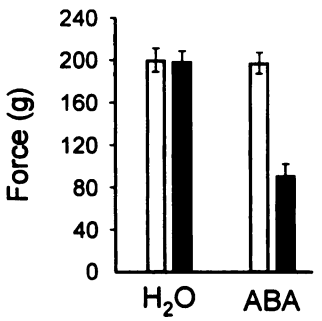

Figure 9. Flower shattering of T2 transgenic plants and the control segregated from the same T0 parent S12 in response of ABA treatment. Force required to pull away flowers from the pedicels was measured at 48 hours after the treatment. Black bar indicates plants with the sh4 transgene white bar indicates plants without the transgene.

Evaluation of Seed ABA Content at Different Developmental Stages

Our *in vitro* experiment demonstrated that ABA could trigger abscission of rice flowers that were either pollinated or not pollinated. Because ABA was found accumulate in developing embryos for the preparation of seed desiccation and dormancy, we tested the hypothesis that ABA synthesized in the developing embryos serves as the source of abscission signal. To test the hypothesis, we

removed stigmas of unopened flowers of the wild species *O. nivara* so that embryo development was blocked. These flowers stayed on the panicle while grains with mature seeds shattered. This suggested that signals from developing rice embryo were required for grain shattering.

We measured ABA content of the developing seeds of *O. nivara*. After anthesis, ABA content per seed increased substantially, especially between 6 to 9 days after pollination. ABA content kept increasing and reached the highest level at 12 days after pollination (Figure 10). This was positively correlated with the expression of *sh4* and negatively correlated with the force required to break away the grain from the pedicel (Li et al. 2006b).

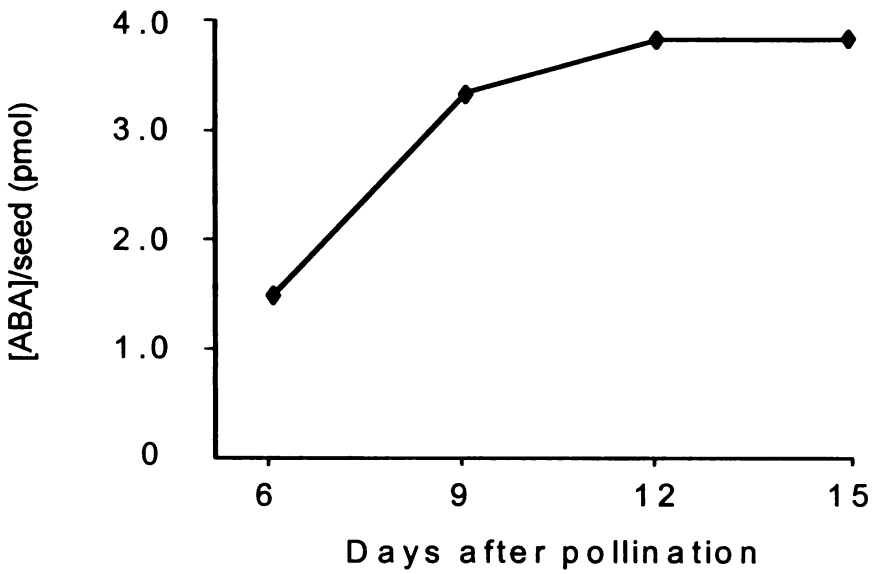

Figure 10. Seed ABA content in *O. nivara*. Seed ABA was measured at 6, 9, 12 and 15 days after pollination.

DISCUSSION

Microarray Analysis

With full genome sequences available, microarray analysis has become one of the most powerful tools to analyze the profiles of gene expression at the genome-wide scale. This method has provided insights into gene function and interactions (Brown and Botstein, 1999; Young 2000; Lockhart and Winzeler, 2000). To deal with such a large amount of data, it is critical to employ statistical methods to reduce the error rate and bias. Although several statistical methods have been recommended for the data analysis, there has been no standard method that works best for every dataset (Clarke and Zhu, 2006).

A popular pre-processing step for two-color array is background correction prior to normalization. The goal of background correction is to correct the foreground intensities for background noise within the spotted region. The most common approach is "Subtract" background correction, which approximates the background at each spot by measuring the local, off-spot signal intensity and subtracting the value from the foreground signal. The "Minimum" or "Half" method subtracts local, off-spot signal intensity from each spot's foreground intensity unless the net value is non-positive. In the latter case, the net intensity is set to 0.5 in "Half" strategy, and one-half the minimum positive net intensity in "Minimum" method (Smyth and Speed, 2003).

I compared several background adjustment options available in the Limma package. The aberrant log-ratio M and log-intensity A plot (MA-plot) after "Subtract", "Minimum" or "Half" background corrections implied that these adjustments were not suitable for the dataset (Figure 7). This may be explained in two ways. Firstly, because the portion of the slide used to estimate background does not have any DNA bound, we are actually estimating the background binding of the spot by using a portion of the slide that might not be very similar (Zhang et al., 2006). Secondly, the simple "Subtract" method excluded the negative background-corrected intensities and lost differential expression information for weakly expressed genes, while spots with small positive intensities corrected by "Minimum" and "Half" were usually unreliable estimates of M and A. These spots had substantially variable log-ratios that challenged the downstream analyses, and might have lost differential expression information of these genes (Zhang et al., 2006). From our MA-plot, it is obvious that a large number of genes were weakly expressed or not expressed at all. Therefore, subtraction based background corrections were not suitable for our dataset.

Although the RMA method yielded better MA-plot than subtract based background correction methods, it was still biased for our dataset, especially for some weakly and moderately expressed genes. The assumption of RMA (Bolstad 2004) is that the observed intensity is composed of the true intensity which is exponentially distributed (and hence positive) and the random noise

which is normally distributed (maybe truncated at zero). Thus this model does not return negative intensity values, which is better than the conventional subtract background correction.

While some studies applied background correction in their microarray analyses, others have proposed ignoring the information in the background intensities (Parmigiani et al., 2003; Qin et al., 2004). Whether to apply background adjustment or not should depend on individual dataset. Prior to using any normalization options, we should always compare the MA-plot before and after various background corrections and determine whether background adjustment is appropriate. In many cases background correction can remove systematic bias, but it is better not to use background correction if the background intensity is low and relatively consistent across arrays.

Gene Expression Regulated by *sh4*

Two categories of genes identified in the present study were previously shown to be associated with abscission. The first category included genes encoding cell wall hydrolytic enzymes and expansins. Dissolution of the middle lamella or even primary cell wall is an essential step in the abscission process. The major components of the cell wall are pectins, cellulose, and hemicellulose (Carpita and Gibeaut, 1993; Chun and Huber, 1998). Abscission involves expression, synthesis and secretion of hydrolytic enzymes. Of the enzymes we identified,

pectinesterases are involved in pectin modification and make the wall more susceptible to other hydrolases (Brown 1997).

It is interesting that the genes encoding cell wall hydrolytic enzymes were up-regulated by *sh4* at the first developmental stage, which is the pollination stage. This suggested that these enzymes were synthesized well before grain abscission occurred. We also identified that two expansin genes were up-regulated by *sh4*. Expansins were originally found to be related to cell elongation and elasticity, the up-regulation at the abscission zone suggested they are associated with abscission (Cho and Cosgrove, 2000; Belfield et al. 2005).

Of various hydrolytic enzymes commonly associated with abscission, genes encoding polygalacturonase (PG) did not exhibit increased expression in *sh4* transgenic plants. Because we were unable to obtain good-quality RNA at the developmental stage very near grain abscission, we cannot rule out the possibility that these genes are expressed at a later stage than what we examined. This hypothesis supported previous finding that the up-regulation of PG was only detected at the time of cell separation (Taylor et al., 1991; Bonghi et al., 1992; Hong and Tucker, 1998), although other studies suggested PG also exhibited increased expression prior to the onset of abscission (Kalaitzis et al., 1997; Gonzalez-Carranza et al., 2002).

The second category is pathogenesis-related genes. The shedding of an organ provides a vulnerable site for pathogen invasion. It is therefore plausible for

pathogen related genes to be up-regulated during abscission process (Roberts et al., 2002). Indeed, defense genes were definitely associated with abscission (reviewed by Patterson, 2001; Roberts et al., 2002). Genes encoding chitinases and beta-1,3-glucanases have been observed to be up-regulated in the abscission zone (Volko et al., 1998; Kubigsteltig et al., 1999). Of the 19 defense related genes, 15 of them were up-regulated by *sh4* at the first developmental stage, suggesting they may play a role in induced resistance of fungal attack after a grain is shed.

In addition to the two categories of abscission related genes found in previous studies, we have identified several other functional groups of genes regulated by *sh4*, such as genes associated with metabolism, signal transduction, transcription factors, membrane, transport, protein biosynthesis and modification, and photosynthesis. Abscission is an active biological process. Previous studies have concluded that RNA and protein synthesis is very active prior to cell separation (Abeles 1968; Abeles and Leather, 1971; Addicott 1982). Thus we expect to see dynamic biochemical activities during the abscission process. Given the hypothesis that cell wall hydrolytic enzymes were synthesized well prior to the onset of abscission, it makes sense that genes involved in protein biosynthesis and modification were up-regulated.

Initiating abscission requires perception of environmental or hormone signals and the signal transduction messengers. It is noticeable that *sh4* regulated a

number of transcription factors. As a transcription factor itself, *sh4* may function relatively upstream in the abscission pathway.

We should keep in mind that rice grain abscission zone is composed of mostly one layer of cells. However, tissue sample for the microarray study is composed of many more layers of cells at both sides. Thus, not all candidate genes identified in this analysis are abscission specific. Recently optimized laser capture microdissection (LCM) methods have been successfully used by the Lashbrook lab at Iowa State University to isolate highly enriched populations of stamen abscission zone cells from paraffin-embedded flowers in *Arabidopsis*. If this method works in rice, abscission-layer specific mRNA may be obtained for future studies.

ABA Regulation of Abscission

Another large category of genes is ABA and/or stress related genes. Most of these genes are inducible by ABA and/or abiotic stresses, such as drought, wound, salt, and cold stresses. Two genes encoding late embryogenesis abundant (LEA) proteins were also up-regulated by *sh4*. The elevated expression of LEA has been observed under both ABA and stress treatment in rice (Rabbani et al., 2003). These proteins act as a desiccation protectant and are involved in protecting macromolecules such as enzymes and lipids (Moons et al., 1997). It has been well documented that ABA is accumulated under abiotic stresses and plays an

important role in the plant response to abiotic stresses (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu 2002). It has also been proposed that stress responsive genes are up-regulated by both ABA-dependent and ABA-independent signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu 2002). *sh4* can induce the expression of ABA and stress responsive genes when plants were not subject to stresses, suggesting it interacts with ABA signaling pathway. This finding was further confirmed by the subsequent ABA experiments. Our results indicated that application of exogenous ABA was capable of promoting rice flower abscission, but only in the wild species *O. nivara* or in *sh4* transgenic cultivar. This suggests that the abscission accelerating activity of ABA requires the participation of *sh4* of the wild species.

Our microarray analysis opens a door to further investigate the function of genes involved in abscission as well as a network within and across the abscission related metabolic pathways. We demonstrated that ABA is the plant hormone that triggers rice grain abscission. This contrasts with many other studies that emphasized the essential role of ethylene in signaling abscission. Our results support that dicotyledons and monocotyledons may have differentiated in hormone signaling for organ abscission (Sargent et al., 1981, 1984). More importantly, this research established the first connection between ABA and a gene regulating plant organ abscission. This sets the stage for future studying hormone signaling network and molecular developmental pathways associated

with organ abscission in rice and other grasses, which remain totally unknown to date.

APPENDIX

Author's Publication List During the Master's Program

Li C, **Zhou A,** Sang T (2006) Rice domestication by reducing shattering. Science **311:**1936-1939

Li C, **Zhou A,** Sang T (2006) Genetic analysis of rice domestication syndrome with the wild annual species, *Oryza nivara*. New Phytologist **170:** 185-194

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