Title: Characterization of a Novel Plantain Asr Gene, MpAsr, that is Regulated in Response to Infection of *F. oxysporum* f.sp. *cubense* and Abiotic Stresses

Running title: *MpAsr* Gene Regulated By *F. oxysporum* and Abiotic Stresses

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Abstract

Asr (abscisic acid, stress, ripening induced) genes are typically up-regulated by a wide range of factors, including drought, cold, salt, abscisic acid (ABA) and injury; in addition to plant responses to developmental and environmental signals. We isolated an Asr gene, MpAsr, from a suppression subtractive hybridization (SSH) cDNA library of cold induced plantain (Musa paradisiaca) leaves. MpAsr expression was up-regulated in Fusarium oxysporum f. sp. cubense infected plantain leaves, peels and roots, suggesting that MpAsr plays a role in plantain pathogen response. In addition, a 581-bp putative promoter region of MpAsr was isolated via genome walking and cis-elements involved in abiotic stress and pathogen-related responses were detected in this same region. Furthermore, the MpAsr promoter demonstrated positive activity and inducibility in tobacco under F. oxysporum f. sp. cubense infection and ABA, cold, dehydration and high salt concentration treatments. Interestingly, transgenic Arabidopsis plants over-expressing MpAsr exhibited higher drought tolerance, but showed no significant decreased sensitivity to F. oxysporum f. sp. cubense. These results suggest that MpAsr might be involved in plant responses to both abiotic stress and pathogen attack.

Key words: abiotic stresses; Asr gene; F. oxysporum f.sp. cubense; plantain; promoter.
Asr is a stress-inducible gene that has been reported exclusively in plants. Iusem et al. (1993) reported the first Asr gene from cultivated tomato and since then Asr genes have been found in various species of dicotyledonous and monocotyledonous plants (Frankel et al., 2006), including Cucumis melo (Hong et al. 2002), lily (Wang et al. 1998), grape (Cakir et al. 2003), Ginkgo biloba (Shen et al. 2005), potato (Silhavy et al. 1995), maize (Riccardi et al. 1998), rice (Vaidyanathan et al. 1999), pummelo (Canel et al. 1995) and loblolly pine (Chang et al. 1996), but surprisingly these genes are not present in Arabidopsis (Yang et al. 2005). The Asr genes in various species are presumed to act as part of a transcription-regulating complex involved in plant development processes such as senescence, fruit ripening, pollen maturation and glucose metabolism (Iusem et al. 1993; Silhavy et al. 1995; Wang et al. 1998; Hong et al. 2002; Cakir et al. 2003; Shen et al. 2005; Frankel et al. 2007) and also respond to different abiotic stress factors, including drought, salt, cold and limited light (Schneider et al. 1997; Huang et al. 2000; Maskin et al. 2001; Jeanneau et al. 2002; Kalifa et al. 2004). Over-expression of the Asr gene (e.g. lily Asr gene) in transgenic Arabidopsis can increase tolerance to drought and salt and decrease a plant’s sensitivity to exogenous abscisic acid (ABA) (Yang et al. 2005).

Banana is an important fruit crop in tropical regions worldwide. Production is increasingly threatened by biotic and abiotic stress, including cold and F. oxysporum f. sp. cubense infection, which has resulted in large-scale decreases in yield. Chemical control is economically cost prohibitive and therefore, breeding programs are presently the only alternative to control fungal infection. A broad comparison among banana cultivars (Lu et al. 2000; Chen et al. 2004) indicates that M. paradisiaca L. ABB Group, one of the banana cultivars has superior cold and disease resistance compared to other cultivars. Therefore, identification of this cold and disease resistance gene from plantain and research into its biological function will provide a new approach to banana breeding programs.

In our previous research, we constructed and screened two suppression subtractive hybridization (SSH) libraries of cold and F. oxysporum f. sp. cubense induced plantain leaves and isolated and identified several cold and disease resistant genes of plantain, including the cold resistance gene *MpRCI* (Feng et al. 2009), F. oxysporum f. sp. cubense resistance gene *MpGlu* (Jin et al. 2007) and Gloeosporium musarum and abiotic stress resistance gene *MpChi-1* (Fan et al. 2007). From the SSH library of cold induced plantain leaves, a new member of the Asr gene family, *MpAsr*, was also isolated. Blast analysis showed high homology between *MpAsr* and the putative Musa acuminata (AA Genomic group) *Asr* gene (gi: 47575680). *MpAsr* gene was up-regulated by cold, ABA, drought and
salt (data unpublished), which is consistent with previous reports of other Asr gene function. Interestingly, MpAsr up-regulation was also observed in plantain infected with *F. oxysporum* f. sp. *cubense*.

In the present study, to further characterize the MpAsr gene, full-length cDNA of the *MpAsr* gene was isolated by RT-PCR using primers designed based on the putative *Musa acuminata* (AA Genomic group) Asr gene cDNA sequence. *MpAsr* expression patterns under *F. oxysporum* f. sp. *cubense* inoculation were performed by northern blot and RT-PCR analyses. The *MpAsr* promoter was also isolated and demonstrated. Furthermore, the biological function of *MpAsr* gene was analyzed by over-expression in *Arabidopsis*.

**Results**

**Isolation and sequence analysis of the *MpAsr* gene**

A *MpAsr* cDNA fragment was isolated from a cold-induced plantain leaf SSH library. The *MpAsr* fragment lacked the 5' end and was 314-bp in length, but contained two highly conserved regions: a putative signal for nuclear targeting and an ABA/WDS signature sequence, described in all ASR proteins by Lu et al. (2007). Considering the highly homologous with the putative *M. acuminata Asr* gene sequence from Genbank, primers were designed using the putative *M. acuminata* (AA Genomic group) *Asr* gene and the full-length cDNA of *MpAsr* were finally obtained using RT-PCR. The full-length *MpAsr* cDNA was 614-bp, containing a 44-bp 5’ untranslated region (UTR), a 138-bp 3’ UTR and a 432-bp ORF. The deduced protein MpASR consisted of 143 amino acids, with a predicted molecular weight of 16.22 kD and a theoretical pI of 5.96, containing abundant His (15.4%), Lys (13.3%), Glu (17.5%) and Ala (14.7%) residues. Two highly conserved MpASR regions were detected. The first region contained a C-terminal region of at least 73 amino acids, with a putative signal for nuclear targeting (KKDAKNEAEASGKK). The second region exhibited an ABA/WDS sequence signature as follows: GAFALYEKHEAKKDHPDHAKHKIIEEIAAVA (Figure 1A). In addition, the MpASR protein has highly homologous with ASR proteins from other species (Figure 1B). Therefore, the *MpAsr* gene likely encodes a novel plantain ASR protein.
**MpAsr expression patterns**

MpAsr gene expression patterns under *F. oxysporum* f. sp. *cubense* inoculation were further investigated by northern blot and RT-PCR. Figure 2 indicates MpAsr mRNA accumulation time profiles in plantain leaves, peels and roots exposed to *F. oxysporum* f. sp. *cubense*. Generally, following inoculation, mRNA levels were up-regulated in a time-dependent manner. In leaves and peels, transcription of MpAsr was highly up-regulated by *F. oxysporum* f. sp. *cubense* at 24 h and maintained at an elevated level for up to 72 h. A decline in expression was then observed at 96 h (Figure 2A, 2B). In roots, MpAsr mRNA expression was up-regulated at 12 h and reached a maximum level at 24 h, then demonstrated reduced expression at 72 h (Figure 2C). These results indicated that MpAsr was regulated by *F. oxysporum* f. sp. *cubense* infection and the expression profile varied with different tissues and inoculation times.

**Analysis and characterization of the MpAsr gene promoter sequence**

The putative promoter sequence of the MpAsr gene was cloned and analyzed to characterize the MpAsr gene. The genome walking method isolated a 581-bp putative promoter region, where cis-elements involved in pathogen and stress-related responses were also detected, including three potential ABA-Responsive Elements (ABRE) (Narusaka et al. 2003) at positions -514, -400 and -349 bp from the ATG code that overlapped with G-box elements (Qian et al. 2007); two dehydration responsive elements (DRE) (Narusaka et al. 2003) at positions -474 and -360 bp and one salicylic acid (SA)-inducible motif (Ndamukong et al. 2007) at position -265 bp (Figure 3A). To investigate the pathogen and stresses regulation of the putative MpAsr gene promoter, promoter fragments were fused to the GUS reporter gene and introduced into tobacco plants (*N. tabacum* cv. *Xanthi*) by *A. tumefaciens* mediated transformation. GUS histochemical assays were performed in leaves of transgenic tobacco plants treated with cold temperature (4°C), 250 mM ABA, 20% (w/v) PEG8000, 400 mM NaCl and *F. oxysporum* f. sp. *cubense* infection (OD\textsubscript{600}=1.0 suspension). The results of these treatments demonstrated that the gus gene can be driven by the promoter and expressed under cold, ABA, PEG, NaCl and *F. oxysporum* f. sp. *cubense*. (Figure 3B) This confirmed that the promoter was inducible by cold, ABA, PEG, NaCl and *F. oxysporum* f.sp. *cubense*
Over-expression of *MpAsr* and Tolerance of transgenic *Arabidopsis* to *F. oxysporum* f. sp. *cubense* infection and drought

To characterize the function of *MpAsr* gene, ORF of *MpAsr* gene under control of the CaMV35S promoter was transformed into *Arabidopsis* plants. The transformation experiments yielded 34 T1 and 215 T2 hygromycin-resistant lines. Two T3 homozygous lines were selected for more detailed analyses. Northern blot analysis confirmed that *MpAsr* gene transcripts were present in both T3 homozygous transgenic lines, whereas no expression was detected in wild-type plants (Figure 4).

Sixty two-week-old transgenic and wild-type *Arabidopsis* plants (Col-0) were inoculated with *F. oxysporum* f. sp. *cubense* for 3-weeks in both transgenic and wild-type plants. Results found 75-89% of the plants had yellowish spots on their leaf surfaces and the symptoms and degree of symptoms were not significantly different between the wild-type and transgenic plants (Figure 5). The results suggested that over-expression of the *MpAsr* gene had no positive influence on *Arabidopsis* sensitivity to *F. oxysporum* f. sp. *cubense*.

Furthermore, sixty two-week-old transgenic plants were used to assess drought tolerance. All wild-type plants exhibited 100% mortality after three weeks from water holding. However 30% and 45% of each respective transgenic line survived, growth continued after resuming a watering regime, plants produced siliques and ultimately set seed (Figure 6). These results demonstrated that transgenic *Arabidopsis* exhibited enhanced drought tolerance consistent with a previous report by Yang et al. (2005).

Discussion

To date, more than 20 *Asr* orthologs have been isolated in different plant species (Frankel et al. 2006). Each of these *Asr* genes play important roles in plant responses to developmental and environmental signaling processes, including but not limited to senescence, fruit ripening, pollen maturation and glucose metabolism (Iusem et al. 1993; Silhavy et al. 1995; Wang et al. 1998; Hong et al. 2002; Cakir et al. 2003; Shen et al. 2005;...
Frankel et al. 2007). In addition, *Asr* genes are typically up-regulated by water deficits, salt imbalances, cold, limited light, injury and ABA exposure; and are involved in plant responses to abiotic stress (Schneider et al. 1997; Huang et al. 2000; Maskin et al. 2001; Jeanneau et al. 2002; Kalifa et al. 2004). In the present study, based on an EST from a cold-induced differential gene expression cDNA library of plantain, we isolated a novel *Asr* gene, *MpAsr*, from plantain. Two highly conserved ASR protein regions, a nuclear targeting signal and an ABA/WDS motif were present (Figure 1A). Alignment analysis of *MpASR* with ASR proteins from other plant taxa further allied *MpAsr* with the ASR protein family (Figure 1B).

Plant defense in response to pathogen attack is usually regulated through a complex network of signaling pathways that include three signaling molecules: salicylic acid (SA), jasmonate (JA) and ethylene (ET) (Dong 1998; Reymond and Farmer 1998; Schenk et al. 2000; Spoel et al. 2003). These pathways do not function independently, but interact with each other through an intricate network (Kunkel and Brooks 2002). Gene accumulation in the defense network may rapidly increase when plants suffer injury or pathogen attack (Broekaert et al. 2006). Subsequently, one or more defense responses are induced (Penninckx et al. 1998; Schenk et al. 2000). In the present study, expression profile analysis indicated that *MpAsr* transcripts accumulated in plantain seedlings under *F. oxysporum f. sp. cubense* infection. In addition, our experiments revealed that the *MpAsr* promoter contains several stress and pathogen related SA response motifs, and can drive *gus* gene expression in tobacco under exogenous ABA, cold, NaCl, dehydration and *F. oxysporum f. sp. cubense* treatments. These results clearly suggested *MpAsr* is involved in plant responses to both abiotic stress and pathogen attack.

Interestingly, our study showed over-expression of *MpAsr* in transgenic *Arabidopsis* enhanced drought tolerance (Figure 5), suggesting utility in crop improvement using a transgenic approach. However, when transgenic *Arabidopsis* were inoculated with *F. oxysporum f.sp. cubense*, no significant difference in disease symptoms or degree was detected between the wild-type and the transgenic plants (Figure 6). A partial explanation is that as a co-regulator among pathogen defense pathways and abiotic stress signaling pathways, the *MpAsr* gene has different downstream factors. In *Arabidopsis*, *Asr* homologs
have not been detected (Yang et al. 2005), therefore MpAsr gene downstream factors in the
MpAsr-mediated pathogen defense pathway are likely absent. Therefore, the abduction
signal cannot be transferred and the MpAsr-mediated pathogen defense pathway cannot be
activated in Arabidopsis. As a result, transgenic Arabidopsis plants show no increased
tolerance to F. oxysporum f. sp. cubense. On the other hand, the drought abduction signal
might be transferred through the MpAsr-mediated ABA signaling pathway in transgenic
Arabidopsis, and drought tolerance is improved by MpAsr over-expression (Schenk et al.
2000; Yang et al., 2005). Further investigation into the function of MpAsr in pathogen
defense pathways and the transformation of F. oxysporum f. sp. cubense sensitive plantain
cultivars using the MpAsr gene is currently in progress.

In summary, we isolated and characterized the plantain Asr gene MpAsr and determined
the gene is responsive to abiotic stress and F. oxysporum f. sp. cubense. To our knowledge,
this is the first report of Asr gene induction following F. oxysporum f. sp. cubense
treatments. The results clearly suggest that MpAsr may be integral in plant response to
pathogen attack. Further research in the physiological and biochemical functions of the
MpAsr gene will serve to elucidate the function of MpAsr in pathogen defense pathways.

Materials and methods
Plant materials and inoculation treatments
The roots of six-week-old plantain plants were wounded and immersed in a Fusarium
oxysporum f. sp. cubense suspension (OD600=1.0) for 0, 12, 24, 36, 48, 72 or 96 h. The
roots, peels and leaves were cut and immediately frozen by submersion in liquid nitrogen
and stored at -80°C until retrieved for RNA preparation.

Cloning and DNA sequence analysis of the MpAsr gene
Total RNA was extracted from plant material with Trizol reagent (Takara) and a
subsequent treatment with DNaseI. The following specific primers were designed based on
the Musa acuminata Asr gene sequence (gi 47575680): forward primer
5’-AGCCACCCGTCGCAAACCACT-3’ and reverse primer
5’-CCAAGCATCCCACACTCAACA-3’. RT-PCR was performed using Takara’s
superscript one-step RT-PCR kit. PCR was performed at 95°C for 5 min; followed by 30
amplification cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 50 s; and an 8-min step at 72°C. PCR products were ligated with the pMD18-T vector and sequenced.

The sequences obtained were compared against the sequences in NCBI Genbank database using the online BLAST program. Protein sequences were aligned using the Clustal W Program in Bioedit.

**Semi-quantitative RT-PCR**

Total RNA isolated from root and peel specimens was subjected to semi-quantitative RT-PCR. The same primers and PCR parameters described above were employed with the exception of 25 PCR cycles. Plantain Actin was amplified as a control using the forward primer 5’-TGTAGGTGATGAGGCCCAAT-3’ and the reverse primer 5’-ATACCTGTGGTACGTCCGCT-3’. PCR products were analyzed on 1% (w/v) agarose gels.

**Northern-blot analysis**

Total RNA aliquots (30μg) from plantain leaves were electrophoresed on 1% (w/v) formaldehyde agarose gel and transferred onto Hybond-N+ nylon membrane (Amersham) following standard procedures. The membranes were hybridized to α-32P-dCTP labeled *MpAsr* open reading frame DNA. Hybridization was conducted at 42°C for 16 h. The DNA membranes were washed under high-stringency conditions and exposed to X-ray film. Actin-hybridized bands confirmed equal sample loading.

**MpAsr promoter isolation and analysis**

Plantain megabase-size DNA was isolated using the method of Liu and Whittier (1994). The GenomeWalker™ kit was chosen to isolate the *MpAsr* gene promoter DNA according to the manufacturer’s instructions (Invitrogen). The DNA was ligated into the pMD18-T vector and sequenced. Sequence analysis and motif searches were conducted with PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and Proscan (Version 1.7; http://www-bimas.cit.nih.gov/molbio/proscan/).

To construct the chimeric genes comprised of the GUS coding sequence driven by the *MpAsr* promoter, 581-bp promoter fragment with additional restriction sites (a *HindIII* site...
at the 5’ end and a *Bam*HI site at the 3’ end) was subcloned into the pMD18-T simple vector (Invitrogen). The resulting constructs were digested with *Hind*III / *Bam*HI and cloned into the corresponding binary expression vector pCAMBIA1391Z sites. Following sequencing verification, the construct was transformed into *A. tumefaciens* strain LBA4404 by electroporation. Tobacco (*Nicotiana tabacum* cv. *xanthiwn*) was transformed with the *A. tumefaciens* strain LBA4404 containing promoter following Yang et al. (2000). The transformed plants were exposed to cold (4°C), 250 mM ABA, 20% (w/v) PEG8000, 400 mM NaCl or *F. oxysporum* f. sp. *cubense* infection (OD_{600}=1.0 suspension) for 10 h. Histochemical staining for beta-glucuronidase (GUS) activity was performed according to the method of Jefferson et al. (1987). Transgenic plants were incubated in GUS reaction buffer (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 100 mM phosphate buffer, pH 7.0, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6 and 10 mM EDTA) at 37°C for 24 h. Tissue visualization was enhanced by rinsing leaves for 3 h at room temperature with an ethanol series to remove chlorophyll. Leaves were subsequently mounted for microscopy. Promoter biological activity and inducibility were confirmed by *gus* gene transient expression.

**Over-expression of *MpAsr* in *Arabidopsis***

The *MpAsr* ORF was ligated with cauliflower mosaic virus 35S (CaMV35S) promoter and subsequently subcloned into pCAMBIA1301 vector (CAMBIA) digested with *Kpn*I and *Pml*I. *Arabidopsis* (*Arabidopsis thaliana*) plants were grown at 22°C. Transformation of *Arabidopsis* (Col-0) was performed according to the floral dip method using *Agrobacterium tumefaciens* strain LBA4404. Seeds were harvested and plated on hygromycin (50 mg/L) selection medium to identify transgenic plants. T3 progenies homozygous for hygromycin resistance were used for further studies.

**Exposure of transgenic *Arabidopsis* to *F. oxysporum* f. sp. *cubense* infection and drought stress**

Sixty two-week-old transgenic and wild type *Arabidopsis* (Col-0) seedlings were inoculated with infectious *F. oxysporum* f. sp. *cubense* suspension (OD_{600}=1.0) using a
root-dip inoculation method. Drought treatments and RNA blot analyses were performed according to the methods of Yang et al. (2005).

Acknowledgments

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References


gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. Plant Mol Biol. 63, 289-305.


Figure legends

Figure 1. *MpAsr* gene deduced amino acid sequence alignment relative to ASR proteins from other plant taxa using the Clustal W Program in Bioedit. 

(A) The *MpAsr* gene deduced amino acid sequence, the nuclear target signal is denoted with a single underline and the ABA/WDS signature is denoted with a double underline.


Figure 2. Analysis of *MpAsr* mRNA expression 0, 12, 24, 36, 48, 72, or 96 h after *F. oxysporum* f. sp. *cubense* inoculation.

(A) Northern blot hybridization of *MpAsr*; 30μg aliquots of total RNA from leaves were loaded into each lane. Equal loading was confirmed relative to the *Actin* gene expression. 

(B-C) Semi-quantitative RT-PCR was used to analyze *MpAsr* expression in peels (B) and roots (C). Plantain *Actin* was amplified as an internal control.

Figure 3. *MpAsr* gene promoter sequence and activity analysis.

(A) *MpAsr* gene promoter sequence analysis. Stress-inducible promoter motifs are marked with underlining. G-box: (C)ACGT(G); ABRE: ACGTGG; DRE: CCGAC; SA-inducible motif: T(C)GACG.

(B) GUS transient expression in tobacco driven by the *MpAsr* promoter under *F. oxysporum* f. sp. *cubense* and different abiotic stress factors. The transgenic tobacco plants were exposed to cold (4℃), 250 mM ABA, 20% (w/v) PEG8000, 400 mM NaCl or *F. oxysporum* f. sp. *cubense* infection (OD600=1.0 suspension) for 10 h. The untreated transgenic tobacco leaves served as the control.

Figure 4. Expression of *MpAsr* in two transgenic lines and the wild type (WT) *Arabidopsis* under normal conditions. TL-1: Transgenic line 1; TL-2: Transgenic line 2; WT: wild type, Col-0.
Figure 5. Yellowish spots rates of wild-type (WT) and transgenic *Arabidopsis* plants. Transgene line TL-1 and TL-2 were estimated following 3-weeks of *F. oxysporum* f. sp. *cubense* inoculation treatment. Inoculation treatments were applied to two-week-old transgenics and WT plants under similar experimental conditions. (A) Two-week-old wild-type and transgenic *Arabidopsis* were inoculated with *Fusarium*. Photographs were taken at 7 d, 10 d and 21 d after inoculation. (B) The yellowish spots indicate the disease rate of sixty wild-type and transgenic *Arabidopsis* plants. Results are averages of three replicates±SD.

Figure 6. Estimation of survival rates of wild-type WT and transgenic *Arabidopsis* plants (lines TL-1 and TL-2) after drought stress treatment. (A) Two-week-old wild-type and transgenic *Arabidopsis* were treated with drought and re-watered. Photographs were taken at 7 d, 14 d after drought stress and 3 d after re-watering. (B) The survival rate of sixty wild-type and transgenic *Arabidopsis* plants. Results are averages of three replicates±SD.
Figure 2

A

\[0, 12, 24, 36, 48, 72, 96\] h

\(-MpAsr\)
\(-Actin\)

B

\(-MpAsr\)
\(-Actin\)

C

\(-MpAsr\)
\(-Actin\)
Figure 3

A

-558 TACTATAGGGCACGGTGCTGCTTCGAGCGGGCGGCTGGCTGAGATAGGATGGAGATGGGTTTGGTC
-496 GTGTTCTCGAGGAAAGACAGGCCAGCTGCTTCTTGCTGCTCTGAGTTACATCGTGCGC
-434 GTCCGGTCTAGAGCGTGCGGTCGCTCGCTCGGGCCAGCGTCCGAGCTGCTGCCCTTGCGTCTC
-372 CAAAGAAGAAAGGCCAACCCACACACACGTCGCTGGCGGCCCACCTCCAAGTCTCAATTGCTTCACCCT
-310 TCCTTTGGTCCTGGCGACGCTGACACTCGAAGACGCTCCACGTCGTGGCCGCGCTCTTCTTAGAC
-248 CCATATCCGGGCCGTCCGATTATAAGGTATATCAGAATCTGACACGCGTGGCTCAGATCGGATTGG
-186 GGGTGACCTCGAGCATCTATCTCAAGTTGGTTCCAAACCCACCGGAAGATACCGGACCTGC
-124 CACTCGGTCGACTGGACGGCCCCTTCCAAGCTTTCGCAACCGCTACATAACCCCCCGCCCTCCTC
-62 CTTCCGATCTCAAGGGCAAGCCACCGCTCGAATACCTGTTTCTAGTCGACTCGAAACCG

1 ATG GCC GAG AAG AAA CAC CAC CAC CTC

MA E E K HH H H L

B

F. oxysporum  Cold  ABA  NaCl  PEG  Untreated
Figure 4

LT-1  LT-2  WT

MpAsr

Actin
Figure 5

A

B

yellowish spots rate (%)
Figure 6

A

WT

LT-1

7d (drought)

WT

LT-1

14d (drought)

WT

LT-1

3d (after rewater)

B

Survival rate (%)

50

45

40

35

30

25

20

15

10

5

0

WT

LT-1

LT-2