THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Botany)

DEGREE

Botany

FIELD

DEPARTMENT

TITLE: Characterization of Gene in Drought-Tolerant Maize (*Zea mays* L.) under Drought Stress

NAME: Miss Phanatchakorn Banthaophit

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Niran Juntawong, Dr.nat.tech.)

COMMITTEE MEMBER

(Associate Professor Prasart Kermanee, Ph.D.)

COMMITTEE MEMBER

(Associate Professor Ravie Sethpakdee, Ph.D.)

DEPARTMENT HEAD

(Associate Professor Srunya Vajrodaya, Dr.rer.nat.)

APPROVED BY THE GRADUATE SCHOOL ON

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

CHARACTERIZATION OF GENE IN DROUGHT-TOLERANT MAIZE (*Zea mays* L.) UNDER DROUGHT STRESS

PHANATCHAKORN BANTHAOPHIT

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Botany)
Graduate School, Kasetsart University
2011

Phanatchakorn Banthaophit 2011: Characterization of Gene in Drought-Tolerant Maize (*Zea mays* L.) under Drought Stress. Master of Science (Botany), Major Field: Botany, Department of Botany. Thesis Advisor: Associate Professor Niran Juntawong, Dr.nat.tech. 98 pages.

Plants have various defensive mechanisms against drought in order to reduce water use. Molecular response of plant to drought implicated in up-regulation of gene expression. Almost 2000 drought-responsive genes were identified under progressive drought stress. In this study, an expression of gene in two maize lines; KSX 4605 (drought-sensitive) and SW 2301 (drought-tolerant) was reported. Under water deficit by 15% PEG in half Hoagland solution, the 2-week-old seedling of SW 2301 is more tolerant to drought than that of KSX 4605. There is a difference in the PCR product of amplified shoot cDNA by using VP14 primers with extra one band in 729 bp in SW 2301 but not in KSX 4605.

The 792-bp-fragment was subjected to RACE-PCR for the full length identification. The 1607-bp-fragment was obtained with Open Reading Frame (ORF) from 56 to 1345bp. The sequence showed high similarity with mRNAs of an unknown protein in *Zea may*, *Sorghum bicolor* and *Oryza sativa* var. Japonica. The protein was highly similar to the conserved domain (KU70) of Ku protein in NCBI database. In the cluster analysis, this full length sequence showed close similarity to monocot groups. This protein required further study for its expression, function and its connection to drought tolerance in the future.

_____ _____ ___ /___ /___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I would like to grateful thank and deeply indebted to my academic advisors Associate Professor Dr. Niran Juntawong advisor for supervision, encouragement and valuable suggestion for completely

writing of thesis. I would sincerely like to thank Associate Professor Dr. Prasart Kermanee burn for his guidance and corrections during writing of this thesis, and gratefully thank to Associate Professor Dr. Ravie Sethpakdee for his guidance, corrections and kind support throughout this study. I would like to greatly indebted to Associate Professor Pattana Srifah Huehne for her advice.

This research is a subproject in the Kasetsart University Corn and Sorghum Master Research Project (KUcosmap) and was supported by a grant from Kasetsart University Research and development Institute (KURDI). The authors would like to thank Dr. Chokechai Aekatasanawan for providing the maize seeds to carry out this research work.

I am especially appreciated my parents, my brother for their continuing encouragements and deeply indebted to Dr. Suwit Vutthisutmethavie, Mr. Akhom Khatfan and Ms. Nukoon Jomchai for their advice. Finally thank my friends who always gave me encouragement throughout my graduate study.

Phanatchakorn Banthaophit

March 2011

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

aa	=	amino acid
AAO3	=	Abscisic aldehyde oxidase in *Vicia faba*
ABA	=	Abscisic acid
AhNCED1	=	*Arachis hypogaea* L NCED-1 in peanut
atha	=	A.thaliana
AtHD2C	=	Histone deacetylase 2C in *Arabidopsis thaliana*
AtNCED3	=	*Arabidopsis thaliana* NCED-3
BiP	=	binding protein
bp	=	base pairs
btra	=	B.taurus
cDNA	=	complementary deoxyribonucleic acid
cele	=	C.elegans
cint	=	C.intestinalis
cm	=	centimeter
crei	=	C.reinhardtii
ºC	=	degree Celsius
DDBJ	=	DNA Data Bank of Japan
ddis	=	D.discoideum
DEPC	=	Dietyl pyrocarbonate
dmel	=	D.melanogaster
DNA	=	deoxyribonucleic acid
DNA-PK	=	DNA-dependent protein kinase
dNTP	=	deoxynucleotide triphosphate
drer	=	D.rerio
DSBs	=	Double-strand breaks
DTT	=	DL-dithiothreitol
EDTA	=	ethylenediamine tetraacetic acid
EST	=	Expressed Sequence Tags
EtBr	=	Ethidium bromide

LIST OF ABBREVIATIONS (Continued)

Fig	=	Figure
ggal	=	G.gallus
gmax	=	G.max
GSP-F	=	forward gene specific primer
GSP-R	=	reverse gene specific primer
GSPs	=	gene specific primers
GSS	=	Genome Survey Sequences
h	=	hour
HaDhn	=	*Helisnthus annuus* L.
HDAC	=	HD2-type histone deacetylase HDAC
HTGS	=	High Throughput Genomic Sequences
hum	=	H.sapiens
hvul	=	H.vulgare
HVA1	=	*Hordeum vulgare* (Himalaya)
kb	=	kilobase pairs
l	=	liter
LB	=	Luria-Bertani medium
LEA	=	*Late Embryogenesis Abundant*
LeNCED1	=	*Lycopersicon esculentum* NCED-1
LeZEP1	=	*Lycopersicon esculentum* ZEP-1
LpLtp	=	*Lycopersicon pennellii* Lipid-Transfer Protein
M	=	molar
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
min	=	minute
mM	=	millimolar
mm	=	millimetre
MgCl2	=	magnesium chloride

LIST OF ABBREVIATIONS (Continued)

mous	=	M.musculus
MPa	=	Mega pascal
mRNA	=	Masseger ribonucleic acid
mtru	=	M.truncatula
NCBI	=	The National Center for Biotechnology Information
NCE	=	neoxanthin cleavage enzyme
NCED	=	9-cis-epoxycarotenoid dioxygenase
ng	=	nanogram
nm	=	nanometre
no	=	Number
nsLtp	=	Nonspecific Lipid-Transfer Protein
ORF	=	Open Reading Frame
osat	=	O.sativa
PaNCED	=	*Persea americana* Mill. cv Lula NCED in avocado
PvNCED1	=	*Phaseolus vulgaris* L. NCED-1 in bean
RACE	=	Rapid Amplification cDNA Ends
RNA	=	ribonucleic acid
RNase	=	ribonuclease
rnor	=	R.norvegicus
RT-PCR	=	Reverse transcriptase polymerase chain reaction
sec	=	second
slyc	=	S.lycopersicum
STS	=	Sequence Tagged Sites
TAE	=	Tris-acetate-EDTA
taes	=	T.aestivum
TE	=	Tris-EDTA
Temp	=	temperature
U	=	unit
VP14	=	viviparous mutant gene locus (*vp14*) of maize

LIST OF ABBREVIATIONS (Continued)

VuABA1	=	*Vigna unguiculata* encodes zeaxanthin epoxydase
VuNCED1	=	*Vigna unguiculata* NCED-1 in cowpea
xlae	=	X.laevis
xtro	=	X.tropicalis
ZEP	=	Zeaxanthin epoxidase
Zm	=	*Zea mays* L.
zmay	=	Z.mays

CHARACTERIZATION OF GENE IN DROUGHT-TOLERANT MAIZE (*Zea mays* L.) UNDER DROUGHT STRESS

INTRODUCTION

Growth, development and reproductive system of plants is greatly affected by environmental stress, such as drought, salinity, chilling, freezing, high temperature and flooding, etc. (Bray, 1997; Shinosaki and Yamaguchi-Shinosaki, 1997; Xing and Zhu, 2002). To avoid these stresses, plants have various biochemical and physiological responses and defensive mechanism. Recent studies revealed that there is expression of various genes under drought stress including *Lea* protein, *HVA1* (Ried and Walker-Simmons, 1993; Xu *et al*., 1996; Han *et al*., 1997; Oraby *et al*., 2005), *VP14* (Schwarz *et al*., 1997; Tan *et al*., 1997; Schwartz *et. al*., 2003), BiP (Alvin *et al*., 2001), *AtHD2C* (Sridha and Wu, 2006) and *NCED groups* (Chernys and Zeevaart, 2000; Iuchi *et al*., 2000, 2001; Wan and Li, 2006; Lu *et al*., 2007; Melhorn *et al*., 2008). Increasing in glycine betaine, proline and sugars in drought stress plants was reported (Voetberg and Sharp, 1991; Nakamura *et al*., 1996; Naidu, 1998; Xing and Rajashekar, 2001; Chen and Murata, 2002).

Abscisic acid (ABA) has been reported to be involved in drought stress. ABA is a plant hormone and plays an important role on responses of plants to environmental stress, such as drought and high salinity (Iuchi *et al*., 2001). Cornish and Zeevaart (1984) reported that ABA accumulation increased in drought stress leaves of *Xanthium strumarium* L. For the response to dehydration, ABA levels increased dramatically in root, shoot, and scutellar tissue of wheat (Ried and Walker-Simmons, 1993). An increased level of ABA causes stomatal closure to prevent water loss. Udomprasert *et al*. (1999) found that water stress caused an increase in proline and ABA levels in maize.

Molecular responses of ABA are involved in gene expression and protein synthesis, such as *Late Embryogenesis Abundant* (LEA) protein in mature seed and in

desiccation-tolerant seedling (Hughes *et al*., 1991; Ried and Walker-Simmons, 1993; Han *et al*., 1997) and *HVA1* gene from barley. In transgenic rice, the increase of stress tolerance correlated with the increased level of the *HVA1* protein (Xu *et al*., 1996; Babu *et al*., 2004). The ABA biosynthesis involves in the expression of multi-gene families. The expression of the genes controls ABA biosynthesis in different organs or tissues. Different environmental stimuli or developmental stages of growth controlled an expression of the gene. These multi-gene families encode different protein enzymes in ABA biosynthesis pathway, such as ZEP (Zeaxanthin epoxidase), AAO (Arabidopsis Aldehyde Oxidase) and NCED (9-cis-epoxycarotenoid dioxygenase). The NCED enzyme has a key regulated step in catalyzed NCED to xanthoxin which is a precursor of ABA (Iuchi *et al*., 2001; Bray, 2002; Oono *et al*., 2003; Tan *et al*., 2003). In maize, *VP14* gene expression is regulated by water stress (Schwartz *et al*., 1997; Tan *et al*., 1997). The *PvNCED1* of bean (*Phaseolus vulgaris* L.) slowly induced by water stress (Qin and Zeevaart, 1999), *VuNCED1* in cowpea strongly induced by drought stress (Iuchi *et al*., 2000) and *PaNCED1* in avocado induced by water stress (Chernys and Zeevaart, 2000). In tomato, LeNCED1 and LeZEP1 increased NCED mRNA and ZEP mRNA during drought stress (Thompson *et al*., 2000). The *rd22* gene in *Arabidopsis* is induced by dehydration stress, high-salt condition and application of exogenous ABA (Abe *et al*., 1997) and the *AAO3* (Abscisic aldehyde oxidase) gene in *Vicia faba* is catalyzed by regulatory enzymes AAO in the final step of ABA biosynthesis pathway (Melhorn *et al*., 2008).

In Thailand, drought stress is a major cause of maize yield reduction which relates to rainfalls. Limiting of growth and yield of the crop may involve in drought signaling which is up or down-regulated during drought induction. However, few studies of genes involving in drought response were carried out between drought-sensitive and drought-tolerance maize lines.

OBJECTIVES

This research aims to study an expression and characterization of gene involved in drought response of drought-sensitive (KSX 4605) and drought-tolerant (SW 2301) maize lines.

LITERATURE REVIEWS

1. Role of ABA in water stress

ABA is a plant hormone and involved in plant growth and development, embryogenesis, root and shoot development, stomatal function, seed dormancy, leaf water relation and adaptation to environmental stress such as cold, drought and high salinity (Behl and Hartung, 1984; Bonetta and McCourt, 1998; Rock and Patrick NG, 1999; Eckardt, 2001). Guerrero and Mullet (1986) found that in pea plants were rapidly dehydrated to turgor pressure ABA levels increased 100-fold, indicating that dehydration induced synthesis of ABA. Damptey *et al*. (1978) also conducted in several cultivars of sweet corn during water deficit of early tassel development and found that endogenous ABA concentration increased both in tassel and leaf in the first two days of water deficit.

ABA distributes along leaf and root tissues and might play a role as the signal molecule carrying information of water availability in the soil from root to leaf tissues. Behl and Hartung (1984) found ABA at high concentration in cytoplasm and vacuolar contents of leaf in stress-tolerant barley cultivar. Zhang and Davies (1991) studied an increase of ABA in xylem sap from un-watered maize plants and tested for anti-transpirant activity. They stated that maize stomatal opening, at least, responded to soil drying.

2. Molecular changes of plants in response to water deficit

Xu *et al*. (1996) reported that an increase of stress tolerance correlated with the level of *HVA1* protein accumulating in the transgenic rice plants, and have hypothesized that *LEA* protein plays an important role in protection of plants under water deficit or salt-stress conditions (Xu *et al*., 1996). In castor bean (*Ricinus communis* L.) seeds *LEA* transcripts were first detected during mid-development and peaked to the onset of desiccation (Han *et al*. (1997). The third generation of transgenic oat (*Avena sativa* L.) expressed *HVA1* activity when compared to the non-

transgenic control plants. The transgenic R3 plant growth were greater and significant increased in tolerance to salt stress conditions (200 mM NaCl) (Oraby *et al*., 2005).

The cDNA encode for putative neoxanthin cleavage enzyme (NCE). The similar protein were found in the wilt-related tomato (*Lycopersicon esculentum* Mill.) library. The tomato cDNA and derived amino acid sequence have been compared to *VP14* of maize and found that mRNA levels of this *VP14* homologue increased dramatically in response to water stress (Burbidge *et al*., 1997). Burbidge *et al.* (1999) used probe for screening of wilt-related tomato cDNA library and identified a cDNA full length sequence which is specific to the *not* gene locus and ORFs of the tomato cDNA was very similar to *VP14* sequence of maize.

Under drought condition the binding protein (BiP) levels in leaf correlated with the maintenance of the shoot turgidity and water content. Over expression of BiP prevented cellular dehydration, the stomatal conductance and transpiration rate in drought leaves were compared with control. They suggested that over-expression of BiP in plants may prevent endogenous oxidative stress (Alvin *et al*., 2001).

An *Arabidopsis* HD2 protein, AtHD2C involved in abscisic acid (ABA) and abiotic responses. Expression of *AtHD2C* was repressed by ABA and in addition *35S:AtHD2C-GFP* transgenic plants display reduced transpiration and enhanced tolerance to salt and drought stresses when compared with wild-type plants. These result demonstrated that AtHD2C could modulate ABA and stress responses (Sridha and Wu, 2006).

The ABA is one signaling important molecule in the regulation of gene expression during water deficit, whereas many indicator gene expressions to response do not ABA for expression. The induction of the genes corresponding to these cDNA by ABA varied, for drought stress could induce expressions of various genes that involved in stress tolerance and responses. Recent studies were emphasized on gene encoded for the 9-cis epoxycarotenoid dioxygenase (*NCED*) enzyme which catalyzes

the biosynthesis of abscisic acid (ABA) in plants. The mechanisms of drought tolerance have been studied in many plant species. Previous studies reported that the drought signal is controlled by changes of abscisic acid through an expression of 9-*cis* epoxycarotenoid dioxygenase (*NCED*) gene. ABA is formed by the oxidative cleavage of an epoxy-carotenoid in maize and the *VP*14 gene has been identified and cloned. The recombinant VP14 protein catalyzes the cleavage of 9-*cis*-epoxy-carotenoids to form C_{25} apo-aldehydes and xanthoxin, a precursor of ABA biosynthesis. In maize found that expression of *VP14* was up regulated by water stress **(**Schwarz *et al*., 1997; Tan *et al*., 1997; Schwartz *et. al*., 2003)**.**

Expression of *AtNCED3* in transgenic *Arabidopsis* was induced by drought stress which plays a key role in ABA biosynthesis. Iuchi *et al*. (2001) improved that drought tolerance by gene of *AtNCED3* caused the accumulation of endogenous ABA level. *VuNCED1* in cowpea (*Vigna unguiculata*) plants has a homology to 9-cis-epoxycarotenoid dioxygenase (NCED) which involved in ABA biosynthesis. The accumulation of ABA and expression of *VuNCED1* were strongly induced by drought stress in cowpea plant and did not expression of *VuABA1* gene that encodes zeaxanthin epoxydase, and have discussed that the *VuNCED1* cDNA encoding a 9-*cis*-epoxycarotenoid dioxygenase and the product was key role to ABA biosynthesis under water stress in drought tolerance cowpea (Iuchi *et al*., 2000).

The expression of *AhNCED1* gene in peanut plants is significantly up-regulated by dehydration and high salinity. The up-regulation of *AhNCED1* expression by exogenous application of ABA suggests a positive feedback control of ABA biosynthesis which in wild-type *Arabidopsis* expression of the *AhNCED*1 gene results in ABA accumulation increasing in response to drought stress. These results indicate that the expression of *AhNCED1* gene plays an important role in the regulation of ABA level during water stress (Wan and Li, 2006).

In avocado fruit, *PaNCED1* and *PaNCED2* strongly induced the fruit ripened and *PaNCED1* was induced by water stress. Results indicated that ABA biosynthesis in avocado regulated the level of carotenoid cleavage (Chernys and Zeevaart, 2000).

Lu *et al*. (2007) studied the role of microtubule in the induction of ABA biosynthesis in root maize in response to osmotic stress, and found that ABA significantly accumulated in maize root cells. The degree of stimulating differently affected on the expression of the *Vp14* gene when the plants were treated with PEG and PEG/oryzalin, and taxol treatment as compared to the control treatment.

3. The tissue or organs in gene response during water stress

In maize plants, Tan *et al*. (1997) found that the *Vp14* mRNA expressed in embryos and roots, and was strongly induced in leaves under water stress. The VuNCED1 in cowpea is strongly induced in stem and leaves but less in root under drought stress (Iuchi *et al*., 2000). Nell *et al*. (1998) cloned cDNA from *Arabidopsis* suspension culture cDNA library encoding NCED and found that AtNCED1 mRNA was present in turgid shoot tissue and rapidly underwent dehydration resulting in AtNCED1 mRNA accumulation. In *Arabidopsis*, *AtNCED3* was dominant in stress leaves and non stress leaves. The *AtNCED3* was the major stress-induced gene in leaves and increased mRNA levels of all NCED and can be detected within 10 min after detachment. The *AtNCED3* was resembled *VP14* of maize, expressing in root and stress induction in leaves, are consistent with the hypothesis that *AtNCED3* and *VP14* are orthologs, but *AtNCED3* appeared not to be a dominant NCED expression in developing embryos, whereas *VP14* accounts for about 70% of developmental regulation of ABA synthesis in seed and 35% of the stress induced ABA synthesis (Tan *et al*., 2003).

Melhorn *et al*. (2008) transformed ABA biosynthesis enzyme 35s *pro:AtNCED3-GFP* and *AAO3-GFP* into guard cells of broad bean leaves for studied in *Arabidopsi*s. The result found that ABA biosynthesis was stimulated by heterologous expression of AtNCED3 and *Arabidopsis* aldehyde oxidase 3 (AAO3) protein, both genes likely regulate ABA biosynthesis enzyme which active in guard cells.

The abscisic aldehyde oxydase (*AAO1* and *AAO3*) of *Arabidopsis* strongly expressed in stem (Seo *et al*., 2000). The AhNCED1 transcript and endogenous ABA accumulated predominantly in leaves and stem of peanut in response to dehydration (Wan and Li, 2006).

The transcriptional profiles of leaves and roots of three-leaf stage seedling of maize under soil stress were regulated by 296 genes under stress and 206 genes were specific to leaves and 90 genes were specific to roots. Sixty stress-regulated genes were regulated by salt stress. Among these genes, 27 genes were specific to leaves and 33 genes were specific to roots (Qing *et al*., 2009).

In wheat (*Triticum aestivum* L.), Ried and Walker-Simmons (1993) reported that a late embryogenesis abundant (LEA) protein responsed to dehydration by increasing ABA levels. ABA levels after 24 h dehydration were at the highest levels in the shoot while at 48 h dehydration ABA levels in both shoots and scutellar were slow down and in the roots was rapidly increased 4-fold. Group 3 LEA mRNAs were induced in root, shoot and scutellar tissues. However, group 3 LEA proteins were detected only in shoot and scutellar tissues but it was not found in root. Xu *et al.* (1996) found *HVA1* gene accumulated at high-levels in leaves and roots of transgenic rice plants under water deficit.

In maize seedling, water deficit significantly induced ABA accumulation in leaf and root tissues (Hu *et al*., 2005) and the intensity of ABA was a signal as a root-to-shoot and the strongest ABA signal originated from the root cap and the meristematic root tip in maize root (Schraut *et al*., 2004). Ren *et al*. (2007) proposed that the xanthophyll was a precursor pool but not be able to sustaining the ABA accumulation. The ABA around the plant accumulated in different compartments and different tissues. ABA hormone synthesized both in the leaves and the roots of plant, and move rapidly through the plant in both the xylem and the phloem (Sauter *et al*., 2001).

In maize plant, response of the reproductive tissue to water deficit was reported. At low water potentials from -0.5, -0.75 and -1.0 MPa, growth of stem, silks and leaves were stopped, respectively. The root growth were decreased only when water potential was lower than -1.4 MPa. These results indicated that a high flow resistance had developed in the xylem. The water potential gradient controlled water uptake by roots but did not in leaves at low water potential. The decrease of turgor in silks and the loss of the water potential gradient in leaves probably contributed to the high sensitivity of these organs to increase in root/shoot ratios (Westgate and Boyer, 1985).

Maize growth in drying soil showed consistently higher root dry weights than well-watered plants. A bulk soil water potential between -0.2 and -0.3 MPa resulted in the ABA increase (Zhang and Davies, 1989).

At low water potentials of maize seedling, ABA content reduced and associated with inhibition of root elongation and promotion of shoot elongation, when compared with control and wild-type seedling at the same water potential, indicating that ABA accumulation played direct roles on the maintenance of primary root elongation and the inhibition of shoot elongation (Saab *et al*., 1990).

Munns and King (1988) found that the inhibitory activity was partly triggered both in leaf and root when water deficits. In soil, drying results in some type of chemical signaling between roots and shoots. In some plants, root-sourced ABA can apparently influence shoot physiology and growth of shoot water relation. ABA application could affect on plants at the plasmamembrane and on gene expression (Davies *et al*., 1990).

Trevino and Connell (1998) studied the Nonspecific Lipid-Transfer Protein (*nsLtp*) gene family in *Lycopersicon pennellii* plants by using gene specific probes to separately describe the expression of three member of the *nsLtp* gene family in different organs of normal and wilt plants. Plants in well-watered, *LpLtp1* and *LpLtp2* transcripts were decreased in the matured leaves, while *LpLtp3* transcription was

detectable only in young leaves at very low levels. Drought and ABA induced *LpLtp1* and *LpLtp2* genes in different patterns of expression in fruit but *LpLtp3* was regulated in leaves tissue.

4. Plant developmental stages and cultivars in response to water deficit

In *Xanthium strumarium* L the highest ABA contents was higher in young leaves than in mature leaves at well watering and showed contrast result when water stressed (Cornish and Zeevaart, 1984). In tomato (*Lycopersicon esculentum*) when water stressed, the ABA highly accumulated in young leaves and highly produced in the mature leaves. Moreover, it found that the ABA deficient mutant of tomato synthesizes ABA at low levels during water deficit when compared to the wild type (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) (Zeevaart and Boyer, 1984). Cohen and Bray (1990) observed correlation between mRNAs and endogenous ABA in wild type leaves. They found that the drought induced both theirs throughout periods of water deficit.

In leaves tissue of two *Wilty* mutants and wild-type genotype maize, Rock and NG (1999) reported that the sensitivities of the two genotypes to ABA were in the same manner after treatment with ABA. The ABA concentrations were similar in mutant and wild-type, all unstressed and drought-stressed tissue.

Landi *et al*. (2001) studied in two maize lines and found that high leaf ABA concentration plant was more susceptible to drought than low leaf ABA concentration. This results indicated that selection for low leaf ABA concentration led to populations with better agronomic performance than did selection for high leaf ABA concentration as a selection criterion to improve drought tolerance. Maize is the most sensitive to water deficit during the reproductive stages from anthesis to silking (Denmead *et al*., 1960; Westgate and Thomson Grant, 1989; Otegui *et al*., 1995).

Water stress at tassel initiation of maize were greater influenced than at anthesis on proline and ABA levels and yield. The increase of proline and ABA

levels were contrast to the yield in both Ki3 and Ki11 maize varieties. Under water stress condition at tassel initiation and at anthesis, Udomprasert *et al*. (1999) found that increased in both maiz varieties. However, proline and ABA levels in Ki3 (drought-sensitive variety) was greater than those in Ki11 (drought-tolerant variety).

Saab *et al*. (1990) measured ABA content in the root growth zone, 10 cm below the tip. They found that low water potential induced a large increase ABA content in wild-type seedling whereas in mutant (vp5) seedling, the accumulation of ABA was little increased by low water potential. The shoots of mutant seedlings were still capable of more rapid elongation than wild-type at low water potential. The time course of ABA content in the shoot growing zone, by measured at the 15 mm upper mesocotyl. Low water potential caused a large increase in ABA content in wild-type seedlings whereas mutant seedling had little effect on ABA levels. The results indicated that accumulation of ABA at low water potential in mutant seedling associated with less growth inhibition.

Jovanovic *et al*. (2000) study the response of root of drought-resistant and drought-susceptible maize lines under osmotic stress at -1.0 MPa. They found that osmotic stress induced different distribution of ABA within cell compartment in drought-resistant maize line but this osmotic stress did not cause any significant effect on pH gradient and compartmental ABA content, whereas in drought-susceptible maize line ABA increased the transport into intracellular and effect on pH gradient.

After 15 days pollination of maize, Andjelkovic and Thompson (2006) used a maccroarray of 2500 maize cDNA for determining transcription changes during water and salt stress. The proportions of cDNA transcripts significantly increased of maize kernels under water stress and salt stress more than 2-fold compared to the control. Under drought, root and shoot fresh weight were decreased. Nevertheless, protein in leaf was induced in both varieties but there was no relation between protein changes and drought tolerance (Mahammadkhani and Heidari, 2007).

Water deficit affected on 12-15% grain yield reduction of maize when water stressed at vegetative growth. The effect was rose to 53-75% at silking stage and then was lowered to 30% at three weeks after silking stage (Claassen and Shaw, 1970). Myers *et al*. (1990) found that during early kernel development of maize, ABA elevation in leaves decreased the rate of cell division by limit the storage capacity of endosperm of the kernel.

Cellier *et al*. (1998) study gene expression in sunflowers (*Helisnthus annuus* L.) between drought-tolerant (R1) and drought-sensitive (S1) lines. They found that leaves of R1 plants accumulated of *HaDhn1* and *HaDhn2* transcriptions. Stomata of both lines displayed similar sensitivity to ABA and ABA-induced accumulation of *HaDhn2* which was higher in the tolerant than in the sensitive genotype, suggesting that drought gene expression might exist in tolerant plants.

5. RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) is the preferred technique utilized to detect and quantify mRNA. RT-PCR may also be used in cloning, constructing a cDNA library, amplifying signal during in situ hybridizations, and synthesizing probes and wide variety of transcripts can be detected with highly sensitive method for the detection of mRNA expression levels in small samples of cells or tissue. Traditionally RT-PCR involves two steps, by reverse transcription (RT reaction) of the target RNA and followed by PCR amplification of its cDNA to detect exactly when, and in which type of cell genes are expressed. RNA is first reverse transcribed into cDNA using a reverse transcriptase; the resulting cDNA is used as templates for subsequent PCR amplification using primers specific for one or more genes (Richert *et al*., 1996; Lorkowski and Cullen, 2003).

RT-PCR can also be carried out as one-step or single tube, in particular its sensitivity allows for the detection of weakly expressed mRNAs or the detection of mRNAs from a small pool of tissue mRNA. RT-PCR is accuracy and precision enables the researcher to detect small differences in mRNA abundance between

samples. Although one-step RT-PCR offers simplicity and convenience and minimizes the possibility for contamination, the resulting cDNA cannot be repeated as in two steps RT-PCR. The mRNA population represents how these genes expressed under any given set of conditions. RNA can be analyzed by a number of technologies, including northern blotting and DNA microarray analysis or by a variety of RT-PCR base method, can provide a good reflection of an organism of gene expression profile (Lorkowski and Cullen, 2003).

Reverse Transcription (RT reaction) is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA or poly(A) RNA, a reverse transcriptase enzyme, a primer, dNTPs and an RNase inhibitor. The resulting cDNA can be used in RT-PCR reaction. RT reaction is also called first strand cDNA synthesis. Three types of primers can be used for RT reaction: oligo (dT) primers, random hexamers and gene specific primers. Viral RNA template or nonpolyadenylated RNA can be copied using random hexamers or specific target primers (Brenner *et al.*, 1997).

MATERIALS AND METHODS

Materials

1. Plant materials

Seeds of two maize lines, KSX 4605- a drought sensitive and SW2301- a drought tolerant lines.

2. General solutions

- Hoagland solution
- Polyethylene glycol (PEG, Fluka, molecular weight 6,000)
- Agarose (Gene Pure LE, ISC Bio Express)
- Ice
- Liquid nitrogen
- Chloroform
- Chloroform: isoamyl alcohol (24: 1)
- DEPC-treat water: 0.1% Dietyl pyrocarbonate (DEPC) in distilled water
- Ultra pure distilled water DNase, RNase free (Invitrogen, USA)
- Distilled water
- 75% and 95% Ethanol
- Isopropyl alcohol
- TRIzol® Reagent (Invitrogen, Carlsbad CA, USA)
- SuperScript™ III First-Strand (Invitrogen™, USA)
- Gene JET™ Plasmid Miniprep Kit (Fermentas, EU)
- pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen™, USA)
- DNA Extraction Kit ((PureExtreme™ Fermentas, EU)
- LB agar: 1% tryptone, 0.5% yest extrac, 1% NaCl, 1.5% agar
- LB medium: 1% tryptone, 0.5% yest extrac, 1% NaCl
- Sodium acetate 3M, pH 5.2 (RNase-free)

- 50x TAE (Tris-acetate EDTA) buffer
- GeneRacer™ Kit (Invitrogen™, Carlsbad, CA, USA)
- TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0)
- Generuler™ 1kb DNA Ladder (0.5µg/ul 50µg, Fermentas, EU)
- 6x Loading dye (Fermentas, EU)

3. General equipments

- Petri dishes
- Centrifuge (Butterfly)
- Microcentrifuge (Centuron Scientific Ltd.)
- Power pack
- Electrophoresis tank
- Gel plates and comb
- PCR machine (Gene Amp PCR system 2400, Perkin Elmer)
- Px2 Thermal Cycler (Thermo Electron Corporation, USA)
- Gel Documentation (Gel Doc 2000, Bio-Rad, USA)
- Gel electrophoresis set (Bio-Rad)
- Micropipettes (Gilson, France)
- Micropipette tips (RNase-free, sterile)
- Microcentrifuge tubes (RNase-free, sterile)
- Incubator shaker (OPTIC ivymen® SYSTEM)
- Vortex apparatus
- pH meter
- Spectrophotometer (UV/visible) and cuvette
- Water bath
- Microwave
- Gloves
- Magnetic stirrer
- Hot Plate Stirrer
- Fume hood

Methods

1. Plants and Plant Growth materials

Seeds of two maize lines, KSX 4605, a drought sensitive and SW 2301, a drought tolerant line (Chutkaew *et al*., 1988), were soaked in distilled water for one day and grown on Petri dish for two days. On the fourth day the seed was transferred and grew with vermiculite in small plastic pots and was watered daily with half-strength Hoagland nutrient solution.

After one week, plants with two fully-expanded leaves were grown hydroponically on half-strength Hoagland nutrient solution. For water stress induction, two-week-old plants were subjected to drought by the application of 15% (w/v) polyethylene glycol 6,000 (PEGs-6000, Fluka) solution, which had water potential of -0.5 MPa (Todorov *et al*., 1998). After treated with PEG for different times at 0, ½, 1, 2, 4, 6, 24 and 48 h, the plants underwent wilting and the third young leaves from terminal shoots, the shoots and the roots segments were collected. All tissues were stored in 1.5 ml microcentrifuge tubes and kept at -80º C until future extraction and analysis. For remainder tissues were obtained water content in plant by fresh weight and dry weight and calculate by;

$$\text{Water content (\%)} = \frac{(\text{Fresh weight} - \text{Dry weight})}{\text{Fresh weight}} \times 100$$

2. RNA Isolation

2.1 RNA extraction

Total RNA was extracted from young leaves, shoot and roots of maize using TRIZOL® Reagent (Invitrogen™, Carlsbad CA, USA). The tissue was homoginized in 1 ml TRIZOL® Reagent by liquid nitrogen to a fine powder using a mortar and pestle. In the isolation step the powder was suspended in 1 ml TRIZOL® Reagent and transferred to 1.5 ml microcentrifuge tubes. The homogenate samples were incubated at 15 to 30º C for 5 min and were phase separated by addition with 200 µl of chloroform per 1 ml TRIZOL® Reagent. The sample tubes were capped securely and were shaken vigorously by hand for 15 sec and incubated at 15 to 30º C for 2 to 3 min. Centrifuge the samples at no more than 12,000 x g for 15 min at 2 to 8º C. RNA remained exclusively in the aqueous phase. The volume of the aqueous phase was about 60% of the volume of TRIZOL® Reagent and transferred into a new tube. RNA precipitation from the aqueous phase was mixed with 500 µl of isopropyl alcohol and incubated samples at 15 to 30º C for 10 min. Centrifuge at 12,000 x g for 10 min at 8º C. The RNA precipitate, often invisible before centrifugation, formed a gel-link pellet on the side and bottom of the tube. The supernatant was washed the RNA pellet once with 1 ml of 75% ethanol, mixed the sample and centrifuged at 7,500 x g for 4 min at 2 to 8º C. Ethanol was removed and the pellet was allowed to air dry at room temperature. The air-dried RNA pellets were dissolved in 40 µl ultra pure water and incubated at 55 to 60º C for 10 min. The RNA was quantified by measuring absorbance at 260 nm. The total RNA was stored at -80º C until use.

2.2 RNA quantification

The concentration of total RNA was spectrophotometrically determined by measuring the absorbance at 260 nm (A_{260}). An absorbance of one unit at 260 nm corresponded to approximately 40 µg of RNA per ml (Sambrook and Russel, 2001). The RNA concentration of each sample was estimated by the following equation:

Concentration of RNA sample = A260 × dilution factor × 40 µg /ml

Purity of RNA was determined by the ratio of the reading at 260 nm and 280 (A260/A280) provides an estimate of purity of RNA with respect to contaminants. The ratio of A260/A280 ranging from 1.8 to 2.0 indicated good quality of the RNA. The total RNA was stored at -80º C until further analysis.

RNA quality was determined by gel electrophoresis to check total RNA; by running with 1.2% agarose gel in 0.5x TAE buffer. The RNA loading sample was prepared by mixing 1µg RNA sample mixed with 1 µl of 6x loading. The mixed sample was loaded slowly into the well of the submerged gel. The RNA samples were gel running at 80 volts in 0.5x TAE buffer until the fastest dye has moved to 2/3 of the gel length. After finishing, the gel was stained in 0.5 µg/ml ethidium bromide (EtBr) solution for 30 min and de-stained the gel for 30 min. The RNA bands in the gel were visualized by using gel documentation system (Bio-Rad, USA) under 254 nm UV trans illuminator and then taken a photographed. The 1 kb ladder was used as marker to size products.

3. Gene expression study by RT-PCR

3.1 Primer design

To amplify the first strand cDNA, specific primers for *HVA1* gene (LEA) (accession no X78205.1), *Lea1* (accession no NM_001111879.1), *Lea2* (accession no NM_001111828.1), *Lea3* (accession no X55388.1), *Leax* (accession no NM_001111959.1), *VuNCED1* (accession no AB030293.1) and *VP14* genes (accession no U95953.2) were designed from nucleotide sequences in the gene bank (WWW.ncbi.nl.nih.gov) by using FastPCR software. The sequences of primers were shown in the Table 1. These primer sequences were synthesized and purchased from the Bioservice unit, National Science and Technology Development Agency (NSTDA), Bangkok.

Table 1 Oligonucleotide forward and reverse primers for cDNA PCR amplification

Name	Oligonucleotide primer	Expected size
HVA1	forward 5′-CAACAGCCTAAAGCGAGTCC-3′ reverse 5′ -CGAGTGTCCTCGTTATCC-3′	1028 bp
Lea1	forward 5′-TGCTTGTTAGTTTGGGGACG-3′ reverse 5′ -ATCAGATCTAGGACTTGGTCCTG-3′	363 bp
Lea2	forward 5′-CGCGAACGTTTCAGCCGATC-3′ reverse 5′ -CAAACAGGGTCACGGACGACAG-3′	707 bp
Lea3	forward 5′-AGACAACACTCACCGATAGCAAG-3′ reverse 5′ -ATCCACACACACGTACCTGTG-3′	561 bp
Leax	forward 5′-CAGACAACACTCACCGATAGC-3′ reverse 5′ -TGATCGCCTACATGCATGCT-3′	527 bp
VuNCED1	forward 5′-ATTGAATTCATGCCTTCATCAGCTTCAAAC-3′ reverse 5′ -ATTGGATCCCAAAAGCTACACGCTGGTCCCC-3′	836 bp
VP14	forward 5′-ATGCAGGGTCTCG-3′ reverse 5′ -TCAGGCCGCCT-3′	1815 bp

3.2 First-strand cDNA synthesis

The reverse transcription of mRNA to first strand cDNA was performed with a two-step kit of SuperScript™ III First-Strand. In a 20 µl reaction, approximately 1 ng to 5 µg of total RNA or 1 ng to 500 ng of mRNA was used. Ten microlitres of mixture containing added the following components to nuclease-free microcentrifuge tube, 2 µl total RNA (50 ng/µl), 1 µl of 50 µM oligo $(dT)_{20}$, 1 µl of 10 mM dNTP mix and 6 µl DEPC-treated water was prepared and incubated the mixture at 65º C for 5 min and quick chill on ice for at least 1 min. The RNA mixture content in the tube was collected by brief centrifugation and the following cDNA synthesis by added 2 µl of 10x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl RNase OUT™ (40U/µl) and 1 µl Superscript™ III RT (200U/µl) to the total RNA mixture and mix well by pipetting gently and collected by brief centrifugation and incubated the mixture at 50º C for 50 min. The reaction was terminated by heating at 80º C for 5 min, then chilled on ice and collected the reaction by brief centrifugation. To removed RNA, 1 µl of *E. coli* RNase H (2U/µl) was added to the

mixture and incubated at 37º C for 20 min. The synthesized first strand cDNA was used as a template for PCR reaction immediately.

3.3 PCR amplification of cDNA

The actin sequence of *Zea mays* was designed from NCBI database GeneBank: U60514 (Maz56) gene. The actin primer were used to amplify the cDNA of maize as a control. The primer pairs of *Zea mays* actin gene was described as following:

Forward primer 5´-AGTCCAAGAGAGGCATTCTG-3´
Reverse primer 5´-TGCAGCAACGTAACGTACCA-3´
Tm 57º C, expected size 291 bp

Oligonucleotide primers in Table 1 were used to amplify cDNA fragments. The PCR amplifications were performed using first-strand cDNA from leaves, shoots and roots of maize as a template. Twenty microliters of reaction volume contained 1.6 µl of 25 mM $MgCl_2$, 2.0 µl Mg-free PCR buffer (10X), 1 µl dNTP mix (2.0 mM), 0.2 µl Taq polymerase (5U/µl), 2.0 µl first strand cDNA (50 ng/µl), 1 µl primer mix (10 µM each). For actin amplification, 0.2 µl primer mix (10 µM each) and 12.2 µl double distilled water were used. The reaction mixture was gently mixed and briefly centrifuged and then was amplified in a thermocycler (Perkin Elmer, Gene Amp PCR system 2400).

The PCR amplification conditions were optimized. The amplification was performed for 35 cycles (Table 2). The reaction started with 3 min initial denaturation step (94º C). The reaction was terminated with a final extension step at 72º C for 7 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis. The DNA was stained with 0.5 µg/ml ethidium bromide (EtBr) solution and visualized and photographed with gel documentation system (Bio-Rad, USA) under 254nm UV Tran illuminator. The 1 kb ladder was used as marker to size

products. The DNA fragment was purified from agarose gel using DNA Extraction Kit (PureExtreme™ Fermentas, EU).

Table 2 Optimized PCR condition for the cDNA amplification as amount of cycles running, denaturing, annealing, extension temperature and time

Primers	Cycles	Denaturing Temp/Time	Annealing Temp/Time	Extension Temp/Time
HVA1	35	94º C/1 min	55º C/1 min	72º C/2 min
Lea1	35	94º C/30 sec	66º C/30 sec	72º C/1 min
Lea2	35	94º C/30 sec	59º C/30 sec	72º C/1 min
Lea3	35	94º C/30 sec	57º C/30 sec	72º C/1 min
Leax	35	94º C/30 sec	61º C/30 sec	72º C/1 min
VuNCED1	35	94º C/30 sec	52º C/30 sec	72º C/1 min
VP14	35	94º C/30 sec	60º C/30 sec	72º C/1 min
Zm-Actin	30	94º C/30 sec	58º C/30 sec	72º C/1 min

3.4 Purification of DNA fragment from agarose gel

The PCR products from agarose gel electrophoresis were purified by using DNA Extraction Kit (PureExtreme™, Fermentas, EU), by slicing the gel about 0.4 g weight and place the slice into 1.5 ml microcentrifuge tube. Three volume of Biding solution to 1 volume of gel were used to dissolute the agarose gel and incubated at 55º C for 5 min. Silica powder; 2 µl per 1 µg of DNA (used 10 µl in this study) was added to the suspension and incubated at 55º C for 15 min, mixing every 2 min with vortex to keep silica powder in suspension. Maximum speed in a conventional table-top microcentrifuge was used to collect the pellet for 5 sec. The pellet was washed by 500 µl of ice cold washing buffer by vortexing and spinning for 5 sec and discarded the supernatant, washing repeat tree times. After removing the supernatant from the last wash, the tube was spinned again and removed the remaining liquid with pipette. Airdried the pellets for 10-15 min and re-suspended the pellet in an aliquot of sterile distilled water by incubated at 55º C for 5 min.

Analytical DNA was obtained by 0.8 % (w/v) gel electrophoresis and stored at -20º C until use.

4. Cloning and DNA sequencing

4.1 Cloning DNA into plasmid vector and bacterial transformation

DNA cloning fragment was inserted into plasmid vector by using the pCT8®/GW/TOPO® TA Cloning® Kit (Invitrogen™, USA) by vector view map see Appendix Figure 4A. For expression of the gene were interested by transform the recombinant into One Shot® competent into *E. coli*. The TOPO® Cloning reaction was performed by using chemically competent *E. coli* the procedure following, final volume 6 µl containing 0.5 to 4 µl of fresh PCR product, 1 µl salt solution, 1 µl TOPO® vector and sterile distilled water was added to a final volume of 6 µl. The reaction was gently mixed and was incubated at room temperature (20-23º C) for 5 min (can be varied from 30 sec to 30 min) and place the reaction on ice for transforming One Shot® competent *E. coli*.

The bacterial transformation proceed were followed, the TOPO® Cloning reaction was added 2 µl into a vial of One Shot® chemically competent *E. coli* and mix gently. The mixture was incubated on ice for 3 to 30 min and heat-shock the cells at 42º C for 30 sec without shaking immediately was transferred the tube to ice and was added 25 µl of room temperature S.O.C. medium. Incubation the cells were shook the tube horizontally 200 rpm at 37º C for 1 h. Spread plate was used 10 to 50 µl from each transformation on a pre-warmed LB plates which containing 100 µg/ml spectinomycin and was incubated overnight at 37º C (recommend to spread two different volumes for one plate will have well-space colonies).

The recombinant clones were analyzed positive clones by picked 2-6 colonies in LB plates for PCR using M13 primer (Appendix Figure 4B) which oligonucleotide were followed:

Forward primer 5′-TTGTAAAACGACGGCCAGTC-3′
Reverse primer 5′-CAGGAAACAGCTATGACCATG-3′
Tm 47º C

For sequencing plasmid was prepared by subculture of a single colony as possible in LB medium containing 100 µg/ml spectinomycin and were incubated at 37º C for 12 to 16 h before isolation plasmid.

4.2 Plasmid extraction and DNA sequencing

For plasmid isolation was used PureExtreme™ GeneJET™ Plasmid Miniprep Kit (Fermentas, EU) and analyzed the plasmids by PCR again to confirm the presence and correct of the insert fragment by M13 and VP14 primer. The sequencing plasmid was done at Genome Institute, BIOTEC Thailand. The computer analysis of the amino acid sequences after sequencing, the nucleotide sequences of two bands as NR1 and NR2 which interested were carried out using the program at the National Center for Biotechnology Information Services (NCBI, Bethesda MD) for quickly identifying segments of a nucleotide sequences that may be of vector origin. VecScreen searches a query sequence for segments that match any sequence in a specialized non-redundant vector database (UniVec) were used online internet network from (URL) http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html. For determination the function of the sequence were obtained by analysis of an unknown sequence is to perform homology search, which is a search for sequence similarity with known sequence in the database by using BLASTN2.2.24 program from GenBank (Zhang *et al*, 2000) at (URL) http://blast.ncbi.nlm.nih.gov/Blast.cgi and DNA Data Bank of Japan (DDBJ) (Altschul *et al*, 1997) at (URL) http://blast.ddbj.nig.ac.jp/top-e.html databases exchange their data daily to update the sequence information**.**

Homology search of nucleotide sequences both NR1 and NR2 fragments were used BLASTN 2.2.24 program from database of NCBI at (URL) http://blast.ncbi.nlm.nih.gov/Blast.cgi and coordinate with all GenBank+EMBL+DDBJ+PDB sequences but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences. The database of DDBJ (DNA Data Bank of Japan) at (URL) http://blast.ddbj.nig.ac.jp/top-e.html was searched for the ESTs similarity in database to identical DNA by selected DNA database of plant and all ESTs (est_atha+est_btra+est_cele+est_cint+est_crei+est_ddis+est_dmel+est_drer+est_ggal+est_gmax+est_hum+est_hvul+est_mous+est_mtru+est_osat+est_rnor+est_slyc+est_taes+est_xlae+est_xtro+est_zmay+est_rest).

Analysis of nucleotide sequence is the last and may be the most important step in nucleotide sequencing. The goal is to determine the function of the sequence obtained. The most frequent analysis of an unknown sequence is to perform homology search, which is a search for sequence similarity with known sequence in the database. These are two international DNA databases available, which provide sequence information as no cost over the internet. All databases exchange their data daily to update the sequence information.

5. Rapid Amplification cDNA Ends Fragment (RACE) PCR for full-length

For interesting DNA fragment as name NR1 was performed the full-length cDNA using the GeneRacer™ Kit (Invitrogen™, Carlsbad, CA, USA) both 5′ and 3′ RACE. The procedure was shown as Appendix Figure 5 and 6 a kit according to the manufactur's instructions. The gene specific primers (GSPs) were designed for 5′ and 3′ ends and the position were shown as Appendix Figure 7

To perform the full-length nucleotide sequences for 5′ and 3′ ends of interested fragment were known sequence. For RACE-PCR was performed using GeneRacer™ Kit. This study, generate full-length cDNA using 5 µg of total RNA isolated from the shoot tissue of SW 2301 maize line at two weeks old planting was

ligated the GeneRacer™ RNA Oligo to the 5′ end linkers (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′). Approximately 5 µg of oligonucleotide ligated mRNA was obtained and used for the cDNA synthesis using Superscript III reverse transcriptase (RT) following the instruction of the vendor (Invitrogen™, Carlsbad, CA, USA). The oligo-dT primers (5′- GCTGTCAACGATACGCTACGTAACGGCATGACAGTG $(T)_{24}$ -3′) was annealed to mRNA to synthesize cDNA. To amplify 5′ end cDNA, reverse gene specific primer (GSP-R) 5′- GTAGATCATGTGCATGCCAGGCGGTTC -3′ were designed and used in combination with GeneRacer™ 5′ primer (5′-CGACTGGAGCACGAGGACACTGA -3′) (Invitrogen™, Carlsbad, CA, USA). To obtain 3′ ends, the first strand of cDNA was amplified with forward gene specific primer (GSP-F1) 5′- TGCCATCTGCTGGTGATGAGC -3′, GSP-F2 5′-GCTCTACTTCAGGATGCACAGACAC -3′ and GSP-F3 5′-ACTCGACCACAACTCATAGCCCTTGTTG -3′ amplification with the GeneRacer™ 3′ primer (5′- GCTGTCAACGATACGCTACGTAACG -3′) and GeneRacer™ 3′ Nested primer (5′- CGCTACGTAACGGCATGACAGTG -3′) (Invitrogen, Carlsbad, CA, USA).

The PCR condition were used PCR reaction 20 µl final volume containing 1.5 µl GeneRacer 5′ or 3′ (10 µM), 1 µl gene specific primer reverse or forward (10 µM), 1 µl RT template, 2.0 µl (10x) i-*Taq*™ plus $MgCl_2$ free PCR buffer, 1.5 µl dNTP mix (2 mM each), 0.2 i-*Taq*™ plus DNA polymerase (5U/µl) (iNtRON Biotechnology) and sterile distilled water 12.8 µl. The PCR amplification were used preheat at 94º C 3 min and followed by 5 cycles of 94º C for 30 sec, 72º C for 1 min and followed by 5 cycles at 94º C for 30 sec, 70º C for 1 min and followed by 20 to 25 cycles of denaturing temperature at 94º C for 30 sec, annealing temperature at 63º C for 30 and extension temperature at 70º C for 1 min. The reaction was terminated with a final extension at 70º C for 10 min. The PCR products were obtained by 1 % (w/v) gel electrophoresis.

The PCR products from RACE-PCR both 5′ and 3′ ends fragment were purified DNA from gel by using DNA Extraction Kit (PureExtreme™ Fermentas, EU) and was transformed by using pCT8®/GW/TOPO® TA Cloning® Kit (Invitrogen™, USA) and selected colony for analyze clones by PCR. The plasmid isolation used GeneJET™ Plasmid Miniprep Kit (Fermentas, EU) and analyzes the plasmids by PCR to confirm the presence. The sequencing plasmid was done at Genome Institute, BIOTEC Thailand.

For identication the nucleotide sequences after sequencing PCR products of 5′ and 3′ ends fragments were carried out using the program at the NCBI for quickly identifying segments of a nucleotide sequences that may be of vector origin. VecScreen searches a query sequence for segments that match any sequence in a specialized non-redundant vector database (UniVec) in online website at (URL) http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html. For link up of 5′ end and 3′ end nucleotide sequences each fragments were used the CLUSTAL 2.0.10 multiple sequence alignment program from (URL) www.ebi.ac.uk/Tools/clustalw2.

The full-length cDNA by nucleotide sequences was analyzed the similarity from database by using BLASTN (2.2.24) program from NCBI database at http://blast.ncbi.nlm.nih.gov/Blast.cgi and DDBJ (DNA Data Bank of Japan) at http://blast.ddbj.nig.ac.jp/top-e.html. The unknown of nucleotide sequence after have got full-length was obtained function and homology search by blastp or blastx to find gene and seek homologous proteins. For future study an open reading frame (ORF) was used the ORF Finder (Open Reading Frame Finder) is a graphical analysis tool which finds all open reading frames of a selectable minimum size in a user's sequence or in a sequence already in the database. This tool identifies all open reading frames using the standard or alternative genetic codes sequence database using the WWW BLAST server which was defined as a sequence stretch that begins with an initiation codon, usually ATG (alternatively GTG or TTG), and ends with a termination codon (TAA, TAG or TGA).

The homology search was used when an amino acid sequence translated from an ORF located in the DNA sequence. For predict gene of eukaryote were used GeneMark.hmm (ES-3.0) protein translations program (Borodovsky and Lukashin, 2008; Lomsadze *et al.*, 2005) version 3.9 at (URL) http://opal.biology.gatech.edu/GeneMark/ by selected GeneMark-E and GeneMark.hmm-E and GeneFinder, and Gene finder for predict possible part of gene which is database of both nucleotides and amino acid sequences by using Sotfberry (2.6) program at (URL) http://www.softberry.com/berry.phtml?topic=gfindb. All of BLASTP or BLASTX, GeneMark and GeneFinder were estimated to predicted gene and for protein sequence analysis in the future study. Multiple alignment and phylogenetic tree were analyzed by clustal_W2 program at (URL) http://www.ebi.ac.uk/Tools/services/web_clustalw2/.

Place and duration of the Experimental

The research work was carried out at the Department of Botany, Faculty of Science Kasetsart University, Bangkhen, Bangkok, from May 2007 - September 2010.

RESULTS AND DISCUSSION

1. Response of KSX 4605 and SW 2301 maize lines to drought

The responses of the two-week-old maize lines, KSX 4605 and SW 2301 to drought by using 15% PEG (6000) was observed at 0 (control), 30 min, 1, 2, 4, 6, 24 and 48 h after induction. Although wilting symptom was observed in both two maize lines, however the KSX 4605 showed a mild wilting after 1 h of drought induction and showed moderate wilting after 4 h, severe wilting after 24 to 48 h. In contrast, the SW 2301 maize line exhibited wilting symptom at 2 h after stress induction and showed wilting at 4 h. However, after 6 h SW 2301 was slightly recovered from wilting. (Figure 1 and Table 3).

The reason why SW 2301 maize line recovered from wilting after 6 h was the time of sampling at predusk (17:45 and 18:00), the sunlight was weakened. SW 2301 was clearly a drought lolerance that could recover quicker than KSX 4605 which was considered as a drought sensitive one.

By comparing between two- and four-week-old seedlings, the drought responses in the two crossed cultivars were similar. However, the results obtained in four-week-old (KSX 4605 and SW 2301) seedling the wilt observation was more severe than the two-week-old (KSX 4605 and SW 2301) seedling (Figure 2 and Table 4).

It is possible that the demand of water uses in two stage of growth played an important role in drought response. Elder seedlings required more water consume than the younger. The younger seedlings were also better adapted to water deficit (Kang *et al*., 2000).

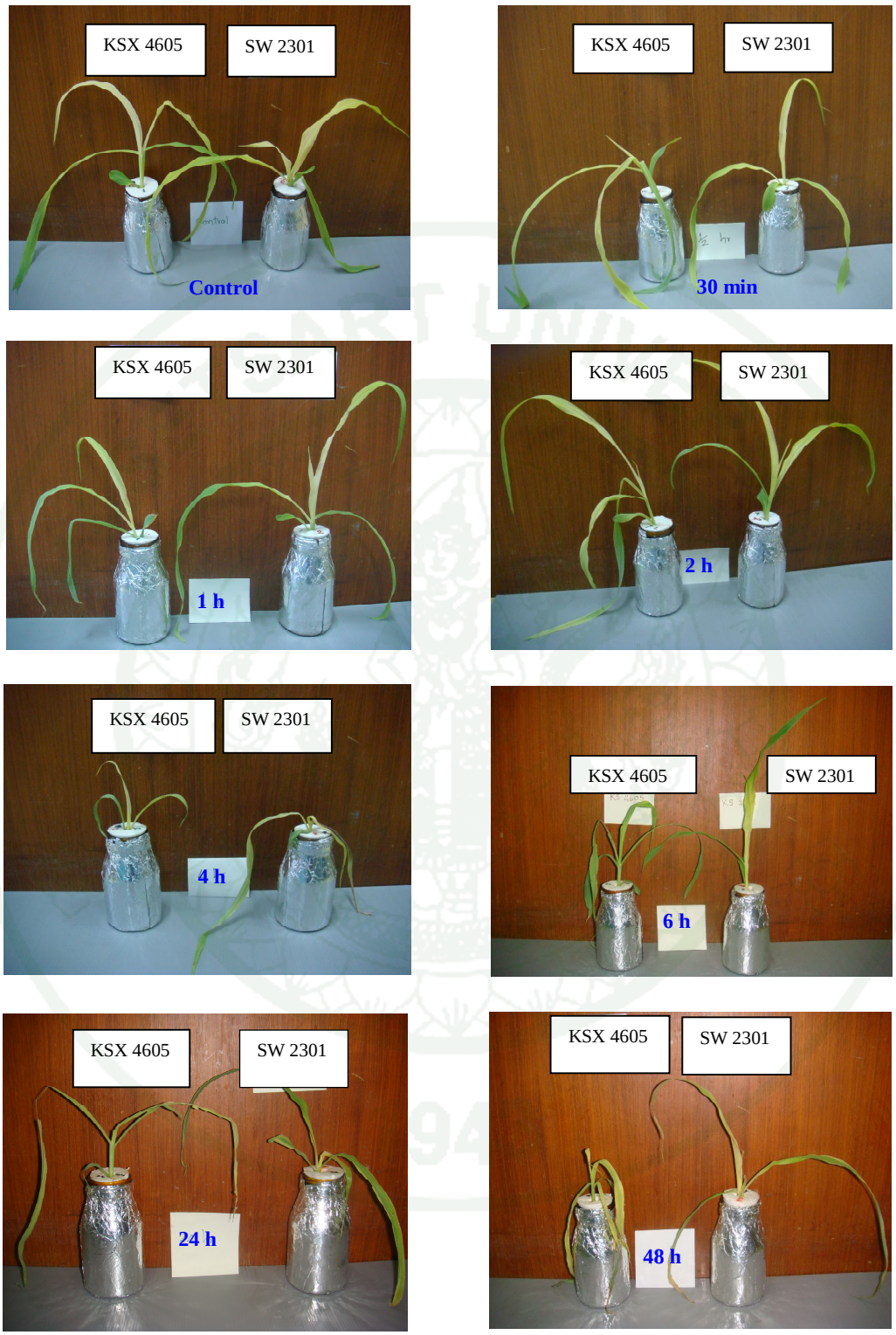


Figure 1 Wilting of 2-week-old seedlings of KSX 4605 and SW 2301 lines after the beginning of drought induction at 0, ½, 1, 2, 4, 6, 24 and 48 h by PEG.

Table 3 Drought response of 2-week-old seedlings of KSX 4605 and SW 2301 lines after the beginning of drought induction by 15% PEG.

Maize lines	Time after drought induction (h)							
	control	**1/2**	**1**	**2**	**4**	**6**	**24**	**48**
KSX 4065	×	×	√	√	√√	√√	√√√	√√√
Water content %)	92.24	92.72	90.76	91.50	86.80	90.68	91.56	84.95
SW 2301	×	×	×	√	√√	×	√	√
Water content (%)	92.69	93.72	92.13	92.54	89.16	91.84	88.91	90.03
Sampling time	11:30	12:30	13:00	14:00	16:00	18:00	12:00	12:00

Drought response (wilting level);

× = normal

√ = mild

√√ = moderate

√√√ = severe (moderate = can be recovered after rehydration)

Average water content (%)

KSX 4605 = 90.15

SW 2301 = 91.38

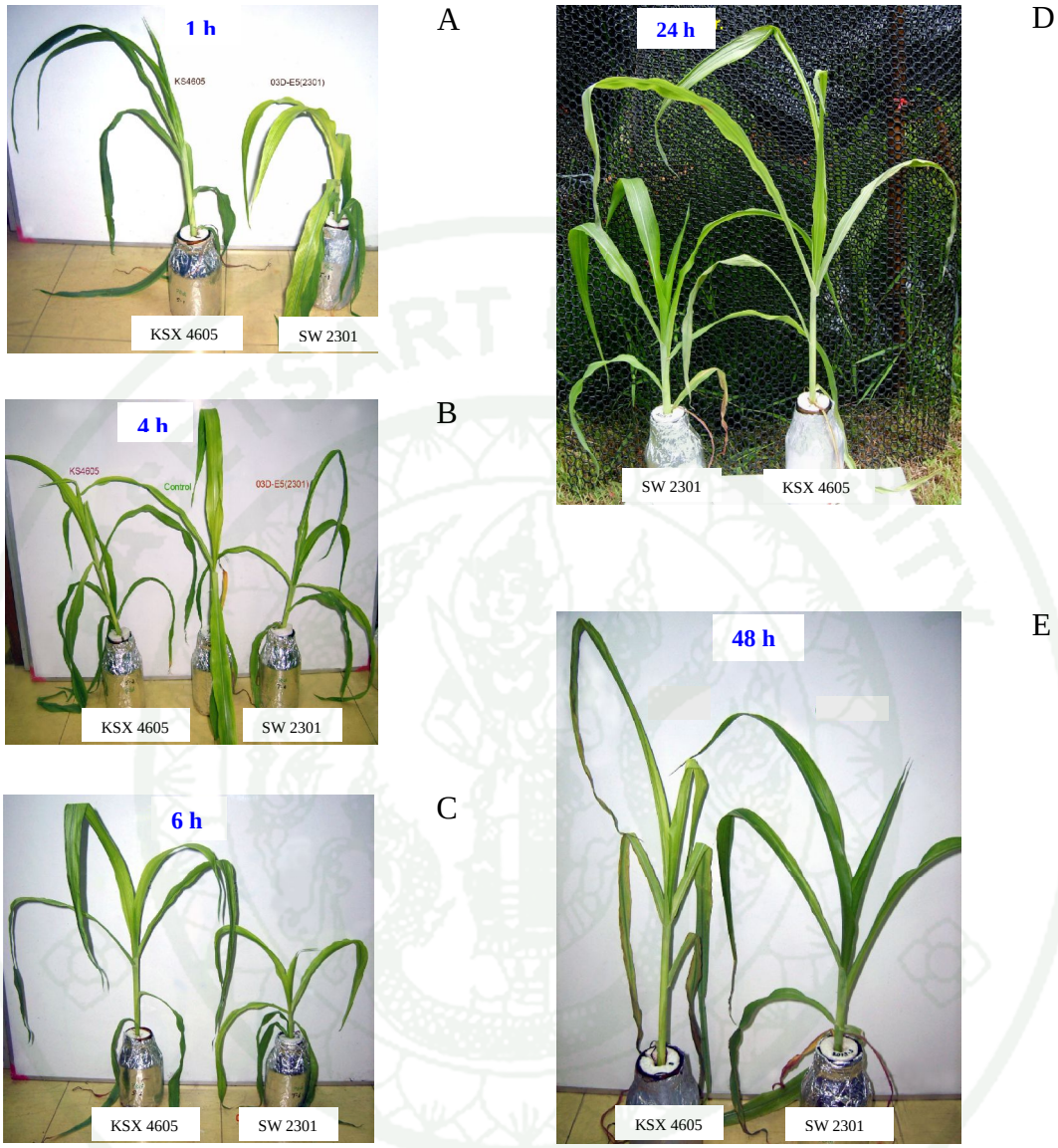


Figure 2 Wilting of 4-week-old seedlings of KSX 4605 and SW 2301 maize lines after the beginning of drought induction time at 1 (A), 4 (B), 6 (C), 24 (D) and 48 (E) h by 15% PEG.

Table 4 Drought response of 4-week-old seedling s of KSX 4605 and SW 2301 lines after the beginning of drought induction by 15% PEG.

Maize lines	Time after drought induction (h)						
	control	**1**	**2**	**4**	**6**	**24**	**48**
KSX 4065	×	√	√	√√	√√	√√√	√√√√
Water content (%)	93.50	89.42	91.27	92.08	91.40	90.56	90.21
SW 2301	×	×	√	√√	√	√	√
Water content (%)	93.21	92.61	92.75	92.60	93.33	-	91.08
Sampling time	12:45	12:45	13:45	15:45	17:45	11:45	11:45

Drought response (wilting level);

× = normal

√ = mild

√√ = moderate

√√√ = severe (moderate = can be recovered after rehydration)

√√√√ = rigid (may can not be recovered after rehydration)

Average water content (%)

KSX 4605 = 91.20

SW 2301 = 92.60

2. Gene profile in KSX 4605 and SW 2301 maize lines under drought

Total RNA were extracted from leaves, roots and shoots of the two maize lines at two weeks old by using TRIZOL® reagent. The result found the A260:A280 ratios of the leaves tissue between 1.6-1.8, the roots between 1.6-2.0 and the shoots between 1.6-2.0 respectively. This result showed well quality of the total RNA.

One microgram of total RNA from root, shoot and leaf tissues were analyzed by 1.2% (w/v) agarose gel electrophoresis for checking the intensity and quality of the RNA. There is no degradation of RNA in the agarose gel electrophoresis (Appendix Figure 2).

The cDNA was amplified and were tested with different primers and with corresponding annealing temperature. There is no difference in the PCR product banding pattern of leaves, root and shoot in all the primers (Figure 3), except VP14 primer. There is a difference in the shoot cDNA amplified banding pattern (Figure 4).

There is a difference in the PCR product of amplified shoot cDNA by using VP14 primers with extra one band in 800 bp which was not detectable in the root and leaf cDNA. All amplified cDNA with the VP14 primer showed a common band of 600 bp (Figure 4).

Both obtained bands 600 bp and 800 bp were cloned into the pCT8®/GW/TOPO® TA Cloning® Kit (Invitrogen™, USA). The cloned 600 bp and 800 bp were sequenced (Figure 5). The exact base pair of the cloned band is 595 bp (NR2) and 792 bp (NR1) after sequencing (Figure 6). Then the sequences were BLASTN in the NCBI and DDBJ for the identification of homology.

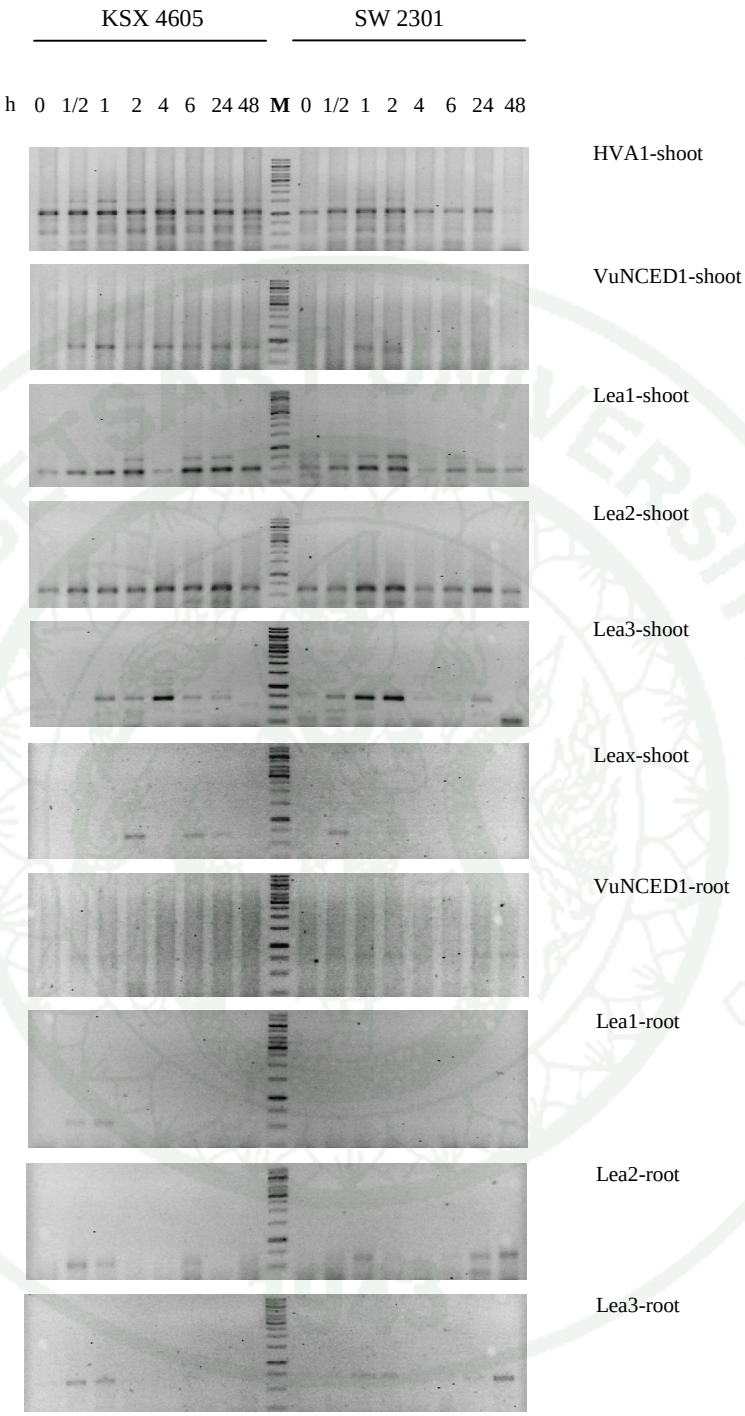


Figure 3 cDNA amplified with HVA1,VuNCED1, Lea1, Lea2, Lea3 and Leax primer of two maize lines after drought induction for 0, 1/2, 1, 2, 4, 6, 24 and 48 h by running 1% agarose gel electrophoresis, **M** is 1kb DNA ladder.

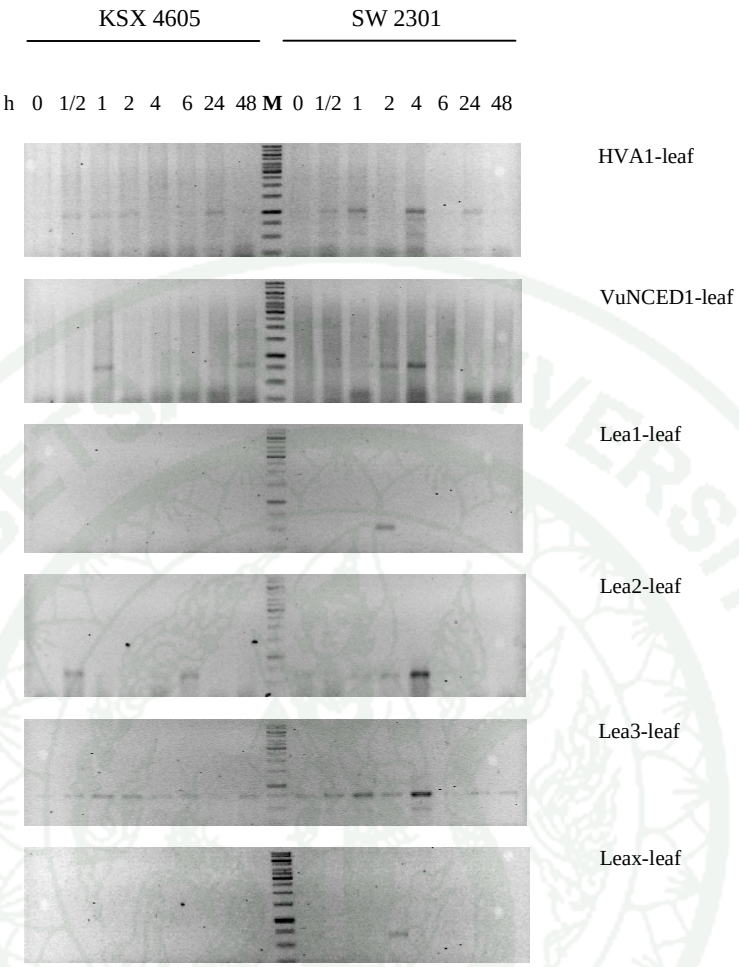


Figure 3 (Continued)

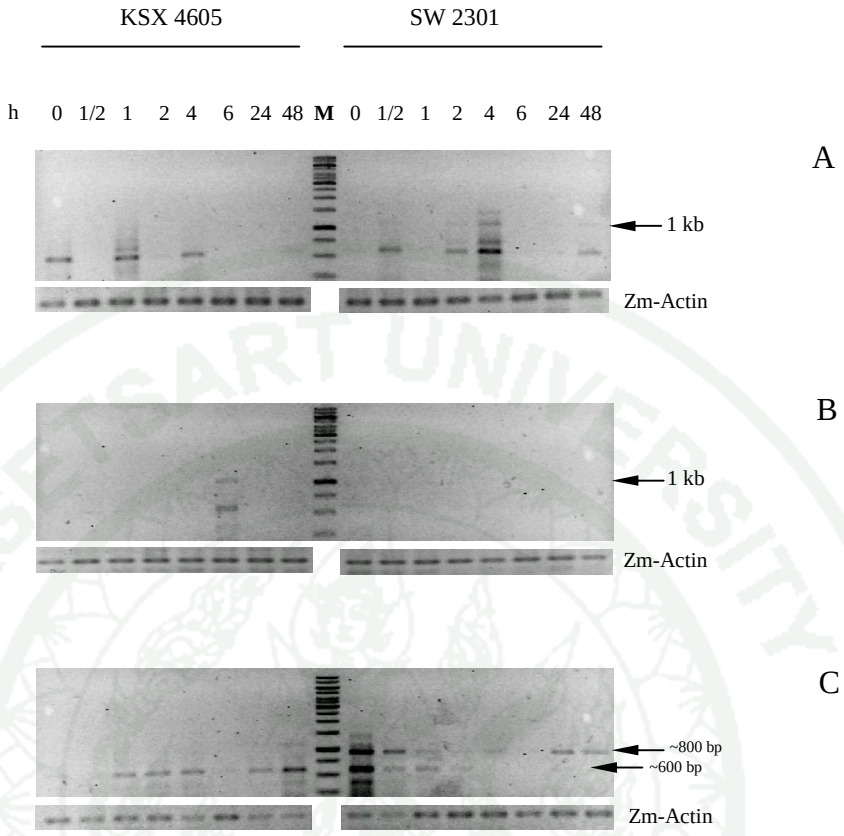


Figure 4 cDNA amplified with VP14 primer from leaf (A), root (B) and shoot (C) tissues of two maize lines after drought induction for 0, 1/2, 1, 2, 4, 6, 24 and 48 h by running 1% agarose gel electrophoresis, **M** is 1kb DNA ladder.

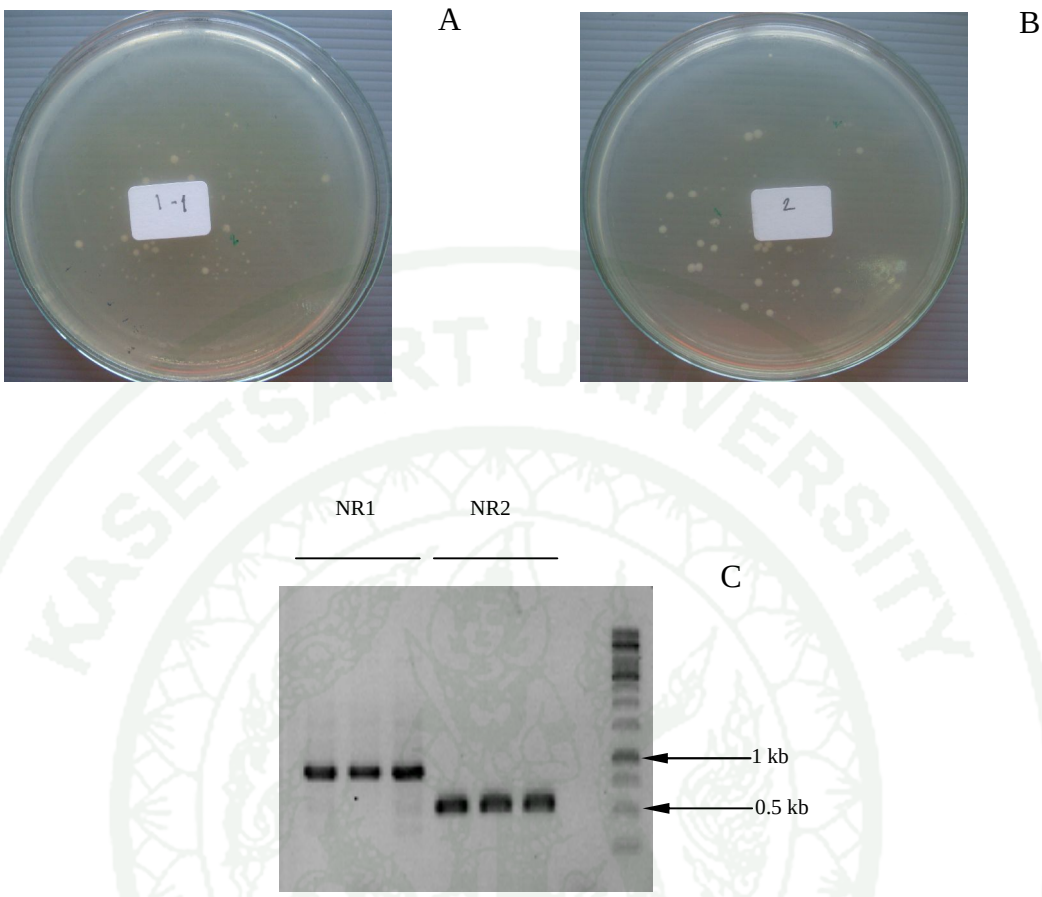


Figure 5 Recombinant colonies on LB agar plate (spectinomycin 100 µg/ml) of NR1 (A) NR2 (B) and PCR products of plasmid amplified with VP14 primer (C) 1kb DNA ladder as a marker.

A

LOCUS 792 bp

BASE COUNT 221 A 166 C 166 G 228 T 11 others

ORIGIN

```
      1 TCAGGCCGCC TGATGACCAG TTCAACATGT ACCTGTTTTA TGCAGATTTG
     51 ATTGGTCTGG ATGGAGATGA GATGACCGAA TATTTGCCAT CTGCTGGTGA
    101 TGAGCTAGAG GATATGACTA ATCAACTGAG AAAACGGATA ATGAAGAAGC
    151 GCAGAGTCAA AACTCTTTCA TTTGCGATTA CCAATGATGT GTGCATAGAA
    201 GTCAATACAT ATGCGCTGGT CCGTCCTACT ACTACAGGGA CAATCACATG
    251 GCTTGATTCA CTAAGTAACC TCCCATTAAA GGTTGAGAGG TCTTTCATAT
    301 GCAATGATAC TGGGGCTCTA CTTCAGGATG CACAGACACG TTTCCNGATG
    351 TACAATGACA CAATTGTCAA ATTTTCTGTA CGTGAACTCT CTGAGGTTAA
    401 AAGGGTTGCA AGCCATCATC TTCGCCTTAT AGGTTTCAAG CCATTGGATT
    451 GCTTGAAAGA TTACCATAAC TTAAGACCAT CGACATTTAT TTATCCGAGT
    501 GATGAGCGTA TATTTGGAAG CACCTGTGTT TTCGTTGCTT TACATAGCTC
    551 AATGTTACGT CTTGGAAGGN TTGCACTTGC ATTTTATGGG AATCCAACTC
    601 GACCACAACT CATAGCCCTT GTTGCTCAAG AAGANGNTAC TTCGTCTGGT
    651 CGTCAGTTTG AACCGCCTGG CATGCACATG ATCTACCTTC CATACTCCGA
    701 TGATATTAGA NATCCTGAAG AAGTTCATGT GACTTCTGAT GATGCNCCNC
    751 GNGCAACANA TGAACAATCA GAAAGCTTCN AAATATCCAN CG
```

B

LOCUS 595 bp

BASE COUNT 128 A 130 C 161 G 162 T 14 others

ORIGIN

```
      1 NGGTTGGATT TCGCCNTTNC AGCCCNCCTG TGAAGTATGT CAGCGCATGA
     51 AGGCCGAGCA TAAGCGGTCT GCAGGTTNGC TGAANCCATT AGAAATCCCA
    101 GAGTGGAAAT GGGAGCATAT CCCCCANGGA TTTTGNGGNG GGTCTACCTC
    151 GTTCCCCCTC GTGNCAGGGA TGCTATTNGG GTGGTAGTGG ATCGCTTGAC
    201 TAAGTCTGCG CATTTTATTC CCATGAAGNC CACAAATTCA GCTTCAGATT
    251 TGGTTCCCTT GTATATGAAG GAAGTAGTCA GGCTTCACGG GGTACCTAAG
    301 TCTATTGTTT CTGATCGAGA CTCCAAATTT GTNTCCAAAT TNTGGGAGGG
    351 TCTCCATAGT GCTTTGGGCA CCAAGCTTTC GCTTAGTGTC GCTTTTCACC
    401 CTCAGACGGA TGGTCAGTCT GAGCGAACAA TCCAAACCTT GGAAGACATG
    451 TTGCGTGCTT GTGTTCTGTC ATGGAAGGGT AGCTGGGAGG ATCATCTTGC
    501 TCTGGCAGAG TTTGCTTATA ACCACAGCTA CCAGGCTAGC ATCAAGGTGG
    551 CACCATTTGA GGCTTTGTAT GGCAGGCGGC CTGAAAGGGC GAATT
```

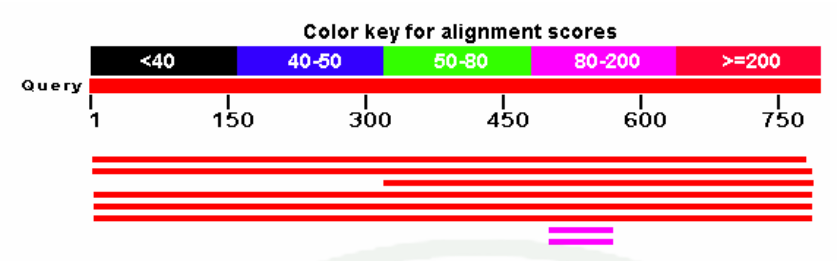
Figure 6 Nucleotide sequences of NR1 (A) and NR2 (B) fragments amplified with VP14 primer.

From the BLASTN results in NCBI (Figure 7A and Appendix Table 2) the 792 bp (NR1) band shows high similarity to *Sorghum bicolor* hypothetical protein mRNA. The accession number is XM_002461257.1 (95% identities) and XM_002459401.1 (94% identities), followed by *Zea mays* hypothetical protein mRNA, the accession numbers is NM_001174336.1 (98% identities). The *Oryza sativa Japonica* Group showed similarity in 3 accession numbers, NM 001065596.1, AK102066.1 and AK099980.1 with identities of 85%, 85% and 84% respectively. Then the same sequence were BLASTN in DDBJ (Figure 7B and Appendix Table 3) and shows high similarity to *Zea mays* cDNA clone, mRNA sequence. The accession number is FL123854, BU093734, DV025081, BM500851, CF036521 and identities (%) of 98, 99, 97, 98 and 97 respectively.

From the BLASTN results in NCBI (Appendix Figure 3A and Appendix Table 4) the 595bp (NR2) band shows high homology to only contiguous genomic DNA sequence comprising the 19-kDa-zein gene family from *Zea mays* complete sequence. The accession numbers is AF546188.1 (90% identities). Because there is no homology sequence of 595 bp to other sequences in the NCBI, it might be possible that the 595 bp sequence is a part of genomic DNA of *Zea mays*.

By comparing to the sequence in NCBI, the 595 bp sequence were BLASTN in DDBJ (Appendix Figure 3B and Appendix Table 5), the result shows less similarity to all obtained sequences.

A



B

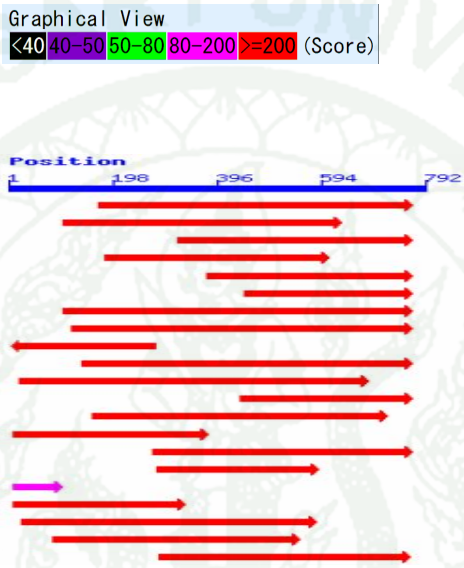


Figure 7 Graphical view of nucleotide sequences alignment of NR1 (792bp) by using BLASTN (2.2.24) program from NCBI (A) and DDBJ database (B).

3. Full-length of VP14 product

The obtained 792 bp fragment (NR1) in the drought tolerant maize line, SW 2301, is renamed to ZM1 and was used for full-length study by GeneRacer™ Kit (Invitrogen™, Carlsbad, CA, USA) with both 5′ and 3′ ends of RACE-PCR. The gene specific primers (GSPs) were designed from the 792 bp sequence (Appendix Figure 7).

The PCR products of 5′ and 3′ ends are shown in Figure 8. The 5′ ends PCR products were ~750 bp. The 3′ ends PCR products were ~1.5kb in size amplified with GSP-F1 primer (Figure 8A) and ~1.3 kb with GSP-F2 (Figure 8C) and ~0.9 kb with GSP-F3. Due to the unclear of ~0.9 kb fragment with GSP-F3 primer on agarose gel (Figure 8D), the nested PCR was carried out. The ~1.3 bp of GSP-F2 PCR product was used as a template with GeneRacer 3′ primer nested and GSP-F3 primer to confirm of the sequence (Figure 8E).

The nucleotide sequence of these fragments were aligned to determine the complete full-length sequence of ZM1 (Appendix Figure 8-10). The full-length sequence of ZM1 with 1607 bp was shown in Figure 9 by cutting poly A at 3′ tail.

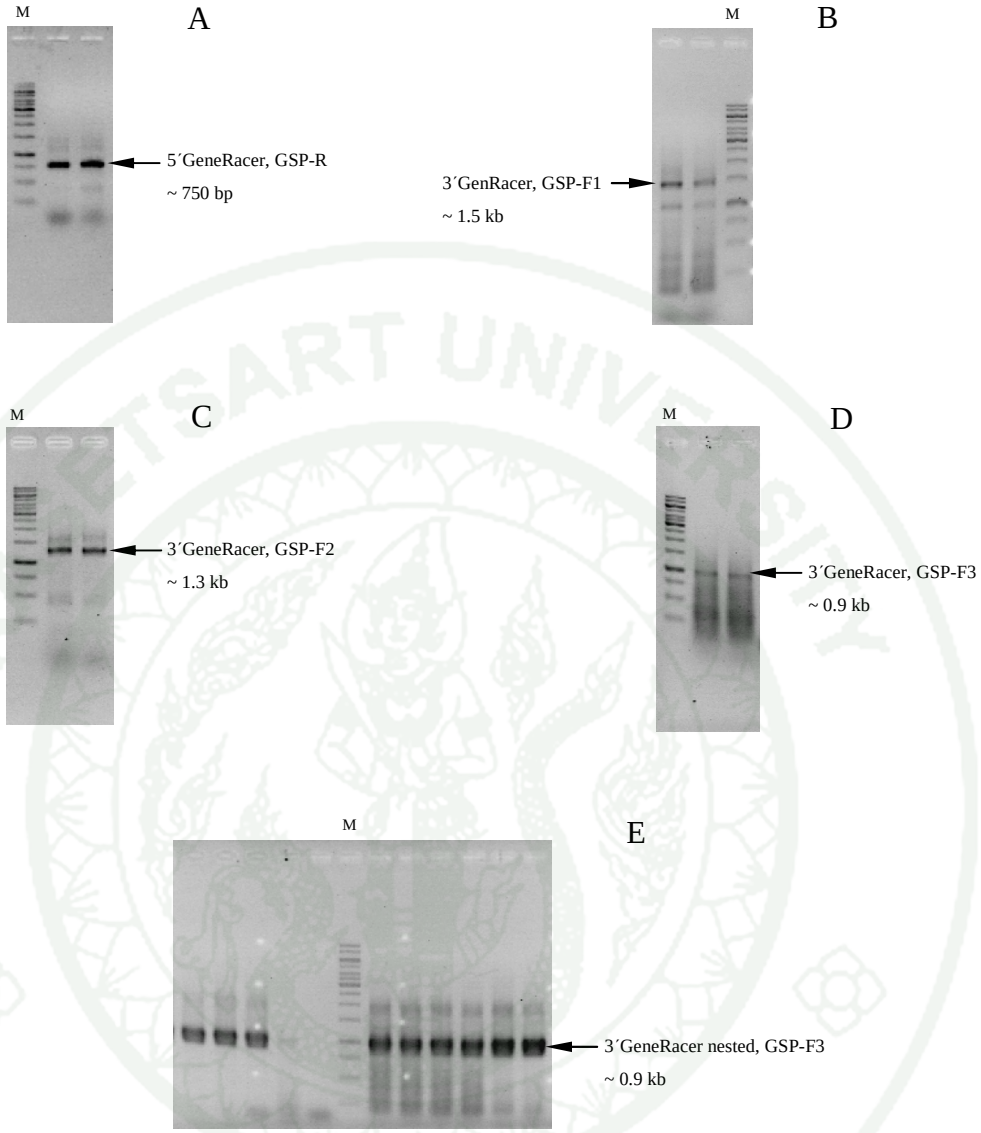


Figure 8 PCR products of 5′ GeneRacer and GSP-R primer (A), 3′ GeneRacer and GSP-F1 primer (B), 3′ GeneRacer and GSP-F2 primer (C), 3′ GeneRacer and GSP-F3 primer (D) and 3′ GeneRacer nested and GSP-F3 (E) obtained by 1% agarose gel electrophoresis, M-1 kb DNA ladder.

LOCUS ZM1 1607 bp

BASE COUNT 454 A 338 C 376 G 439 T

ORIGIN

```
   1 CGACTGGAGC ACGAGGACAC TGATATGATT AGGACAACAC TTCAACGTGC
  51 AAAGGATGCA CAAGATCTTG GCCTGTCGTA TTGAACTTCT TCCACTGAGC
 101 CCGCCTGATG ACCAGTTCAA CATGTCCCTG TTTTATGCAG ATTTGATTGG
 151 TCTGGATGGA GATGAGATGA CCGAATATTT GCCATCTGCT GGTGATAAGC
 201 TAGAGGATAT GACTAATCAA CTGAGAAAAC GGATAATGAA GAAGCGCAGA
 251 GTCAAAACTC TTTCATTTGC GATTACCAAT GATGTGTGCA TAGAAGTCAA
 301 TACATATGCG CTGGTCCGTC CTACTACTAC AGGGACAATC ACATGGCTTG
 351 ATTCACTAAG TAACCTCCCA TTAAAGGTTG AGAGGTCTTT CATATGCAAT
 401 GATACTGGGG CTCTACTTCA GGATGCACAG ACACGTTTCC AGATGTACAA
 451 TGACACAATT GTCAAATTTT CTGTACGTGA ACTCTCTGAG GTTAAAAGGG
 501 TTGCAAGCCA TCATCTTCGC CTTATAGGTT TCAAGCCATT GGATTGCTTG
 551 AAAGATTACC ATAACTTAAG ACCATCGACA TTTATTTATC CGAGTGATGA
 601 GCGTATATTT GGAAGCACCT GTGTTTTCGT TGCTTTACAT AGCTCAATGT
 651 TACGTCTTGG AAGGTTTGCA CTTGCATTTT ATGGGAATCC AACTCGACCA
 701 CAACTCATAG CCCTTGTTGC TCAAGAAGAG GTTACTTCGT CTGGTCGTCA
 751 GTTTGAACCG CCTGGCATGC ACATGATCTA TCTTCCATAC TCCGATGATA
 801 TTAGATATCC TGAGGAAGTT CATGTGACTT CTGATGATGC ACCGCGTGCA
 851 ACAGATGAAC AAATCAAGAA AGCTTCGAAT ATATTCAAAC GTATTGATCT
 901 GATAAATTTC TCTGCATGCC AATTTGCTAA CCCAGCTTTG CAAAGACACT
 951 ATGGGATCTT GGAGGCCTTA GCTTTAGGCG AAGATGAGAT GCCTGATATA
1001 AAGGACGAGA CCCTGCCTGA CGAAGAAGGC TTGTCTAAGC CAGGGGTAGC
1051 CAATGCTATT GAGGAATTCA AGACTTCAGT CTATGGTGAA AATTATGACC
1101 AAGAGGAGGC AGAAGCGGCA GCAGGGAAAG CTTCCCGTGG TAATGCTTCA
1151 AAAAAGCGGA AGGAGGTCAC TGATGCAGCT GCGCAGATAA GTGCTGCTTA
1201 TGACTGGGCA GAACTTGCAG ACAATGGAAA ACTGAAGGAA ATGACCACGG
1251 TGGAATTGAG ATCCTACCTG ACCGCGCATG ATCTCCCGGT TTCTGGTAAG
1301 AAAGAGGTAC TTATCAGCAG GATCTTGACT CACCTGGGTA AGTGAAGCCG
1351 GGTATCCGAA CTGTTAGTTT CCTGAGACCC GACTGACATC TGTAGTGCTG
1401 TCTCACGGAT CCTTGTGGAC CGTAATCCTC CCCCGTGCAG GATACGTGCC
1451 GAATTTTTGT ATAGCGACAG GACAGCACCA GTTGCCAATG TAACCTGTGT
1501 TAAGTTTCGT CTTGTGGTAT CATCTGCTTG TCATTGTCAA CATGAAACAT
1551 AATGTAATTC TGGATACTTA GACCCACAAT ATGGAGCATA ACTGCATAAG
1601 GTATGTT
```

Figure 9 Full-length nucleotide sequences of ZM1 with 1607 bp.

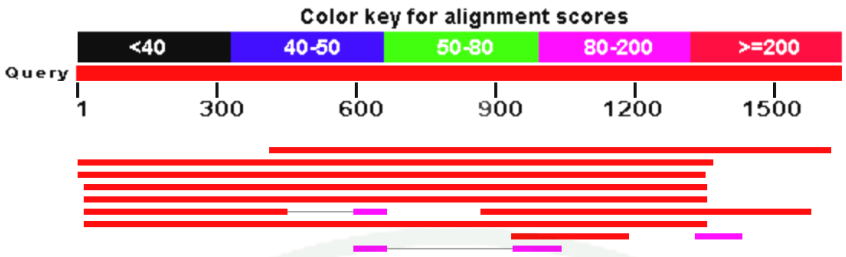
The full-length sequence of ZM1 was BLASTN with NCBI and DDBJ databases. The result is shown in Figure 10 and Appendix Table 6 and 7.

From the BLASTN resulting in NCBI (Figure 10A and Appendix Table 6) of ZM1 shows high similarity to *Zea mays* hypothetical protein (LOC100381505), mRNA, the accession number is NM_001174336.1 (99% identities). The *Sorghum bicolor* hypothetical protein, mRNA showed similarity in 2 accession numbers, XM_002461257.1 (95% identities) and XM_002459401.1 (95% identities). The accession numbers NM_001065596.1 (85% identities) is *Oryza sativa Japonica* Group Os07g0184900 (Os07g0184900) mRNA, complete cds and AK102066.1 (85% identities) is *Oryza sativa Japonica* Group cDNA clone: J033082F01, full insert sequence, followed by *Zea mays* hypothetical protein mRNA, the accession numbers is EU950919.1 (99% identities) and *Oryza sativa Japonica* Group cDNA clone:J013129E14, full inserted sequence, accession number is AK099980.1 (84% identities).

Then the ZM1 sequences were BLASTN in DDBJ (Figure 10B and Appendix Table 7) the result shows high similarity to *Zea mays* cDNA, mRNA sequence. The 19 accession numbers is DV025081, DR959368, CA403179, DR827788, DV621938, DN205349, CF628530, EB813067, FL123854, CF625524, DN214358, DN233109, BG874047, DR792592, CF040139, DR792591, FL442295, CF036521, BU093734 and *Sorghum bicolor* cDNA clone POL1_10_G12_A002 5', mRNA sequence accession number is CF483041 with all 20 accession number identities between 95-100 (%).

From the BLASTN result both from NCBI and DDBJ database, the 1607bp nucleotide sequence shows high similarity to nucleotide sequence of *Zea mays.*

A



B

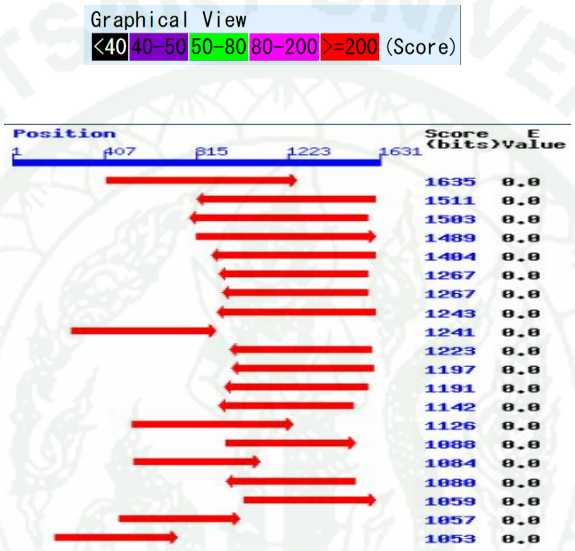


Figure 10 Graphical view of nucleotide sequence alignment of ZM1 full-length (NR1) by BLASTN (2.2.24) program from NCBI (A) and DDBJ database (B).

The prediction of ZM1 sequence by analysis of open reading frame (ORF) in NCBI (Figure 11 and Appendix Figure 11) shows that the ORF contains only one possible open reading frame 1290 bp sequence of base at frame +2 from the position 56 (start codon, ATG) to 1345 (stop codon, TGA), encoding 429 amino acids.

By using GeneMark program for predict sequence of eukaryote (Figure 12), the results showed 44.43% G+C content, matrices in rice and translation to protein 429 amino acids, strand plus, predict length of the exon gene is 1290 bp and it ranges from 56 to 1345. This shows the similar result of NCBI database.

The Softberry program was used for gene finder to predict possible part of the gene. The results were shown in Figure 13. The predicted 1,281 bp gene sequence is found in monocot genomic DNA, the positions of predicted gene is exon 1, starting from 56 to 1337, score 61.89, chain plus, predicted protein mRNA, encoding 427 amino acids.

The prediction of ZM1 full-length from ORF finder and GeneMark showed similar results to 429 amino acids and to 427 amino acids from Softberry. These 3 programs predicted gene exon by strand plus in eukaryote, monocot and protein mRNA but the gene function is not known. This long ORF is a good indication of the present of a gene in the sequence.

The BLASTP form NCBI was used to obtained function and homology search to find gene and seek homologous proteins (Table 5 and Appendix Figure 12). Result the ZM1 full-length (1607 bp) shows high similarity to hypothetical protein SORBIDRAFT_02g004740 [*Sorghum bicolor*], the accession number is XP 002459446.1 (92% identities) and hypothetical protein SORBIDRAFT_02g00480 [*Sorghum bicolor*] the accession number is XP 002461302.1 (91% identities). Followed by Os07g0184900 [*Oryza sativa Japonica* Group], the accession number is NP 001059061.1 (82% identities).

BLAST tree view and graphic summary view of conserved domains (Figure 14A and Figure 14B) were shown as the query sequence providing specific hits with KU70 which it is Ku domain protein Pku70. The result of distance tree view was shown unknown sequence similar to three-leaf-monocots, hypothetical protein LOC100381505 [*Zea mays*] and four-leaf-monocots. The results of BLASTP and ORF could determined the position DNA query sequences (ZM1) to fine gene and seek homologous proteins for future study in gene expression.

The comparision of amino acid sequences ZM1 and all 11 accession numbers from NCBI database (Table 5) were selected for analysing phylogenetic tree with multiple sequence alignment clustalW2 program (Figure 14C and Appendix Figure 13). The result distance of ZM1 sequence shows high similarity to hypothetical protein LOC100381505 [Zea mays] Ku-core domain Ku70 (NP_001167807.1, 99%), hypothetical protein SORBIDRAFT 02g004740 Sorghum bicolor ATP-dependent DNA helicase ii 70 kDa subunit (XP_002459446.1, 90 scores), hypothetical protein SORBIDRAFT 02g000480 [Sorghum bicolor] ATP-dependent DNA helicase ii 70 kDa subunit (XP_002461302.1, 89 scores) and Os07g0184900 [Oryza sativa Japonica Group]ATP-dependent DNA helicase 2 subunit KU70 (NP_001059061.1, 81 scores) respectively.

From this studies the ZM1 is highly similar to Ku70 protein which is Ku protein conserve (Table 5 and Figure14). The characterization and function of Ku70 protein have been demonstrated that it plays important role in the repair of DNA DSBs (double-strand breaks) and in telomere length regulation in *Arabidopsis*, a dicot model plant (Bundock *et al*., 2002; Tamura *et al*., 2002; Downs and Jackson, 2004).

AtKU70 plant was found telomeres significantly longer (>30 kb) than in wild type plants (2-4 kb) (Bundock *et al*., 2002). *VrKu* protein of mung bean function in DNA repair independently of DNA-PK complex (Liu *et al*., 2007). Function of Ku70 in monocot, *OsKu70* is required for the maintenance of chromosomal stability and normal development in rice (*Oryza sativa*). Hong *et al*. (2010) reported that

telomeres in homozygous G2 *osku70* mutant were markedly longer (10-20 kb) than those wild type plants (5-10 kb).

ZM1 was obtained specifically in shoot but not in root and leaf tissues, and (Figure 4). Indicating that the expression of plant Ku70 was tissue specific gene in different species and could be regulated by several distinct way. *VrKu70* and *VrKu80* of mung bean (*Vigna radiate* L.) were expressed the highest levels in hypocotyls and leaves of mung bean (Liu *et al*., 2007). Rice plant *Osku70* was obtained from various organs at different developmental stages under normal conditions (Hong *et al*., 2010). ZM1 was obtained specificly in drought tolerant maize line (SW 2301). Ku70 was probably correlated with normal growth and development in SW 2301 maize line. Figure 4C shows decreasing ZM1 expression when exposed to water stress, ensured water stress affecting on growth and auxin inhibition. Auxin induces various development responses, such as imposing changes in cell, division cell expansion, cell differentiation and organ initiation (Woodward and Bartel, 2005). Implicit by studying of Liu *et al*. (2007) who found *VrKu* gene expression was stimulated by exogenous auxins.

DNA sequence amplified using VP14 primers which designed by using the VP14 gene from the NCBI database in this study was not NCED gene. It was proved to be a part of Ku70 gene and should be further clarified.

```
  56 atgcacaagatcttggcctgtcgtattgaacttcttccactgagc
     M  H  K  I  L  A  C  R  I  E  L  L  P  L  S
 101 ccgcctgatgaccagttcaacatgtccctgttttatgcagatttg
     P  P  D  D  Q  F  N  M  S  L  F  Y  A  D  L
 146 attggtctggatggagatgagatgaccgaatatttgccatctgct
     I  G  L  D  G  D  E  M  T  E  Y  L  P  S  A
 191 ggtgataagctagaggatatgactaatcaactgagaaaacggata
     G  D  K  L  E  D  M  T  N  Q  L  R  K  R  I
 236 atgaagaagcgcagagtcaaaactctttcatttgcgattaccaat
     M  K  K  R  R  V  K  T  L  S  F  A  I  T  N
 281 gatgtgtgcatagaagtcaatacatatgcgctggtccgtcctact
     D  V  C  I  E  V  N  T  Y  A  L  V  R  P  T
 326 actacagggacaatcacatggcttgattcactaagtaacctccca
     T  T  G  T  I  T  W  L  D  S  L  S  N  L  P
 371 ttaaaggttgagaggtctttcatatgcaatgatactggggctcta
     L  K  V  E  R  S  F  I  C  N  D  T  G  A  L
 416 cttcaggatgcacagacacgtttccagatgtacaatgacacaatt
     L  Q  D  A  Q  T  R  F  Q  M  Y  N  D  T  I
 461 gtcaaattttctgtacgtgaactctctgaggttaaaagggttgca
     V  K  F  S  V  R  E  L  S  E  V  K  R  V  A
 506 agccatcatcttcgccttataggtttcaagccattggattgcttg
     S  H  H  L  R  L  I  G  F  K  P  L  D  C  L
 551 aaagattaccataacttaagaccatcgacatttatttatccgagt
     K  D  Y  H  N  L  R  P  S  T  F  I  Y  P  S
 596 gatgagcgtatatttggaagcacctgtgttttcgttgctttacat
     D  E  R  I  F  G  S  T  C  V  F  V  A  L  H
 641 agctcaatgttacgtcttggaaggtttgcacttgcattttatggg
     S  S  M  L  R  L  G  R  F  A  L  A  F  Y  G
 686 aatccaactcgaccacaactcatagcccttgttgctcaagaagag
     N  P  T  R  P  Q  L  I  A  L  V  A  Q  E  E
 731 gttacttcgtctggtcgtcagtttgaaccgcctggcatgcacatg
     V  T  S  S  G  R  Q  F  E  P  P  G  M  H  M
 776 atctatcttccatactccgatgatattagatatcctgaggaagtt
     I  Y  L  P  Y  S  D  D  I  R  Y  P  E  E  V
 821 catgtgacttctgatgatgcaccgcgtgcaacagatgaacaaatc
     H  V  T  S  D  D  A  P  R  A  T  D  E  Q  I
 866 aagaaagcttcgaatatattcaaacgtattgatctgataaatttc
     K  K  A  S  N  I  F  K  R  I  D  L  I  N  F
 911 tctgcatgccaatttgctaacccagctttgcaaagacactatggg
     S  A  C  Q  F  A  N  P  A  L  Q  R  H  Y  G
 956 atcttggaggccttagctttaggcgaagatgagatgcctgatata

     I  L  E  A  L  A  L  G  E  D  E  M  P  D  I
1001 aaggacgagaccctgcctgacgaagaaggcttgtctaagccaggg
     K  D  E  T  L  P  D  E  E  G  L  S  K  P  G
1046 gtagccaatgctattgaggaattcaagacttcagtctatggtgaa
     V  A  N  A  I  E  E  F  K  T  S  V  Y  G  E
1091 aattatgaccaagaggaggcagaagcggcagcagggaaagcttcc
     N  Y  D  Q  E  E  A  E  A  A  A  G  K  A  S
1136 cgtggtaatgcttcaaaaaagcggaaggaggtcactgatgcagct
     R  G  N  A  S  K  K  R  K  E  V  T  D  A  A
1181 gcgcagataagtgctgcttatgactgggcagaacttgcagacaat
     A  Q  I  S  A  A  Y  D  W  A  E  L  A  D  N
1226 ggaaaactgaaggaaatgaccacggtggaattgagatcctacctg
     G  K  L  K  E  M  T  T  V  E  L  R  S  Y  L
1271 accgcgcatgatctcccggtttctggtaagaaagaggtacttatc
     T  A  H  D  L  P  V  S  G  K  K  E  V  L  I
1316 agcaggatcttgactcacctgggtaagtga 1345
     S  R  I  L  T  H  L  G  K  *
```

Figure 11 Nucleotide and deduced amino acid sequence of ZM1, start codon (ATG) and stop codon (TGA) are underlined (length 429 aa).

```
Eukariotyc GeneMark.hmm version bp 3.9 April 25, 2008
Sequence name: Wed Jun  9 06:59:06 EDT 2010
Sequence length: 1607 bp
G+C content: 44.43%
Matrices file: /home/genmark/euk_ghm.matrices/rice_hmm3.0mod
Wed Jun  9 06:59:06 2010

Predicted genes/exons

Gene  Exon  Strand  Exon          Exon Range          Exon       Start/End
  #     #           Type                            Length       Frame


  1    1    +   Single              56       1345       1290          1 3 - -

>gene_1|GeneMark.hmm|429_aa
MHKILACRIELLPLSPPDDQFNMSLFYADLIGLDGDEMTEYLPSAGDKLEDMTNQLRKRI
MKKRRVKTLSFAITNDVCIEVNTYALVRPTTTGTITWLDSLSNLPLKVERSFICNDTGAL
LQDAQTRFQMYNDTIVKFSVRELSEVKRVASHHLRLIGFKPLDCLKDYHNLRPSTFIYPS
DERIFGSTCVFVALHSSMLRLGRFALAFYGNPTRPQLIALVAQEEVTSSGRQFEPPGMHM
IYLPYSDDIRYPEEVHVTSDDAPRATDEQIKKASNIFKRIDLINFSACQFANPALQRHYG
ILEALALGEDEMPDIKDETLPDEEGLSKPGVANAIEEFKTSVYGENYDQEEAEAAAGKAS
RGNASKKRKEVTDAAAQISAAYDWAELADNGKLKEMTTVELRSYLTAHDLPVSGKKEVLI
SRILTHLGK
```

Figure 12 Predicted gene result of ZM1 using GeneMark.hmm Protein Translations program from http://opal.biology.gatech.edu/GeneMark/

```
FGENESH 2.6 Prediction of potential genes in Monocot genomic DNA
Time     :   Wed Jun  9 07:30:31 2010
Seq name:  test sequence
Length of sequence: 1607
Number of predicted genes 1: in +chain 1, in -chain 0.
Number of predicted exons 1: in +chain 1, in -chain 0.
Positions of predicted genes and exons: Variant   1 from   1, Score:45.022882
  G Str    Feature    Start         End     Score           ORF            Len

  1 +     1 CDSf         56 -       1337   61.89         56 -       1336   1281


Predicted protein(s):
>FGENESH:[mRNA]   1   1 exon (s)     56  -   1337  1281 bp, chain +
ATGCACAAGATCTTGGCCTGTCGTATTGAACTTCTTCCACTGAGCCCGCCTGATGACCAG
TTCAACATGTCCCTGTTTTATGCAGATTTGATTGGTCTGGATGGAGATGAGATGACCGAA
TATTTGCCATCTGCTGGTGATAAGCTAGAGGATATGACTAATCAACTGAGAAAACGGATA
ATGAAGAAGCGCAGAGTCAAAACTCTTTCATTTGCGATTACCAATGATGTGTGCATAGAA
GTCAATACATATGCGCTGGTCCGTCCTACTACTACAGGGACAATCACATGGCTTGATTCA
CTAAGTAACCTCCCATTAAAGGTTGAGAGGTCTTTCATATGCAATGATACTGGGGCTCTA
CTTCAGGATGCACAGACACGTTTCCAGATGTACAATGACACAATTGTCAAATTTTCTGTA
CGTGAACTCTCTGAGGTTAAAAGGGTTGCAAGCCATCATCTTCGCCTTATAGGTTTCAAG
CCATTGGATTGCTTGAAAGATTACCATAACTTAAGACCATCGACATTTATTTATCCGAGT
GATGAGCGTATATTTGGAAGCACCTGTGTTTTCGTTGCTTTACATAGCTCAATGTTACGT
CTTGGAAGGTTTGCACTTGCATTTTATGGGAATCCAACTCGACCACAACTCATAGCCCTT
GTTGCTCAAGAAGAGGTTACTTCGTCTGGTCGTCAGTTTGAACCGCCTGGCATGCACATG
ATCTATCTTCCATACTCCGATGATATTAGATATCCTGAGGAAGTTCATGTGACTTCTGAT
GATGCACCGCGTGCAACAGATGAACAAATCAAGAAAGCTTCGAATATATTCAAACGTATT
GATCTGATAAATTTCTCTGCATGCCAATTTGCTAACCCAGCTTTGCAAAGACACTATGGG
ATCTTGGAGGCCTTAGCTTTAGGCGAAGATGAGATGCCTGATATAAAGGACGAGACCCTG
CCTGACGAAGAAGGCTTGTCTAAGCCAGGGGTAGCCAATGCTATTGAGGAATTCAAGACT
TCAGTCTATGGTGAAAATTATGACCAAGAGGAGGCAGAAGCGGCAGCAGGGAAAGCTTCC
CGTGGTAATGCTTCAAAAAAGCGGAAGGAGGTCACTGATGCAGCTGCGCAGATAAGTGCT
GCTTATGACTGGGCAGAACTTGCAGACAATGGAAAACTGAAGGAAATGACCACGGTGGAA
TTGAGATCCTACCTGACCGCGCATGATCTCCCGGTTTCTGGTAAGAAAGAGGTACTTATC
AGCAGGATCTTGACTCACCTG

>FGENESH:   1   1 exon (s)     56  -   1337   427 aa, chain +
MHKILACRIELLPLSPPDDQFNMSLFYADLIGLDGDEMTEYLPSAGDKLEDMTNQLRKRI
MKKRRVKTLSFAITNDVCIEVNTYALVRPTTTGTITWLDSLSNLPLKVERSFICNDTGAL
LQDAQTRFQMYNDTIVKFSVRELSEVKRVASHHLRLIGFKPLDCLKDYHNLRPSTFIYPS
DERIFGSTCVFVALHSSMLRLGRFALAFYGNPTRPQLIALVAQEEVTSSGRQFEPPGMHM
IYLPYSDDIRYPEEVHVTSDDAPRATDEQIKKASNIFKRIDLINFSACQFANPALQRHYG
ILEALALGEDEMPDIKDETLPDEEGLSKPGVANAIEEFKTSVYGENYDQEEAEAAAGKAS
RGNASKKRKEVTDAAAQISAAYDWAELADNGKLKEMTTVELRSYLTAHDLPVSGKKEVLI
SRILTHL
```

Figure 13 Predicted gene result of ZM1 using Softberry program from http://www.softberry.com/berry.phtml?topic=gfindb

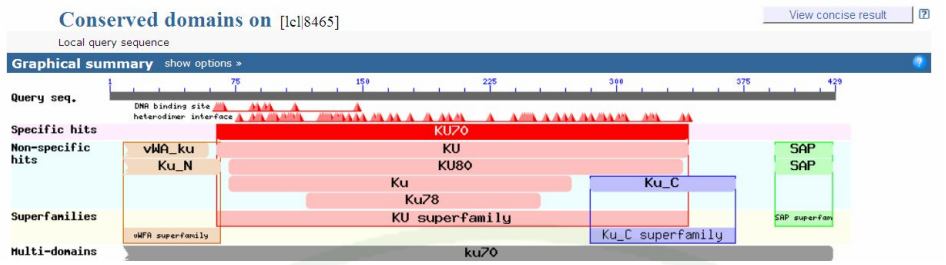
Table 5 Sequences alignments of ZM1 full-length by using BLASTP (2.2.24) program from NCBI database.

Accession number	Description	Identities Nt (%)	Positives (%)	Score (bits)	E value
XP_002459446.1	hypothetical protein SORBIDRAFT_02g004740 [*Sorghum bicolor*])	387/425 (92)	404/425 (96)	799	0.0
XP_002461302.1	hypothetical protein SORBIDRAFT_02g000480 [*Sorghum bicolor*]	385/424 (91)	401/424 (95)	791	0.0
NP_001059061.1	Os07g0184900 [*Oryza sativa Japonica* Group]	348/425 (82)	389/425 (92)	685	0.0
NP_001167807.1	hypothetical protein LOC100381505 [*Zea mays*]	299/300 (99)	300/300 (100)	622	3e-176
XP_002267875.1	PREDICTED: hypothetical protein [*Vitis vinifera*]	284/426 (67)	350/426 (83)	603	2e-170
BAF03493.1	Ku70 homolog [*Populus nigra*]	278/426 (66)	348/426 (82)	593	2e-167
XP_002317447.1	predicted protein [*Populus trichocarpa*]	276/427 (65)	347/427 (82)	580	9e-164
XP_002521532.1	ku P70 DNA helicase, putative [*Ricinus communis*]	263/426 (62)	331/426 (78)	560	2e-15
AAT48365.1	Ku70-like protein [*Vigna radiata*]	251/426 (59)	328/426 (77)	537	1e-150
ABR16802.1	unknown [*Picea sitchensis*]	255/426 (60)	341/426 (81)	535	4e-150

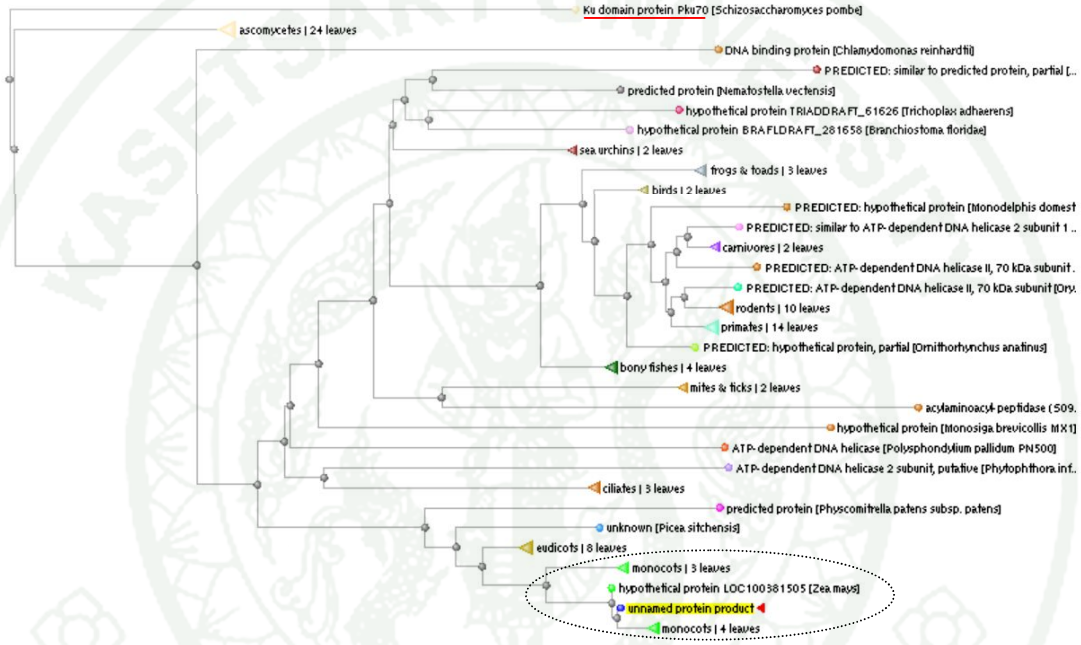
Table 5 (Continued)

Accession number	Description	Identities Nt (%)	Positives (%)	Score (bits)	E value
NP_564012.1	KU70 (ARABIDOPSIS THALIANA KU70 HOMOLOG); double-stranded DNA binding / protein binding [Arabidopsis thaliana]	249/426 (59)	328/426 (77)	523	1e-146
XP_002892931.1	hypothetical protein ARALYDRAFT_471898 [Arabidopsis lyrata subsp. lyrata]	244/427 (58)	327/427 (77)	513	3e-143

A



B



C

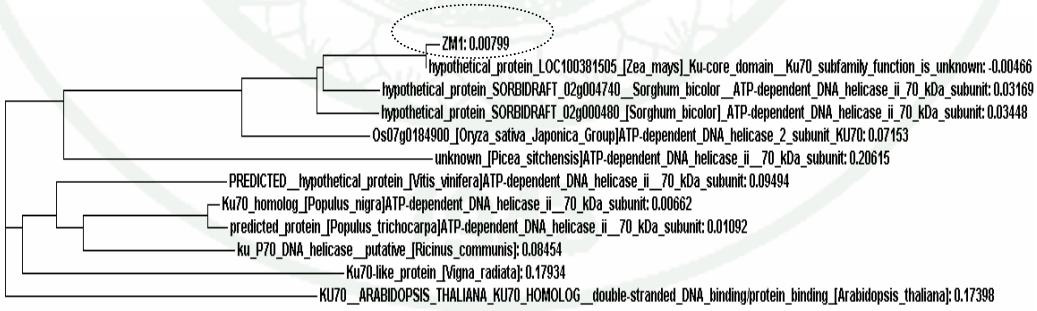


Figure 14 Graphic summary view of conserved domains (A) and distance tree view result in rectangle by BLAST pairwise alignments from NCBI database (B) and clustalW2 was used for alignment phylogram tree to show distance of ZM1 amino acid with NCBI database accession numbers in Table 5 (C).

CONCLUSION

The study of gene expression in two maize lines under drought condition can be summarized as follows:

1. The KSX 4605 (drought sensitive) and SW 2301 (drought tolerance) wilting symptom was observed in both two maize lines, showing a slightly wilting after 1 h of drought induction and showed moderate wilting after 4 h, severe wilting after 24 to 48 h after water stress in KSX 4605. While SW 2301 exhibited wilting symptom at 2 h after stress induction and showed wilting at 4 h. However, after 6 h SW 2301 was slightly recovered from wilting

2. According to RT-PCR technique, a 792 bp fragment was found in the shoot tissue of SW 2301, a drought tolerant line, with VP14 primer.

3. By RACE-PCR, the full-length sequence of 1,607 bp was obtained. The ORF of this sequence contains 1290 bp, starting from the position 56 (start codon, ATG) to 1345 (stop codon, TGA), encoding 429 amino acids. The amono acid sequence was predicted to be a part of Ku70 gene.

LITERATURE CITED

Abe, H., K. Yamaguchi-Shinosaki, T. Urao, T. Iwasaki, D. Hosokawa and K. Shinozaki. 1997. Role of *Arabidopsis* MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. **Plant Cell.** 9:1859-1863.

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Res**. 25: 3389-3402.

Alvin, F.C., S.M.B. Carolino, J.C.M. Cascardo, C.C. Nunes, C.A. Martinez, W.C. Otoni and E.P.B. Fontes. 2001. Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. **Plant Physiol.** 126: 1042-1054.

Andjelkovic, V. and R. Thompson. 2006. Changes in gene expression in maize kernel in response to water and salt stress. **Plant Cell Rep.** 25: 71-79.

Babu, R.C., J. Zhang, A. Blum, T.-H.D. Ho, R. Wu and H.T. Nguyen. 2004. *HVA1*, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protein. **Plant Sci.** 166: 855-862.

Behl, R. and W. Hartung. 1984. Transport and compartmentation of abscisic acid in root of Hordeum distichon under osmotic stress. **J. Exp. Bot.** 36: 1433-1440.

Bonetta, D. and P. McCourt. 1998. Genetic analysis of ABA signal transduction pathways. **Trends Plant Sci.** 3(6): 231-235.

Borodovsky, M. and A. Lukashin. 2008. **Eukaryotic GeneMark.hmm version bp 3.9**. (Computer Program). http://opal.biology.gatech.edu/GeneMark/.

Bray, E.A. 1997. Plant responses to water deficit. **Trends Plant Sci.** 2: 48-54.

Bray, E.A. 2002. Abscisic acid regulation of gene expression during water deficit stress in the era of the *Arabidopsis* genome. **Plant Cell Environ.** 25: 153-161.

Brenner, C.A., A.W. Tam, P.A. Nelson, E.G. Engelman, N. Suzuki, K.E. Fry and J.W. Larrick. 1997. Isolation and construction of DNA clones, pp. 63-74. *In* C.R. Newton and A. Graham, 2nd eds. **PCR.** Bios. Scientific Publishers Limited, 1994, 1997, UK.

Bundock, P.H., H. van Attikum and P. Hooykaas. 2002. Increased telomere length and hypersensitivity to DNA-damaging agents in an *Arabidopsis KU70* mutant. **Nucleic Acids Res.** 30: 3395-3400.

Burbidge, A., T. Grieve, A. Jackson, A. Thompson and I. Taylor. 1997. Structure and expression of a cDNA encoding a putative neoxanthin cleavage enzyme (NCE), isolated from a wilt-related tomato (*Lycopersicon esculentum* Mill.) library. **J. Exp. Bot.** 47(317): 2111-2112.

Burbidge, A., T.M. Grieve, A. Jackson, A. Thompson, D.R. MaCarty and I.B. Taylor. 1999. Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14.* **Plant J.** 17(4): 427-431.

Cellier, F., G. Conejero, J-C. Breitler and F. Casse. 1998. Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower: Accumulation of dehydrin transcripts correlated with tolerance. **Plant Physiol.** 116: 319-328.

Chen, T.H.H. and N. Murata. 2002. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. **Plant Biol.** 5: 250-257.

Chernys, J.T. and J.A.D. Zeevaart. 2000. Characterization of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid Biosynthesis in Avocado. **Plant Physiol.** 124: 343-353.

Chomczynski P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. **Anal. Biochem.** 162(1): 156-159.

Chutkaew C., A. Saimaneerat, C. Ackatasanawan, S. Jampatong and Y. Petratanagool. 1988. Selection for drought resistance in corn, pp. 201-209. *In* **The Proceeding of 26th Kasetsart University Annual Conference 1988**. Kasetsart University, Bangkok.

Claassen, M.M. and R.H. Shaw. 1970. Water deficit effects on corn: Ⅱ Grain components. **Agron. J.** 62: 652-655.

Cohen, A. and E.A. Bray. 1990. Characterization of three mRNAs that accumulate in wilted tomato leaves in response to elevated levels of endogenous abscisic acid. **Planta.** 182: 27-33.

Cornish, K. and J.A.D. Zeevaart. 1984. Abscisic acid metabolism in relation to water stress and leaf age in *Xanthium Strumarium*. **Plant Physiol.** 76: 1029-1035.

Cornish, K. and J.A.D. Zeevaart. 1985. Abscisic acid accumulation by root of *Xanthium strumarium* L. and *Lycopersicon esculentum* Mill. in relation to water stress. **Plant Physiol.** 79: 653-658.

Damptey, H.B., B.G. Coombe and D. Aspinall. 1978. Apical dominance, water deficit and axillary inflorescence growth in *Zea mays*: The role of abscisic Aacid. **Ann. Bot.** 42: 1447-1458.

Davies, W.J., T.A. Mansfield and A.M. Hetherington. 1990. Sensing of soil water status and the regulation of plant growth and development. **Plant Cell Environ.** 13: 709-719.

Denmead, O.T. and R.H. Shaws. 1960. The effects of soil moisture stress at different stages of growth on the development and yield of corn. **Agron. J.** 52: 272-274.

Downs, J.A. and S.P. Jackson. 2004. A means to a DNA end: the many roles of ku. **Nat. Rev., Mol. Cell Biol.** 5: 367-378.

Eckardt, N.A. 2001. Luc genetic screen illuminates Stress-responsive gene regulation. **Plant Cell.** 13: 1969-1972.

Guerrero, F. and J.E. Mullet. 1986. Increased abscisic acid biosynthesis during plant dehydration requires transcription. **Plant Physiol.** 80: 588-591.

Han, B., D.W. Hughes, G.A. Galau, J.D. Bewley and A.R. Kermode. 1997. Changes in late embryogenesis-abundant (LEA) messenger RNAs and dehydrins during maturation and premature during of *Ricinus Communis* L. seeds. **Planta.** 201: 27-35.

Hong, J.-P., M.Y. Byun, K. An, S.-J. Yang, G. An and W.T. Kim. 2010. Osku70 is associated with developmental growth and genome stability in rice. **Plant physiol.** 152: 374-387.

Hu, J-F., G-F. Li, Z-H. Gao, L. Chen, H-B. Ren and W.S. Jia. 2005. Regulation of water deficit-induced abscisic acid accumulation by apoplastic ascorbic acid in maize seedlings. **J. Integ Plant Biol.** 47(11): 1335-1344.

Hughes, D.W. and G.A. Galau. 1991. Developmental and environmental induction of *Lea* and *LeaA* mRNAs and the Postabscission program during embryo culture. **Plant Cell.** 3: 605-618.

Iuchi, S., M. Kobayashi, K. Yamaguchi-Shinozaki and K. Shinozaki. 2000. A stress-inducible gene for 9-*cis*-epoxycarotenoid dioxygenase involved in Abscisic acid biosynthesis under water stress in drought-tolerant cowpea. **Plant Physiol.** 123: 553-562.

Iuchi, S., M. Kobayashi, T. Taji, M. Naramoto, M. Seki, T. Kato, S. Tabata, Y. Kakubari, K. Yamaguchi-Shinozaki and K. Shinozaki. 2001. Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxy carotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. **Plant J.** 27: 325-333.

Jovanovic, Lj., R. Stikic and W. Hartung. 2000. Effect of osmotic stress on abscisic acid efflux and compartmentaion in the roots of two maize lines differing in drought susceptibility. **Biolog. Plantar.** 43(3): 407-411.

Kang, S., W. Shi and J. Zhang. 2000. An improved water-use efficiency for maize grown under regulated deficit irrigation. **Field Crops Res.** 67: 207-214.

Landi, P., M.C. Sanguineti, S. Conti and R. Tuberosa. 2001. Direct and correlated responses to divergent selection for leaf abscisic acid concentration in two maize population. **Crop Sci.** 41: 335-344.

Liu, P.-F., W.-C. Chang, Y.-K. Wang, S.-B. Munsamy, S.-H. Hsu, H.-Y. Chang, S.-H. Wu and R.-L. Pan. 2007. Differential regulation of gene expression in etiolated mung bean hypocotyls by auxins. **Biochim. Biophys. Acta**. 1769: 443-454.

Lomsadze, A., V. Ter-Hovhannisyan, Y. Chernoff and M. Borodovsky. 2005. Gene identification in novel eukaryotic genomes by self-training algorithm. **Nucleic Acids Res.** 33 (20): 6494-6506.

Lorkowski, S. and P. Cullen. 2003. **Analysing Gene Expression Vol.1**: A Handbook of Method: Possibilities and Pitfalls. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Lu, B., Z. Gong, J. Wang, J. Zhang and J. Liang. 2007. Microtubule dynamics in relation to osmotic stress-induced ABA accumulation in *Zea mays* roots. **J. Exp. Bot.** 58(10): 2565-2572.

Melhorn, V., K. Matsumi, H. Koiwai, K. Ikegami, M. Okamoto, E. Nambara, F. Bittner and T. Koshiba. 2008. Transient expression of *AtNCED3* and *AAO3* gene in guard cells causes stomatal closure in *Vicia faba*. **J. Plant Res.** 121: 125-131.

Mohammadkhani, N. and R. Heidari. 2007. Effect of drought stress on soluble protein in two maize varieties. **Turk J. Biol.** 32:23-30.

Munns, R. and R.W. King. 1988. Abscisic acid is not the only stomatal inhibitory in the transpiration stream of wheat plants. **Plant Physiol.** 88: 703-708.

Myers, P.N., T.L. Setter, J.T. Madison and J.F. Thompson. 1990. Abscisic acid inhibition of endosperm cell division in cultured maize kernels. **Plant Physiol.** 94: 1330-1336.

Naidu, B.P. 1998. Separation of sugars, polyols, proline analogues, and betaines in stressed plant extracts by high performance liquid chromatography and quantification by ultra violet detection. **Aust. J. Plant Physiol.** 25: 793-800.

Nakamura, T., M. Ishitani, P. Harinasut, M. Nomura, T. Takabe and Tessuko Takabe. 1996. Distribution of glycinebetaine in old and young leaf blades of salt-stressed barley plants. **Plant Cell Physiol.** 37(6): 873-877.

Neill, S.J., E.C. Burnett, R. Desikan and J.T. Hancock. 1998. Cloning of wilt-responsive cDNA from an *Arabidopsis thaliana* suspension culture cDNA library that encodes a putative 9-*cis*-epoxy-carotenoid dioxygenase. **J. Exp. Bot.** 49(328): 1893-1894.

Oono, Y., M. Seki, T. Nanjo, M. Narusaka, M. Fujita, R. Satou, T. sakura, J. Ishida, K. Akiyama, K. Iida, K. Maruyama, S. Satoh, K. Yamaguchi-Shinosaki and K. Shinosaki. 2003. Monitoring expression profiles of *Arabidopsis* gene expression during rehydration process after dehydration using *ca* 7000 full-length cDNA microsrray. **Plant J.** 34: 868-887.

Oraby, H.F., C.B. Ransom, A.N. Kravchenko and M.B. Sticklen. 2005. Barley *HVA1* gene confers-salt tolerance in R3 transgenic oat. **Crop Sci.** 45: 2218-2227.

Otegui, M.E., F.H. Andrade and E.E. Suero. 1995. Growth, water use, and kernel abortion of maize subjected to drought at silking. **Field Crops Res.** 40: 87-94.

Qin, X. and J.A.D. Zeevaart. 1999. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. **Proc. Natl. Acad. Sci. USA** 96: 15354-15361.

Qing, D-J., H-F. Lu, H-T. Dong, D-F. Dong and T-Z. Li. 2009. Comparative profiles of gene expression in leaves and roots of maize seedling under conditions of salt stress and the removal of salt stress. **Plant Cell Physiol.** 50(4): 889-903.

Ren, H., Z. Gao, L. Chen, K. Wei, J. Liu, Y. Fan, W.J. Davies, W. Jia and J. Zhang. 2007. Dynamic analysis of ABA accumulation in relation to the rate of ABA catabolism in maize tissues under water deficit. **J. Exp. Bot.** 58(2): 211-219.

Richert, J., E. Kranz, H. Lorz and T. Dresselhaus. 1996. A reverse transcriptase-polymerase chain reaction assay for gene expression studies at the single cell level. **Plant Sci.** 114: 93-99.

Ried, J.L. and M.K. Walker-Simmons. 1993. Group 3 late embryogenesis abundant proteins in desication-tolerant seedlings of wheat (*Triticum aestivum* L.). **Plant Physiol.** 102: 125-131.

Rock, C.D. and P.P.F. NG. 1999. Dominant *Wilty* mutants of *Zea mays* (Poaceae) are not impaired in abscisic acid perception or metabolism. **Amer. J. Bot.** 86(12): 1796-1800.

Saab, I.N., R.E. Sherp, J. Prichard and G.S. Voetberg. 1990. Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. **Plant Physiol.** 93: 1329-1336.

Sambrook, J. and D.W. Russell. 2001. **Molecular Cloning: A Laboratory Manual**. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sauter, A., W.J. Davies and W. Hartung. 2001. The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. **J. Exp. Bot.** 52(363): 1991-1997.

Schraut, D., C.I. Ullrich and W. Hartung. 2004. Lateral ABA treansport in maize roots (*Zea mays*): Visualization by immunolocalization. **J. Exp. Bot.** 55(403): 1635-1641.

Schwartz, S.H., B.C. Tan, D.A. Gage, J.A.D. Zeevaart and D.R. McCarty. 1997. Specific oxidative cleavage of carotenoids by VP14 of maize. **Sci.** 276: 1872-1874.

Seo, M., H. Koiwai, S. Akaba, T. Komano, T. Oritani, Y. Kamiya and T. Koshiba. 2000. Anscisic aldehyde oxidase in leaves of *Arabidopsis thaliana*. **Plant J.** 23: 481-488.

Shinosaki, K. and K. Yamaguchi-Shinosaki. 1997. Gene expression and signal transduction in water-stress response. **Plant Physiol.** 115: 327-334.

Sridha, S. and K. Wu. 2006. Identification of AtHD2C as a novel regulator of abscisic acid responses in *Arabidopsis*. **Plant J.** 46: 124-133.

Tamura, K., Y. Adachi, K. Chiba, K. Oguchi and H. Takahashi. 2002. Identification of Ku70 and Ku80 homologues in *Arabidopsis thaliana*: evidence for a role in the repair of DNA double-strand breaks. **Plant J.** 29: 771-781.

Tan, B-C., L.M. Joseph, W-T. Deng, L. Liu, Q-B. Li, K. Cline and D.R. McCarty. 2003. Molecular characterization of the *Arabidopsis* 9-*cis* epoxycarotenoid dioxygenase gene family. **Plant J.** 35: 44-56.

Tan, B-C., S.H. Schwartz, J.A.D. Zeevaart and D.R. McCarty. 1997. Genetic control of abscisic acid biosynthesis in maize. **Proc. Natl. Acad. Sci. USA** 94: 12235-12240.

Thompson, A.J., A.C. Jackson, R.A. Parker, D.R. Morpeth, A. Burbige and I.B. Taylor. 2000. Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxydase and 9-*cis*-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. **Plant Mol. Biol.** 42: 833-845.

Todorov, D., V. Alexieva and E. Karanov. 1998. Effect of putrescine 4-PU-30, and abscisic acid on maize plants grown under normal, drought, and rewatering conditions. **J. Plant Growth Reg.** 17: 197-203.

Trevino, M.B. and M.A. O Connell. 1998. Three drought-responsive members of the nonspecific lipid-transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression. **Plant Physiol.** 116: 1461-1468.

Udomprasert, N., J. Kijjanon, R. Thiraporn and A. Machuay. 1999. Effects of water deficit at tasseling on proline and abscisic acid levels and yield of corn. **Kasetsart J. (Nat. Sci.).** 33:310-316.

Voetberg, G.S. and R.E. Sharp. 1991. Growth of the maize primary root at low water potentials: Ⅲ Role of increased proline deposition in osmotic adjustment. **Plant Physiol.** 96: 1125-1130.

Wan, X-R. and L. Li. 2006. Regulation of ABA level and water-stress tolerance of *Arabidopsis* by ectopic expression of a peanut 9-*cis*-epoxycarotenoid dioxygenase gene. **Biochem. Biophys. Res. Commun.** 347: 1030-1038.

Westgate, M. E. and D.L. Thomson Grant. 1989. Water deficits and reproduction in maize: response of the reproductive tissue to water deficits at anthesis and mid-grain fill. **Plant Physiol.** 91:862-867.

Westgate, M.E. and J.S. Boyer. 1985. Osmotic adjustment and the inhibition of leaf, root, stem and silk growth at low water potentials in maize. **Planta.** 164:540-549.

Woodward, A.W. and B. Bartel. 2005. Auxin: regulation, action and interaction. **Ann. Bot.** 95: 707-735.

Xing, W. and C.B. Rajashekar. 2001. Glycine betaine involvement in freezing tolerance and water stress in *Arabidopsis thaliana*. **Environ. Exp. Bot.** 46: 21-28.

Xiong, L. and J.K. Zhu. 2002. Molecular and genetic aspects of plant responses to osmotic stress. **Plant Cell Environ.** 25: 131-139.

Xu, D., X. Duan, B. Wang, B. Hong, T-H.D. Ho and R. Wu. 1996. Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. **Plant Physiol.** 110: 249-257.

Zeevart, J.A.D. and G.L. Boyer. 1984. Accumulation and transport of abscisic acid and its metabolites in *Ricinus* and *Xanthium*. **Plant Physiol.** 74: 934-939.

Zhang, J. and W.J. Davies. 1991. Antitranspirant activity in xylem sap of maize plants. **J. Exp. Bot.** 42: 317-321.

Zhang, J. and W.J. Davies. 1989. Abscisic acid produced in dehydrating roots may enable the plant to measure the water status of the soil. **Plant Cell Environ.** 12: 73-81.

Zhang, Z., S. Schwartz, L. Wagner and W. Miller. 2000. A greedy algorithm for aligning DNA sequences. **J Comput Biol.** 7(1-2): 203-214.

Zheng, J., J. Zhao, J. Zhang, J. Fu, M. Fu, M. Gou, Z. Dong, W. Hou, Q. Huang and G. Wang. 2006. Comparative expression profiles of maize genes from water stress-specific cDNA macroarray in response to high-salinity, cold or abscisic acid. **Plant Sci.** 170: 1125-1132.

APPENDIX

Appendix Table 1 Sequence alignment of NR1 (792 bp) by using BLASTN 2.2.24 program NCBI database.

Accession number	Description	Strand	Identities Nt (%)	Score (bits)	E value
XM_002461257.1	*Sorghum bicolor* hypothetical protein, mRNA	Plus/Plus	733/778 (95)	1199	0.0
XM_002459401.1	*Sorghum bicolor* hypothetical protein, mRNA	Plus/Plus	737/785 (94)	1194	0.0
NM_001174336.1	*Zea mays* hypothetical protein LOC100381505 (LOC100381505), mRNA	Plus/Plus	455/469 (98)	804	0.0
NM_001065596.1	*Oryza sativa Japonica* Group Os07g0184900 (Os07g0184900) mRNA, complete cds	Plus/Plus	666/787 (85)	782	0.0
AK102066.1	*Oryza sativa Japonica* Group cDNA clone:J033082F01, full insert sequence	Plus/Plus	666/787 (85)	782	0.0
AK099980.1	*Oryza sativa Japonica* Group cDNA clone:J013129E14, full insert sequence	Plus/Plus	668/804 (84)	715	0.0
AK102995.1	*Oryza sativa Japonica* Group cDNA clone:J033116E13, full insert sequence	Plus/Plus	64/72 (89)	86.1	3e-13
AP003861.2	*Oryza sativa Japonic*a Group genomic DNA, chromosome 7, BAC clone:OJ1046_F10	Plus/Plus	64/72 (89)	86.1	3e-13

Appendix Table 2 Sequence alignment of NR1 (792bp) by using BLASTN 2.2.24 program DDBJ database.

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
FL123854	FL123854.1 3500101 CERES-264 *Zea mays* cDNA clone 689879 5', mRNA sequence.	Plus/Plus	584/594 (98)	1116	0.0
BU093734	BU093734.1 1091048F01.y1 1091 - Immature ear with common ESTs screened by Schmidt lab *Zea mays* cDNA, mRNA sequence.	Plus/Plus	528/531 (99)	1033	0.0
DV025081	DV025081.1 ZM BFb0145I11.r ZM_BFb *Zea mays* cDNA 5', mRNA sequence.	Plus/Plus	437/447 (97)	825	0.0
BM500851	BM500851.1 PAC000000000890 Pioneer AF-1 array *Zea mays* cDNA, mRNA sequence.	Plus/Plus	419/424 (98)	793	0.0
CF036521	CF036521.1 QCG32g05.yg QCG *Zea mays* cDNA clone QCG32g05, mRNA sequence.	Plus/Plus	380/390 (97)	710	0.0
CF040139	CF040139.1 QCH9d10.yg QCH *Zea mays* cDNA clone QCH9d10, mRNA sequence.	Plus/Plus	307/317 (96)	565	e-157
GO843410	GO843410.1 CLS CLiFpEfSpn 117a3 1 j11cLibkit5LD E06 LS CLiFpEfSPn plant *Festuca pratensis* cDNA clone 117j11 5', mRNA sequence.	Plus/Plus	564/665 (84)	535	e-148

Appendix Table 2 (Continued)

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
GT040099	GT040099.1 CLS cLiFproots 2a2 1 m18cLibkit5LD G09 CLS cLiFp roots plant Festuca arundinacea cDNA clone 2m18 5', mRNA sequence.	Plus/Plus	550/647 (85)	531	e-147
CD966734	CD966734.1 SEQ 139 GeneTag2 Zea mays cDNA, mRNA sequence.	Plus/Minus	271/273 (99)	525	e-145
GT044595	GT044595.1 CLS cLiFproots 56a1 1 a05cLibkit5LD A03 CLS cLiFp roots plant Festuca arundinacea cDNA clone 56a05 5', mRNA sequence.	Plus/Plus	533/627 (85)	515	e-142
GT819125	GT819125.1 CCYO5320.b1 CCYO Brachypodium distachyon flower+ flower drought Brachypodium distachyon cDNA clone CCYO5320 5', mRNA sequence.	Plus/Plus	561/665 (84)	502	e-138
CF483041	CF483041.1 POL1 10 G12.g1 A002 Pollen Sorghum bicolor cDNA clone POL1 10 G12 A002 5', mRNA sequence.	Plus/Plus	304/325 (93)	486	e-133
CK072814	CK072814.1 76976rsicem_6505.y1 Oryza sativa cv. PA64s panicle ferile cDNAlibrary Oryza sativa Indica Group cDNA 5', mRNA sequence.	Plus/Plus	470/558 (84)	416	e-113

Appendix Table 2 (Continued)

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
FL720173	FL720173.1 CCGB5265.b1 CCGB Panicum virgatum apex + stem (L) Panicum virgatum cDNA clone CCGB5265 5', mRNA sequence.	Plus/Plus	330/372 (88)	398	e-107
GR323920	GR323920.1 CCOS1460.b1 CCOS Avena barbata leaf, grown under high rainfall (H) Avena barbata cDNA clone CCOS1460 5', mRNA sequence.	Plus/Plus	418/494 (84)	394	e-106
CA221635	CA221635.1 SCSGFL4035C09.g FL4 Saccharum hybrid cultivar (mixed) cDNA clone SCSGFL4035C09 5', mRNA sequence.	Plus/Plus	281/307 (91)	392	e-105
		Plus/Plus	84/94 (89)	107	1e-19
FL747576	FL747576.1 CCGC2661.b1 CCGC Panicum virgatum early floral buds + reproductivetissue (H) Panicum virgatum cDNA clone CCGC2661 5', mRNA sequence.	Plus/Plus	293/329 (89)	367	8e-98
CD057063	CD057063.1 HO14A22S HO Hordeum vulgare cDNA clone HO14A22 5-PRIME, mRNA sequence.	Plus/Plus	470/563 (83)	367	8e-98
CK075489	CK075489.1 73660rsicem 7214.y1 Oryza sativa cv. PA64s panicle ferile cDNA library Oryza sativa Indica Group cDNA 5', mRNA sequence.	Plus/Minus	398/470 (84)	363	1e-96
CJ639731	CJ639731.1 Triticum aestivum cDNA clone whec17p04 5', Y.Ogihara unpublished cDNA library Wh EMC, mRNA sequence.	Plus/Plus	398/476 (83)	343	1e-90

Appendix Table 3 Sequences alignment of NR2 (595bp) by BLASTN 2.2.24 program from NCBI database.

Accession number	Description	Strand	Identities Nt (%)	Score (bits)	E value
AF546188.1	Contiguous genomic DNA sequence comprising the 19-kDa-zein gene family from *Zea mays*, complete sequence	Plus/Plus	499/558 (90)	710	0.0
CU302231.4	*S.lycopersicum* DNA sequence from clone LE_HBa-291F9, complete sequence	Plus/Plus	62/72 (87)	76.8	1e-10

Appendix Table 4 Sequences alignment of NR2 (595bp) by BLASTN 2.2.24 program from DDBJ database.

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
CD726282	CD726282.1 MK_1_9 *Pennisetum glaucum* seedlings exposed to drought stress *Pennisetum glaucum* cDNA clone MK 1 9, mRNA sequence.	Plus/Minus	61/68 (89)	79.8	2e-11
CD473890	CD473890.2 nad03-11ms1-f08 Nad03 *Nuphar advena* cDNA clone nad03-11ms1-f08 5',mRNA sequence.	Plus/Plus	60/69 (86)	65.9	3e-07
BI678863	BI678863.1 SWS768 SWS BI678863.1 SWS768 SWS (Sapwood of black locust - Summer) *Robinia pseudoacacia* cDNA, mRNA sequence.	Plus/Plus	60/69 (86)	65.9	3e-07

Appendix Table 4 (Continued)

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
DY911038	DY911038.1 CHAX634.b1 C16.ab1 CHA(XYZ) common wild sunflower *Helianthus annuus* cDNA clone CHAX634, mRNA sequence.	Plus/Plus	49/55 (89)	61.9	5e-06
GH629088	GH629088.1 HYS11582 Hybrid poplar cambium cDNA library during tension woodsy-zgj-shice5 B12.ab1, mRNA sequence.	Plus/Plus	32/33 (96)	58.0	7e-05
		Plus/Plus	52/62 (83)	44.1	1.1
CI296984	CI296984.1 *Oryza sativa Japonica* Group cDNA, clone: 009-M089F-D01, 5'end.	Plus/Plus	86/105 (81)	58.0	7e-05
AM825375	AM825375.1 Nicotiana tabacum EST, clone nt006055001.	Plus/Plus	63/75 (84)	54.0	0.001
BI122809	BI122809.1 I013P31P Populus leaf cDNA library Populus tremula x Populus tremuloides cDNA, mRNA sequence.	Plus/Plus	63/75 (84)	54.0	0.001
		Plus/Plus	53/62 (85)	52	0.004
FG477443	FG477443.1 020911KALA008655HT (KALA) Dormant kiwifruit buds three days after KALAA00865, mRNA sequence.	Plus/Plus	29/30 (96)	52	0.004
		Plus/Plus	25/26 (96)	44.1	1.1
		Plus/Plus	52/62 (83)	44.1	1.1
DV673511	DV673511.1 CGN-9605 Pericarp Coffea canephora cDNA clone cccp21m19 5', mRNAsequence.	Plus/Plus	38/42 (90)	52.0	0.004

Appendix Table 4 (Continued)

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
DV672796	DV672796.1 CGN-8591 Pericarp *Coffea canephora* cDNA clone cccp8i16 5', mRNAsequence.	Plus/Minus	38/42 (90)	52.0	0.004
DV666621	DV666621.1 CGN-4569 Pericarp *Coffea canephora* cDNA clone cccp23d6 5', mRNAsequence.	Plus/Plus	38/42 (90)	52.0	0.004
CV256585	CV256585.1 WS0244.B21_E06 PTxD-ICC-N-A-14 *Populus trichocarpa* x *Populus deltoides* cDNA clone WS0244 E06 3', mRNA sequence.	Plus/Minus	53/62 (85)	52.0	0.004
DB716302	DB716302.1 *Solanum lycopersicum* cDNA, clone: LEFL2022K17, 5' end, express edin fruit.	Plus / Plus	29/30 (96)	52.0	0.004
FG451149	FG451149.1 010602KAFB005213HT (KAFB) *Actinidia deliciosa* buds 3 days afterKAFBB00521, mRNA sequence.	Plus/Plus	49/57 (85)	50.1	0.017
FG451148	FG451148.1 010602KAFB005212HT (KAFB) *Actinidia deliciosa* buds 3 days afterKAFBB00521, mRNA sequence.	Plus/Plus	49/57 (85)	50.1	0.017
FG449197	FG449197.1 010531KAFB002732HT (KAFB) *Actinidia deliciosa* buds 3 days afterKAFBB00273, mRNA sequence.	Plus/Plus	49/57 (85)	50.1	0.017

Appendix Table 4 (Continued)

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
FC889310	FC889310.1 KN0AAQ4YF08RM1 IVIA1 *Citrus clementina* cDNA clone KN0AAQ4YF08, mRNAsequence.	Plus/Plus	55/65 (84)	50.1	0.017
		Plus/Plus	62/75 (82)	46.1	0.27
EY871476	EY871476.1 CL06-C4-500-059-A01-CT.F Rangpur lime root, greenhouse plant *Citrus limonia* cDNA, mRNA sequence	Plus/Plus	58/69 (84)	50.1	0.017
		Plus/Plus	60/72 (83)	48.1	0.069
DY302922	DY302922.1 KN0AAQ4YF08RM1 CitNFL *Citrus clementina* cDNA 5', mRNA sequence.	Plus/Plus	55/65 (84)	50.1	0.017
		Plus/Plus	62/75 (82)	46.1	0.27

Appendix Table 5 Sequences alignments of NR1 full-length (ZM1) by using BLASTN (2.2.24) program from NCBI database.

Accession number	Description	Strand	Identities Nt (%)	Score (bits)	E value
NM_001174336.1	*Zea mays* hypothetical protein (LOC100381505), mRNA gbBT061429.1 *Zea mays* full-length cDNA clone ZM_BFb0145I11 mRNA, complete cds	Plus/Plus	1187/1195 (99)	2163	0.0
XM_002461257.1	*Sorghum bicolor* hypothetical protein, mRNA	Plus/Plus	1282/1356 (95)	2089	0.0
XM_002459401.1	*Sorghum bicolor* hypothetical protein, mRNA	Plus/Plus	1271/1341 (95)	2085	0.0
NM_001065596.1	*Oryza sativa Japonica* Group Os07g0184900 (Os07g0184900) mRNA, complete cds	Plus/Plus	1134/1340 (85)	1303	0.0
AK102066.1	*Oryza sativa Japonica* Group cDNA clone:J033082F01, full insert sequence	Plus/Plus	1134/1340 (85)	1303	0.0
EU950919.1	*Zea mays* clone 645836 mRNA sequence	Plus/Plus	698/705 (99)	1264	0.0
AK099980.1	*Oryza sativa Japonica* Group cDNA clone:J013129E14, full insert sequence	Plus/Plus	1135/1358 (84)	1223	0.0
AY109188.1	*Zea mays* PCO145472 mRNA sequence	Plus/Plus	251/252 (99)	460	9e-126

Appendix Table 5 (Continued)

Accession number	Description	Strand	Identities Nt (%)	Score (bits)	E value
AK102995.1	Oryza sativa Japonica Group cDNA clone:J033116E13, full insert	Plus/Plus	377/440 (86)	453	2e-123
		Plus/Plus	66/74 (90)	89.8	5e-14
EZ097749.1	TSA: Zea mays contig33404, mRNA sequence	Plus/Minus	101/101 (100)	187	2e-43
AP003861.2	Oryza sativa Japonica Group genomic DNA, chromosome 7, BAC clone:OJ1046_F10	Plus/Plus	94/105 (90)	134	2e-27
		Plus/Plus	66/74 (90)	89.8	5e-14

Appendix Table 6 Sequences alignments of NR1 full-length (ZM1) by using BLASTN (2.2.24) program from DDBJ database.

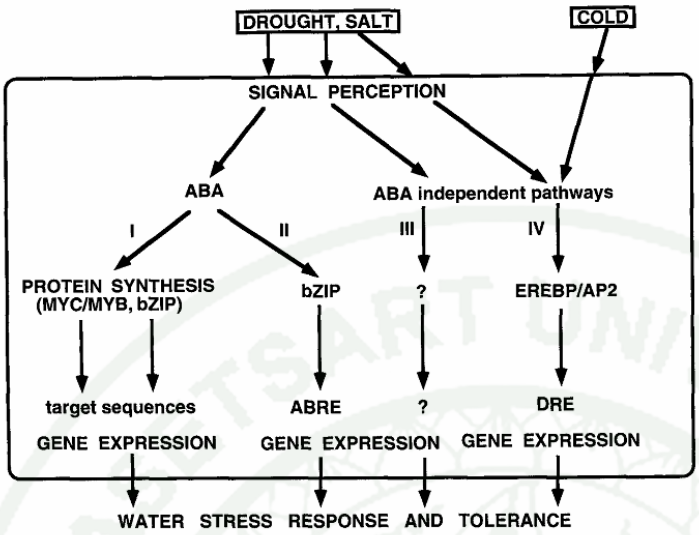
Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
DV025081	DV025081.1 ZM_BFb0145I11.r ZM_BFb *Zea mays* cDNA 5', mRNA sequence.	Plus/Plus	837/841 (99)	1635	0.0
DR959368	DR959368.1 ZM_BFb0071F06.f ZM_BFb *Zea mays* cDNA 3', mRNA sequence.	Plus/Minus	783/790 (99)	1511	0.0
CA403179	CA403179.1 EL01N0448A08.g Endosperm_4 *Zea mays* cDNA, mRNA sequence.	Plus/Minus	786/796 (98)	1503	0.0
DR827788	DR827788.1 ZM_BFb0071F06.r ZM_BFb *Zea mays* cDNA 5', mRNA sequence.	Plus/Plus	780/790 (98)	1489	0.0
DV621938	DV621938.1 IV-1091-406C-A01.T7-1 UGIV-1091-Reseq *Zea mays* cDNA, mRNA sequence.	Plus/Minus	720/724 (99)	1404	0.0
DN205349	DN205349.1 MEST817_B09.T7-1 UGA-ZmSAM-XZ2 *Zea mays* cDNA, mRNA sequence.	Plus/Minus	657/663 (99)	1267	0.0
CF628530	CF628530.1 zmrws48_0A10-006-h07.s2 zmrws48 *Zea mays* cDNA 3', mRNA sequence.	Plus/Minus	642/643 (99)	1267	0.0
EB813067	EB813067.1 ZM_BFb0356O14.f ZM_BFb *Zea mays* cDNA 3', mRNA sequence.	Plus/Minus	678/695 (97)	1243	0.0

Appendix Table 6 (Continued)

Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
FL123854	FL123854.1 3500101 CERES-264 Zea mays cDNA clone 689879 5', mRNA sequence.	Plus / Plus	632/634 (99)	1241	0.0
CF625524	CF625524.1 zmrws05_0A21-001-h08.s3 zmrws05 Zea mays cDNA 3', mRNA sequence.	Plus/Minus	627/629 (99)	1223	0.0
DN214358	DN214358.1 MEST997_A11.T7-1 UGA-ZmSAM-XZ2 Zea mays cDNA, mRNA sequence.	Plus/Minus	629/636 (98)	1197	0.0
DN233109	DN233109.1 MEST927_B09.T7-1 UGA-ZmSAM-XZ2 Zea mays cDNA, mRNA sequence.	Plus/Minus	625/633 (98)	1191	0.0
BG874047	BG874047.1 MEST45-G02.T3 ISUM4-TN Zea mays cDNA clone MEST45-G02 3', mRNA sequence.	Plus/Minus	594/600 (99)	1142	0.0
CF483041	CF483041.1 POL1_10_G12.g1_A002 Pollen Sorghum bicolor cDNA clone POL1_10_G12_A002 5', mRNA sequence.	Plus/Plus	674/708 (95)	1126	0.0
DR792592	DR792592.1 ZM_BFb0012I03.r ZM_BFb Zea mays cDNA 5', mRNA sequence.	Plus/Plus	567/573 (98)	1088	0.0
CF040139	CF040139.1 QCH9d10.yg QCH Zea mays cDNA clone QCH9d10, mRNA sequence.	Plus/Plus	550/551 (99)	1084	0.0

Appendix Table 6 (Continued)

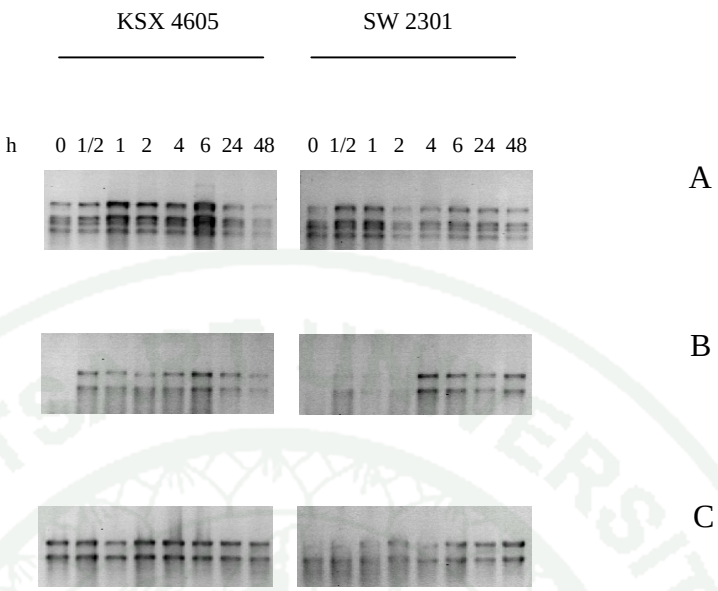
Accession number	Definition	Strand	Identities Nt (%)	Score (bits)	E value
DR792591	DR792591.1 ZM_BFb0012I03.f ZM_BFb *Zea mays* cDNA 3', mRNA sequence.	Plus/Minus	566/573 (98)	1080	0.0
FL442295	FL442295.1 13848083 CERES-264 *Zea mays* cDNA clone 689879 3', mRNA sequence.	Plus/Plus	565/574 (98)	1059	0.0
CF036521	CF036521.1 QCG32g05.yg QCG *Zea mays* cDNA clone QCG32g05, mRNA sequence.	Plus/Plus	533/533 (100)	1057	0.0
BU093734	BU093734.1 1091048F01.y1 1091 - Immature ear with common ESTs screened by Schmidt lab *Zea mays* cDNA, mRNA sequence.	Plus/Plus	534/535 (99)	1053	0.0



Signal transduction pathways between the perception of a water-stress signal and gene expression. At least four signal transduction pathways exist (I–IV): two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein biosynthesis is required in one of the ABA-dependent pathways (I). In another ABA-dependent pathway, ABRE does not require protein biosynthesis (II). In one of the ABA-independent pathways, DRE is involved in the regulation of genes not only by drought and salt but also by cold stress (IV). Another ABA-independent pathway is controlled by drought and salt but not by cold (III).

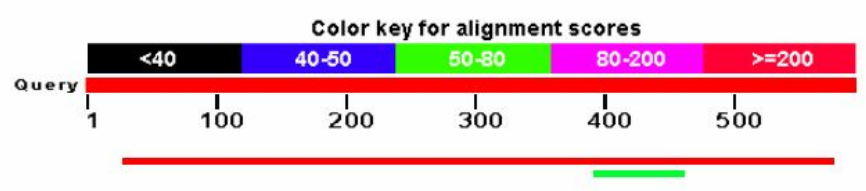
Appendix Figure 1 Show signal transduction pathways between the perception of water-stress signal and gene expression.

Source: Chernys and Zeevaart (2000)



Appendix Figure 2 Total RNA of two maize lines loading 1 µg per lane from leaves (A), roots (B) and shoots (C) tissues of two maize lines by running 1.2% (w/v) agarose gel electrophoresis; control (0h), time of drought stress 30 min(1/2h), 1, 2, 4, 6, 24 and 48 h.

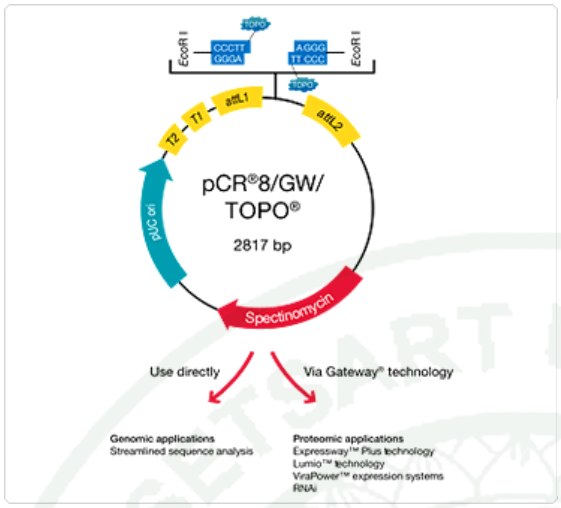
A



B

Appendix Figure 3 Graphical view of nucleotide sequence alignment of NR2 (595 bp) by using BLASTN 2.2.24 program NCBI database (A) and DDBJ database (B).

A

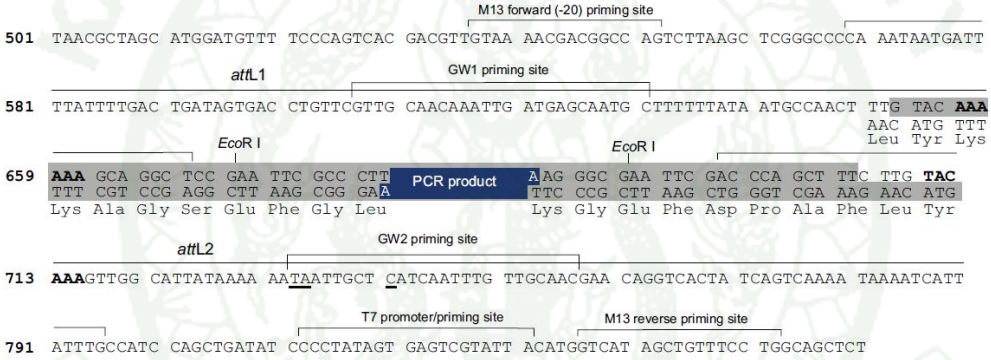


Comments for pCR®8/GW/TOPO®
2817 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*attL*1: bases 569-668
GW1 priming site: bases 607-631
TOPO® recognition site 1: bases 678-682
TOPO® recognition site 2: bases 683-687
*attL*2: bases 696-795
GW2 priming site: bases 733-757
T7 Promoter/priming site: 812-831 (c)
M13 reverse priming site: bases 836-852
Spectinomycin promoter: bases 930-1063
Spectinomycin resistance gene (Spn^R): 1064-2074
pUC origin: bases 2141-2814

(c) = complementary sequence

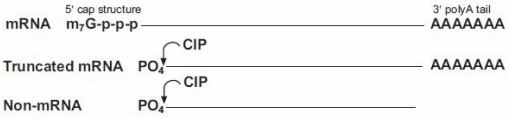
B



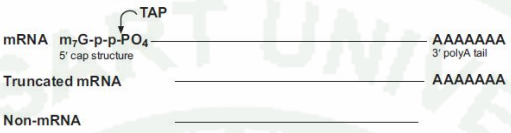
Appendix Figure 4 The structure of pCR®8/GW/TOPO® Entry vector allows TOPO® TA Cloning®for multiple downstream applications, circle map (A) and the promoter sequence diagram (B).

Source: pCR®8/GW/TOPO® TA Cloning® technical manual, www.invitrogen.com

1. Treat total RNA or mRNA with calf intestinal phosphatase (CIP) to remove the 5′ phosphates. This eliminates truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo. **Note**: CIP has no effect on full-length, capped mRNA.

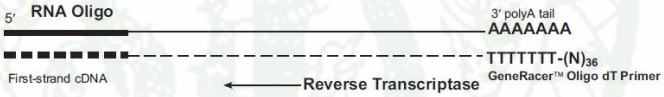


2. Treat dephosphorylated RNA with tobacco acid pyrophosphatase (TAP) to remove the 5′ cap structure from intact, full-length mRNA. This treatment leaves a 5′ phosphate required for ligation to the GeneRacer™ RNA Oligo.

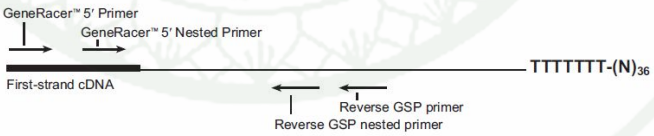


3. Ligate the GeneRacer™ RNA Oligo to the 5′ end of the mRNA using T4 RNA ligase. The GeneRacer™ RNA Oligo will provide a known priming site for GeneRacer™ PCR primers after the mRNA is transcribed into cDNA.

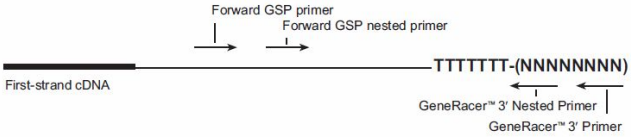
4. Reverse transcribe the ligated mRNA using Cloned AMV RT or SuperScript™ III RT and the GeneRacer™ Oligo dT Primer to create RACE-ready first-strand cDNA with known priming sites at the 5′ and 3′ ends. (If you are only interested in the 5′ ends, you can reverse transcribe using random primers or a gene-specific primer. If you are only interested in the 3′ ends, reverse transcribe the original, unligated mRNA or total RNA using the GeneRacer™ Oligo dT Primer.)



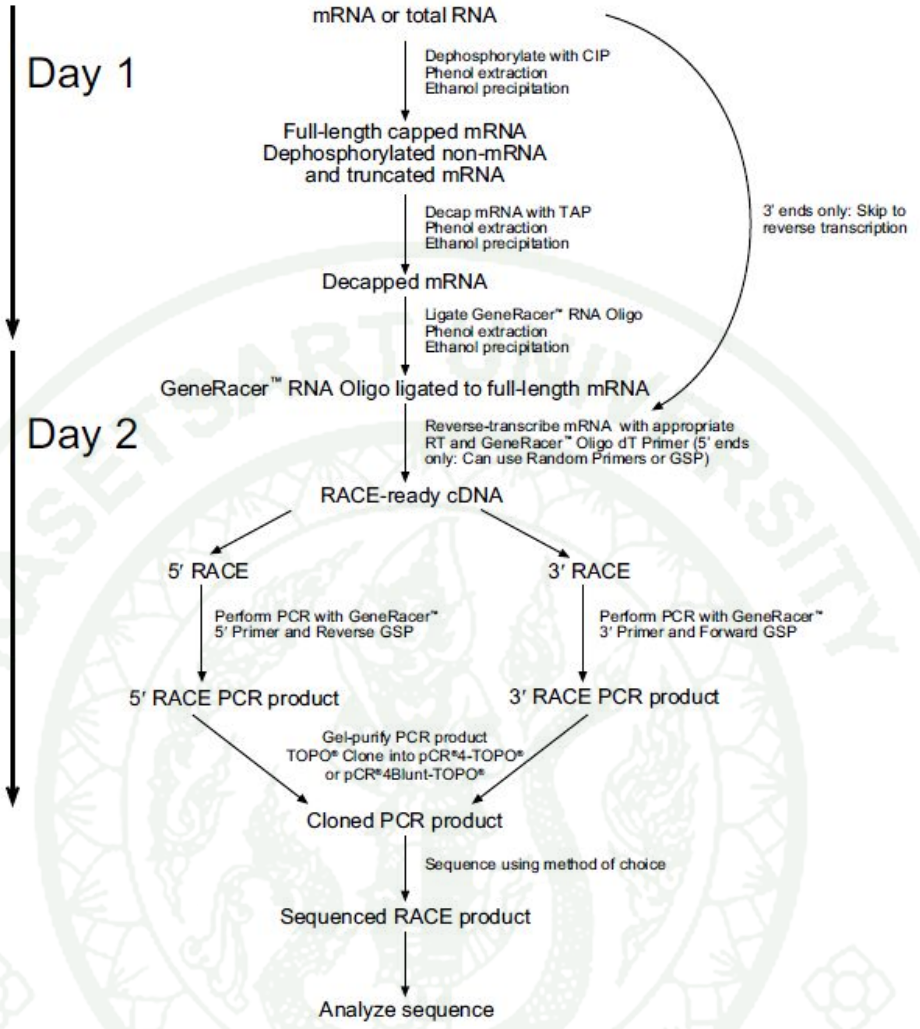
5. To obtain 5′ ends, amplify the first-strand cDNA using a reverse gene-specific primer (Reverse GSP) and the GeneRacer™ 5′ Primer (homologous to the GeneRacer™ RNA Oligo). Only mRNA that has the GeneRacer™ RNA Oligo ligated to the 5′ end AND is completely reverse transcribed will be amplified using PCR. If needed, perform additional PCR with nested primers.



6. To obtain 3′ ends, amplify the first-strand cDNA using a forward gene-specific primer (Forward GSP) and the GeneRacer™ 3′ Primer (homologous to the GeneRacer™ Oligo dT Primer). Only mRNA that has a polyA tail and is reverse transcribed will be amplified using PCR. If needed, perform additional PCR with nested primers.



Appendix Figure 5 Overview of the GeneRacer™ methods by using technique RLM- RACE and RACE for 5′ end. (www.invitrogen.com)



Appendix Figure 6 The flowchart shows the GeneRacer™ experimental outline. Day 1 ligating the GeneRacer™ RNA Oligo to decapped RNA. For Day 2, perform the reverse transcriptase (RT) reaction, (www.invitrogen.com)

```
  1 TCAGGCCGCC TGATGACCAG TTCAACATGT ACCTGTTTTA TGCAGATTTG
 51 ATTGGTCTGG ATGGAGATGA GATGACCGAA TATTTGCCAT CTGCTGGTGA
101 TGAGCTAGAG GATATGACTA ATCAACTGAG AAAACGGATA ATGAAGAAGC
151 GCAGAGTCAA AACTCTTTCA TTTGCGATTA CCAATGATGT GTGCATAGAA
201 GTCAATACAT ATGCGCTGGT CCGTCCTACT ACTACAGGGA CAATCACATG
251 GCTTGATTCA CTAAGTAACC TCCCATTAAA GGTTGAGAGG TCTTTCATAT
301 GCAATGATAC TGGGGCTCTA CTTCAGGATG CACAGACACG TTTCCNGATG
351 TACAATGACA CAATTGTCAA ATTTTCTGTA CGTGAACTCT CTGAGGTTAA
401 AAGGGTTGCA AGCCATCATC TTCGCCTTAT AGGTTTCAAG CCATTGGATT
451 GCTTGAAAGA TTACCATAAC TTAAGACCAT CGACATTTAT TTATCCGAGT
501 GATGAGCGTA TATTTGGAAG CACCTGTGTT TTCGTTGCTT TACATAGCTC
551 AATGTTACGT CTTGGAAGGN TTGCACTTGC ATTTTATGGG AATCCAACTC
601 GACCACAACT CATAGCCCTT GTTGCTCAAG AAGANGNTAC TTCGTCTGGT
651 CGTCAGTTTG AACCGCCTGG CATGCACATG ATCTACCTTC CATACTCCGA
701 TGATATTAGA NATCCTGAAG AAGTTCATGT GACTTCTGAT GATGCNCCNC
751 GNGCAACANA TGAACAATCA GAAAGCTTCN AAATATCCAN CG
```

Appendix Figure 7 Show position of designed gene specific primer (GSPs) for full-length of NR1 fragment, 792 bp using RACE-PCR.

TGCCATCTGCTGGTGATGAGC : GSP-F1

GCTCTACTTCAGGATGCACAGACAC : GSP-F2

ACTCGACCACAACTCATAGCCCTTGTTG : GSP-F3

GAACCGCCTGGCATGCACATGATCTAC : GSP-R(reverse antisense)

Appendix Figure 8 Results nucleotide alignment of 5′ end (GSP-R), NR1 and 3′end (GSP-F1) by CLUSTAL 2.0.10 multiple sequence alignment.

```
GSP-R-5         TCATCTCCGCCTTATAGGTTTCAAGCCATTGGATTGCTTGAAAGATTACCATAACTTAAG 715

GSP-R           TCATCTTCGCCTTATAGGTTTCAAGCCATTGGATTGCTTGAAAGATTACCATAACTTAAG 550
NR1             TCATCTTCGCCTTATAGGTTTCAAGCCATTGGATTGCTTGAAAGATTACCATAACTTAAG 617
GSP-F1-3        TCATCTTCGCCTTATAGGTTTCAAGCCATTGGATTGCTTGAAAGATTACCATAACTTAAG 365
                ****** **************************************************

GSP-R-5         ACCATCGACATTTAATTATCCGAGCGATGAGCGTATATTTGGAAGCACCTGTGTTTTCGT 775
GSP-R           ACCATCGACATTTATTTATCCGAGTGATGAGCGTATATTTGGAAGCACCTGTGTTTTCGT 610
NR1             ACCATCGACATTTATTTATCCGAGTGATGAGCGTATATTTGGAAGCACCTGTGTTTTCGT 677
GSP-F1-3        ACCATCGACATTTATTTATCCGAGTGATGAGCGTATATTTGGAAGCACCTGTGTTTTCGT 425
                ************** ********* **********************************

GSP-R-5         TGCTTTACATAGCTCAATGNTACGTCNTGGAANGNTTGCACTTGCATTTNATGGG-AATC 834
GSP-R           TGCTTTACATAGCTCAATGTTACGTCTTGGAAGGTTTGCACTTGCATTTTATGGGGAATC 670
NR1             TGCTTTACATAGCTCAATGTTACGTCTTGGAAGGNTTGCACTTGCATTTTATGGG-AATC 736
GSP-F1-3        TGCTTTACATAGCTCAATGTTACGTCTTGGAAGGTTTGCACTTGCATTTTATGGG-AATC 484
                ****************** ****** ***** * ************** ***** ****

GSP-R-5         CAACTCGACCACAACTCATAGCCCTTGTTGCTCAAGAANAG-TTACTNCGNCTGGTCGTC 893
GSP-R           CAACTCGACCACAACTCATAGCCCTTGTTGCTCAAGAAGAGGTTACTTCGTCTGGTCGTC 730
NR1             CAACTCGACCACAACTCATAGCCCTTGTTGCTCAAGAAGANGNTACTTCGTCTGGTCGTC 796
GSP-F1-3        CAACTCGACCACAACTCATAGCCCTTGTTGCTCAAGAAGAGGTTACTTCGTCTGGTCGTC 544
                ************************************ *   **** ** *********

GSP-R-5         AGTTTGA-CCGCCTG-GCATGCN-ATGA-TCTACAGGGCGAATTCANCCAGCTTCTGNA- 948
GSP-R           AGNTTGAACCGCCTGCATGNACA-ATGAATCTACAAANNNNNNN---------------- 773
NR1             AGTTTGAACCGCCTG-GCATGCACATGA-TCTACCTTCCATACTCCGATGATATTAGANA 854
GSP-F1-3        AGTTTGAACCGCCTG-GCATGCACATGA-TCTATCTTCCATACTCCGATGATATTAGATA 602
                ** **** *******      *  **** ****

GSP-R-5         -CAAGTGGNATATAAAAANATTGCTCACATTGN--TGCACGAANNG-GCNTATCNNNNAA 1004
GSP-R           ------------------------------------------------------------
NR1             TCCTGAAGAAGTTCATGTGACTTCTGATGATGC--NCCNCGNGCAACANATGAACAATCA 912
GSP-F1-3        TCCTGAGGAAGTTCATGTGACTTCTGATGATGCACCGCGTGCAACAGATGAACAAATCAA 662

GSP-R-5         NAAATCTT--ATTTGCNTCNGCTGAANNCCCCNNAGGGNNCGATTNN------------- 1049
GSP-R           ------------------------------------------------------------
NR1             GAAAGCTT--CNAAATATCCANCGTATNGATCTGANAATTCCTGCTGCCANATGCTACCC 970
GSP-F1-3        GAAAGCTTCGAATATATTCAAACGTATTGATCTGATAAATTTCTCTGCATGCCAATTTGC 722

GSP-R-5         ------------------------------------------------------------
GSP-R           ------------------------------------------------------------
NR1             CCTTGCAAAACCTTGGANCTTGGAGCCTACTTAGCNANAAAGNGCNGAATAAGACANACC 1030
GSP-F1-3        TAACCCAGCTTTGCAAAGACACTATGGGATCTTGGAGGCCTTAGCTTTAGGCGAAGATGA 782

GSP-R-5         ------------------------------------------------------------
GSP-R           ------------------------------------------------------------
NR1             NGCTAGGNAATCCCCCCTTCTGNCANGTGGATTNAAAAATGNCNCATTGTGCCNANGGCC 1090
GSP-F1-3        GATGCCTGATATAAAGGACGAGACCCCTGCCTGACGAAAAAGGCTTGTCTAAANCCAGGG 842

GSP-R-5         ------------------------------------------------------------
GSP-R           ------------------------------------------------------------
NR1             TCCCAAAAACTTTTGCCCCNGATCCNANNGGNATNNGCANCGTCGGCCNGGCCGTC---- 1146
GSP-F1-3        GGTAGCCAATGCTATTGAGGAATTCAAGACTTCNGTCTATGGNGNAAAATTATGACCAAG 902

GSP-R-5         ---
GSP-R           ---
NR1             ---
GSP-F1-3        AGG 905
```

(length 1110 bp)

Appendix Figure 8 (Continued)

```
GSP-F2-nested       ------------------------------------------------------------
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              ------------------------------------------------------------
GSP-F2-M13F         NGNNNAATAATGGATTTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATG 60

GSP-F2-nested       ------------------------------------------------------------
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              ------------------------------------------------------------
GSP-F2-M13F         AGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCCGAATTCGCCCTTG 120

GSP-F2-nested       ------------------------------------------------------------
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              ------------------------------------------------------------
ZmF2-M13F           CTCTACTTCAGGATGCACAGACACGTTTCCAGATGTACAATGACACAATTGTCAAATTTT 180

GSP-F2-nested       ------------------------------------------------------------
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              ------------------------------------------------------------
GSP-F2-M13F         CTGTACGTGAACTCTCTGAGGTTAAAAGGGTTGCAAGCCATCATCTTCGCCTTATAGGTT 240

GSP-F2-nested       ------------------------------------------------------------
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              ------------------------------------------------------------
Zm0903-53-M13F      TCAAGCCATTGGATTGCTTGAAAGATTACCATAACTTAAGACCATCGACATTTATTTATC 300

GSP-F2-nested       ------------------------------------------------------------
zmF2-m13r-puc       ------------------------------------------------------------
GSP-F3              ------------------------------------------------------------
GSP-F2-M13F         CGAGTGATGAGCGTATATTTGGAAGCACCTGTGTTTTAGTTGCTTTACATAGCTCAATGT 360

GSP-F2-nested       ------------------------------------------------------------
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              ------------------------------------------------------------
GSP-F2-M13F         TACGTCTTGGAAGGTTTGCACCTGCATTTTATGGGAATCCAACTCGACCACAACTCATAG 420

GSP-F2-nested       ---------CNNANNAAGAGGTTACTTCGTCTGGTCGTCAGTTTGAACCGCCTGGCATGC 51
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              ---------GNNTNACNCGATGTACATATGCCGGTTGTCAGCTGGAGTTCTCTGTCGGGG 51
GSP-F2-M13F         CCCTTGTTGCTCAAGAAGAGGTTACTTCGTCTGGTCGTCAGTTTGAACCGCCTGGCATGC 480

GSP-F2-nested       ACATGATCTATCTTCCATACTCCGATGATATTAGATATCCTGAGGAAGTTCATGTGACTT 111
GSP-F2-m13r-puc     ------------------------------------------------------------
GSP-F3              GCTTAG-CAAAGGTCTTTGCTCGGATTATAGTAGCTAGCCTGAGGAAGTCCGTGGGACTT 110
GSP-F2-M13F         ACATGATCTATCTTCCATACTCCGATGATATTAGATATCCTGAGGAAGTTCATGTGACTT 540

GSP-F2-nested       CTGATGATGCACCGCGTGCAACAGATGAACAAATCAAGAAAGCTTCGAATATATTC-AAA 170
GSP-F2-m13r-puc     ------------------------TGAACAAATCAAGAAAGCTTCGAATATATTCCAAA 35
GSP-F3              CTGATGATGCACCGCGTGCAACAGATGAACAAATCATGAAAGTTACGAATATATTC-AAA 169
GSP-F2-M13F         CTGATGATGCACCGCGTGCAACAGATGAACAAATCAAGAAAGCTTCGAATATATTC-AAA 599
                                           *********** ***** * *********** ***

GSP-F2-nested       CGTATTGATCTGATAAATTTCTCTGCATGCCAATTTGCTAACCCAGCTTTGCAAAGACAC 230
GSP-F2-m13r-puc     CGTATTGATCTGATAAATT-CTCTGCATGCCAATTTGCTAACCCAGCTTTGCAAAGACAC 94
GSP-F3              CGTATTGATCTGATAAATTTCTCTGCATGCCAATTTGCTAACCCAGCTTTGCAAAGACAC 229
GSP-F2-M13F         CGTATTGATCTGATAAATTTCTCTGCATGCCAATTTGCTAACCCAGCTTTGCAAAGACAC 659
                    ****************** ****************************************
```

Appendix Figure 9 Results nucleotide alignment of 3′end from GSP-F2 and GSP-F3 fragments by CLUSTAL 2.0.10 multiple sequence alignment.

```
GSP-F2-nested       TATGGGATCTTGGAGGCCTTAGCTTTAGGCGAAGATGAGATGCCTGATATAAAGGACGAG 290
GSP-F2-m13r-puc     TATGGGATCTTGGAGGCTTTAGCTTTAGGCGAAGATGAGATGCGTGATATAAAGGACGAG 154
GSP-F3              TATGGGATCTTGGAGGCCTTAGCTTTAGGCGAAGATGAGATGCCTGATATAAAGGAAGAG 289
GSP-F2-M13F         TATGGGATCTTGGAGGCCTTAGCTTTAGGCGAAGATGAGATGCCTGATATAAAGGACGAG 719
                    ***************** ************************ ************ ***

GSP-F2-nested       ACCCTGCCTGACGAAGAAGGCTTGTCTAAGCCAGGGGTAGCCAATGCTATTGAGGAATTC 350
GSP-F2-m13r-puc     ACCCTGCCTGACGAAGAAGGCTTGTCTAAGCCAGGGGTAGCCAATGCTATTGAGGAATTC 214
GSP-F3              ACCCTGCCTGACGAAGAAGGCTTGTCTAAGCCAGGGGTAGCCAATGCTTTTGAGGAATTC 349
GSP-F2-M13F         ACCCTGCCTGACGAAGAAGGCTTGTCTAAGCCAGGGGTAGCCAATGCTATTGAGGAATTC 779
                    ********************************************** ***********

GSP-F2-nested       AAGACTTCAGTCTATGGTGAAAATTATGACCAAGAGGAGGCAGAAGCGGCAGCAGGGAAA 410
GSP-F2-m13r-puc     AAGACTTCAGTCTATGGTGAAAATTATGACCAAGAGGAGGCAGAAGCGGCAGCAGGGAAA 274
GSP-F3              AAGACTTCAGGCTATGGTGAAAATTATGACCAAGAGGAGGCAGAAGCGGCTTCAGGGAAA 409
GSP-F2-M13F         AAGACTTCAGTCTATGGTGAAAATTATGACCAAGAGGAGGCAGAAGCGGCAGCAGG-AAA 838
                    ********** ************************************  **** ***

GSP-F2-nested       GCTTCCCGTGGTAATGCTTCAAAAAAGCGGAAGGAGGTCACTGATGCAGCTGCGCAGATA 470
GSP-F2-m13r-puc     GCTTCCCGTGGTAATGCTTCAAAAAAGCGGAAGGAGGTCACTGATGCAGCTGCGCAGATA 334
GSP-F3              GCTTCCCGTGCTAATGCTTCAAAAAAGCGGAGCGAGTTCACTGATGCCGATACGCAAATA 469
GSP-F2-M13F         GCTTCCCGTGGTAATGCTTCAAAAAAGCGGAAGGAGGTCACTGATGCAGCTGCGCAGATA 898
                    ********** ********************  *** ********** * * **** ***

GSP-F2-nested       AGTGCTGCTTATGACTGGGCAGAACTTGCAGACAATGGAAAACTGAAGGAAATGACCACG 530
GSP-F2-m13r-puc     AGTGCTGCTTATGACTGGGCAGAACTTGCAGACAATGGAAAACTGAAGGAAATGACCACG 394
GSP-F3              AGTGCTGCTTGTCACATGTCAAAAGATGTTTTATATCTACAACTTTTGGAACATCCCATC 529
GSP-F2-M13F         AGTGCTGCTTATGACTGGGCAGAACTTGCAGACAATGGAAAACTGAANNAAT-------- 950
                    ********** * **  * ** **  **      **  * ****     **

GSP-F2-nested       GTGGAATTGAGAT-CCTACCTGACCGCGCATGATCTCCCG-GTTTCTGGTAAGAAAGAGG 588
GSP-F2-m13r-puc     GTGGAATTGAGAT-CCTACCTGACCGCGCATGATCTCCCG-GTTTCTGGTAAGAAAGAGG 452
GSP-F3              GGCGATTTATTTTTCCTTACTGACCTATTATGATCCACCGTGTGAATTCAAAAAAAGACC 589
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       TACTTATCAGCAGGATCTTGACTCACCTGGGTAAGTGAAGCCGGGTATCCG--AACTGTT 646
GSP-2-m13r-puc      TACTTATCAGCAGGATCTTGACTCACCTGGGTAAGTGAAGCCGGGTATCCG--AACTGTT 510
GSP-F3              GACATATATCAAATATCTTGTCTC-CCGTTACAACTACGCCTGGTTGCTTACCATTTATT 648
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       AGTTTCCTGAGACCCGACTG-ACATCTGTAGTGCTGTCTCACGGATCCT-TGTGGACCGT 704
GSP-F2-m13r-puc     AGTTTCCTGAGACCCGACTG-ACATCTGTAGTGCTGTCTCACGGATCCT-TGTGGACCGT 568
GSP-F3              ACTTTGGTCACATTCTTCTCCACTACTCAAAAGACGTCAATATAATAGTACACTGGCTAT 708
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       AATCCTCCCCCGTGCA-GGATACGTGC-CGAATTTTTGTATAGACACCGCCCATTGCCAA 762
GSP-F2-m13r-puc     AATCCTCCCCCGTGCA-GGATACGTGC-CGAATTTTTGTATAGCGACAGGACAGCACCAG 626
GSP-F3              ATACTTCTCTCAAGCACGGATATATTTACCTATTTTCGTGACACAACCGGATAATGGTTA 768
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       TGGCACATGTGTCATGTGTCATCTTGCGTCATGAGCTGTCAGCCGCTGGCCATAGGCAAC 822
GSP-F2-m13r-puc     TTGCCAATGTAACCTGTGTTAAGTTTCGTCTTGTGGTATCATCTGCTTGTCATTGTCAAC 686
GSP-F3              CATTCTGTCTCATCCCTCTTGTCATGCAGATCG-GATCCCATGTATAAGTCCAAATACAT 827
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       ATGATGCATTTCGGGATTCTGGA-ACCTAGACCCGCAATATAGATGCTTACTGCATGTTG 881
GSP-F2-m13r-puc     ATGAAACATAATGTAATTCTGGATACTTAGACCCACAATATGGAGCATAACTGCATAAGG 746
GSP-F3              ACACTGCAAGTCCCCATCCTTAG---CTGAATTAATTCCGTATATGATCTATGTCTAATA 884
GSP-F2-M13F         ------------------------------------------------------------
```

Appendix Figure 9 (Continued)

```
GSP-F2-nested       TA-TGTTAANCANAAAAAAAAATAANNNNGTCNTNNGCGNTNNCNNGNNN-NTGGGGNNN 939
GSP-F2-m13r-puc     TA-TGTTAAAAAAAAAAAAAAAAAAAAAAACACTGTCAT--GCCGTTAC-GTAGCGTAT 802
GSP-F3              TGCTACTAAATCAACATGAAGCCATGGTAAGTCGGAATATACACCAGGACCAAAATATAT 944
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       NCCCNNNATCA---------------------------------------------- 950
GSP-F2-m13r-puc     CGTTGACAGCAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAA 862
GSP-F3              ACTCTT--------------------------------------------------- 950
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       ------------------------------------------------------------
GSP-F2-m13r-puc     TAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTAT 922
GSP-F3              ------------------------------------------------------------
GSP-F2-M13F         ------------------------------------------------------------

GSP-F2-nested       ---------------------------
GSP-F2-m13r-puc     TTGCCATCCAGCTGATTCCCCNAANNNN 950
GSP-F3              ---------------------------
GSP-F2-M13F         ---------------------------
```

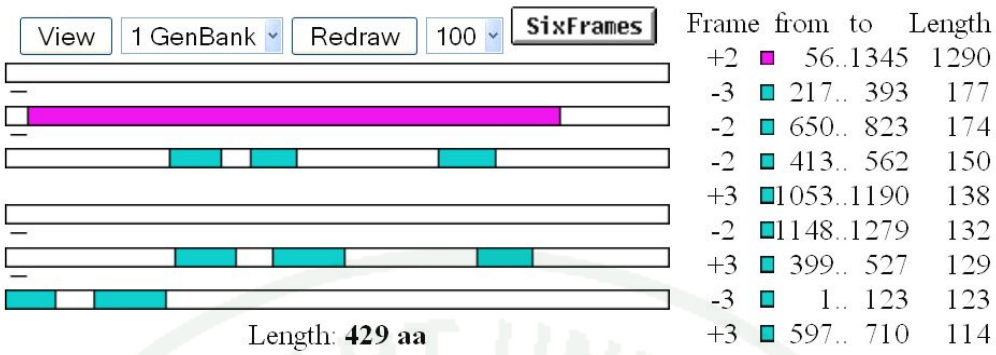
Appendix Figure 9 (Continued)

```
GSP-F2-M13FR,GSP-F3       --------------------------------------------------
NR1, 5′end                CGACTGGAGCACGAGGACACTGATATGATTAGGACAACACTTCAACGTGC 50

GSP-F2-M13FR,GSP-F3       --------------------------------------------------
NR1, 5′end                AAAGGATGCACAAGATCTTGGCCTGTCGTATTGAACTTCTTCCACTGAGC 100

GSP-F2-M13FR,GSP-F3       --------------------------------------------------
NR1, 5′end                CCGCCTGATGACCAGTTCAACATGTCCCTGTTTTATGCAGATTTGATTGG 150

GSP-F2-M13FR,GSP-F3       --------------------------------------------------
NR1, 5′end                TCTGGATGGAGATGAGATGACCGAATATTTGCCATCTGCTGGTGATAAGC 200

GSP-F2-M13FR,GSP-F3       --------------------------------------------------
NR1, 5′end                TAGAGGATATGACTAATCAACTGAGAAAACGGATAATGAAGAAGCGCAGA 250

GSP-F2-M13FR,GSP-F3       -------------------------------------------NGNNNAA 7
NR1, 5′end                GTCAAAACTCTTTCATTTGCGATTACCAATGATGTGTGCATAGAAGTCAA 300
                                                                        **

GSP-F2-M13FR,GSP-F3       TAATGGATTTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTG 57
NR1, 5′end                TACATATGCGCTGGTCCGTCCTACTACTACAGGGACAATCACATGGCTTG 350
                          **          *  * *  *     *   *  *  *   *    ***

GSP-F2-M13FR,GSP-F3       ATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCC 107
NR1, 5′end                ATTCACTAAGTAACCTCCCATTAAAGGTTGAGAGGTCTTTCATATGCAAT 400

GSP-F2-M13FR,GSP-F3       GAATTCGCCCTTGCTCTACTTCAGGATGCACAGACACGTTTCCAGATGTA 157
NR1, 5′end                GA---TACTGGGGCTCTACTTCAGGATGCACAGACACGTTTCCAGATGTA 447
                          **     *    ************************************

GSP-F2-M13FR,GSP-F3       CAATGACACAATTGTCAAATTTTCTGTACGTGAACTCTCTGAGGTTAAAA 207
NR1, 5′end                CAATGACACAATTGTCAAATTTTCTGTACGTGAACTCTCTGAGGTTAAAA 497
                          **************************************************

GSP-F2-M13FR,GSP-F3       GGGTTGCAAGCCATCATCTTCGCCTTATAGGTTTCAAGCCATTGGATTGC 257
NR1, 5′end                GGGTTGCAAGCCATCATCTTCGCCTTATAGGTTTCAAGCCATTGGATTGC 547
                          **************************************************

GSP-F2-M13FR,GSP-F3       TTGAAAGATTACCATAACTTAAGACCATCGACATTTATTTATCCGAGTGA 307
NR1, 5′end                TTGAAAGATTACCATAACTTAAGACCATCGACATTTATTTATCCGAGTGA 597
                          **************************************************

GSP-F2-M13FR,GSP-F3       TGAGCGTATATTTGGAAGCACCTGTGTTTTAGTTGCTTTACATAGCTCAA 357
NR1, 5′end                TGAGCGTATATTTGGAAGCACCTGTGTTTTCGTTGCTTTACATAGCTCAA 647
                          ****************************** ********************

GSP-F2-M13FR,GSP-F3       TGTTACGTCTTGGAAGGTTTGCACCTGCATTTTATGGGAATCCAACTCGA 407
NR1, 5′end                TGTTACGTCTTGGAAGGTTTGCACTTGCATTTTATGGGAATCCAACTCGA 697
                          ********************** ************************

GSP-F2-M13FR,GSP-F3       CCACAACTCATAGCCCTTGTTGCTCAAGAAGAGGTTACTTCGTCTGGTCG 457
NR1, 5′end                CCACAACTCATAGCCCTTGTTGCTCAAGAAGAGGTTACTTCGTCTGGTCG 747
                          **************************************************

GSP-F2-M13FR,GSP-F3       TCAGTTTGAACCGCCTGGCATGCACATGATCTATCTTCCATACTCCGATG 507
NR1, 5′end                TCAGTTTGAACCGCCTGGCATGCACATGATCTACCTTCCATACTCCGATG 797
                          ********************************* ****************
```

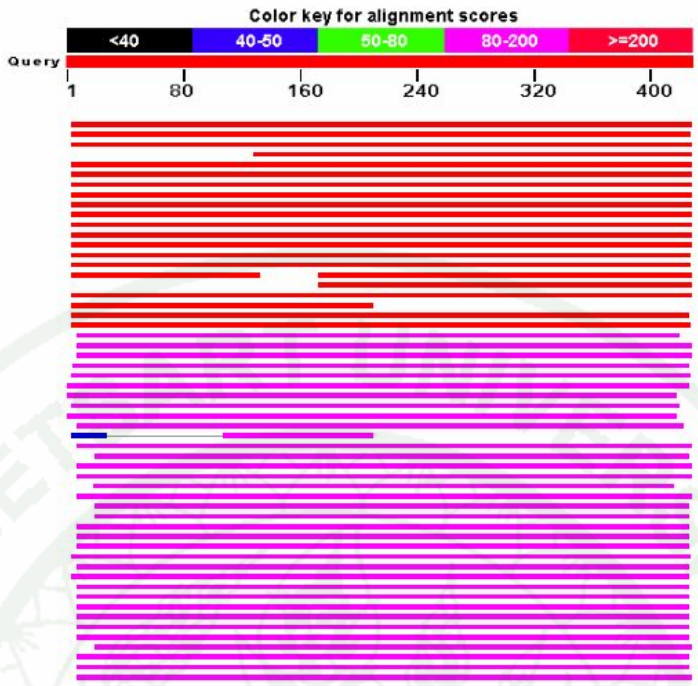
Appendix Figure 10 Result nucleotide alignment of 3′end (GSP-F2-M13F+R,GSP-F3) and NR1-5′end fragments by CLUSTAL 2.0.10 multiple sequence alignment.

```
GSP-F2-M13FR,GSP-F3     ATATTAGATATCCTGAGGAAGTTCATGTGACTTCTGATGATGCACCGCGT 557
NR1, 5'end              ATATTAGATATCCTGAGGAAGTTCATGTGACTTCTGATGATGCACCGCGT 847
                        **************************************************

GSP-F2-M13FR,GSP-F3     GCAACAGATGAACAAATCAAGAAAGCTTCGAATATATTCAAACGTATTGA 607
NR1, 5'end              GCAACAGATGAACAAATCAAGAAAGCTTCGAATATATTCAAACGTATTGA 897
                        **************************************************

GSP-F2-M13FR,GSP-F3     TCTGATAAATTTCTCTGCATGCCAATTTGCTAACCCAGCTTTGCAAAGAC 657
NR1, 5'end              TCTGATAAATTTCTCTGCATGCCAATTTGCTAACCCAGCTTTGCAAAGAC 947
                        **************************************************

GSP-F2-M13FR,GSP-F3     ACTATGGGATCTTGGAGGCCTTAGCTTTAGGCGAAGATGAGATGCCTGAT 707
NR1, 5'end              ACTATGGGATCTTGGAGGCCTTAGCTTTAGGCGAAGATGAGATGCCTGAT 997
                        **************************************************

GSP-F2-M13FR,GSP-F39    ATAAAGGACGAGACCC-TGCCTGACGAAGAAGGCTTGTCTAAGCC--AGG 754
NR1, 5'end              ATAAAGGACGAGACCCCTGCCTGACGAAAAAGGCTTGTCTAAANCCAGGG 1047
                        ***************  **********  ***********   *   **

GSP-F2-M13FR,GSP-F3     GGTAGCCAATGCTATTGAGGAATTCAAGACTTCAGTCTATGGTGAAAA-T 803
NR1, 5'end              GGTAGCCAATGCTATTGAGGAATTCAAGACTTCNGTCTATGGNGNAAAAT 1097
                        **********************************  ********* *** *

GSP-F2-M13FR,GSP-F3     TATGACCAAGAGGAGGCAGAAGCGGCAGCAGGGAAAGCTTCCCGTGGTAA 853
NR1, 5'end              TATGACCAAGAGG---------------------------------------- 1110
                        *************

GSP-F2-M13FR,GSP-F3     TGCTTCAAAAAAGCGGAAGGAGGTCACTGATGCAGCTGCGCAGATAAGTG 903
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     CTGCTTATGACTGGGCAGAACTTGCAGACAATGGAAAACTGAAGGAAATG 953
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     ACCACGGTGGAATTGAGATCCTACCTGACCGCGCATGATCTCCCGGTTTC 1003
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     TGGTAAGAAAGAGGTACTTATCAGCAGGATCTTGACTCACCTGGGTAAGT 1053
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     GAAGCCGGGTATCCGAACTGTTAGTTTCCTGAGACCCGACTGACATCTGT 1103
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     AGTGCTGTCTCACGGATCCTTGTGGACCGTAATCCTCCCCCGTGCAGGAT 1153
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     ACGTGCCGAATTTTTGTATAGCGACAGGACAGCACCAGTTGCCAATGTAA 1203
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     CCTGTGTTAAGTTTCGTCTTGTGGTATCATCTGCTTGTCATTGTCAACAT 1253
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     GAAACATAATGTAATTCTGGATACTTAGACCCACAATATGGAGCATAACT 1303
NR1, 5'end              --------------------------------------------------

GSP-F2-M13FR,GSP-F3     GCATAAGGTATGTTAAAAAAAAAAAAAAAAAAAAAAAAACACTGTCATGCC 1353
NR1, 5'end              --------------------------------------------------
```

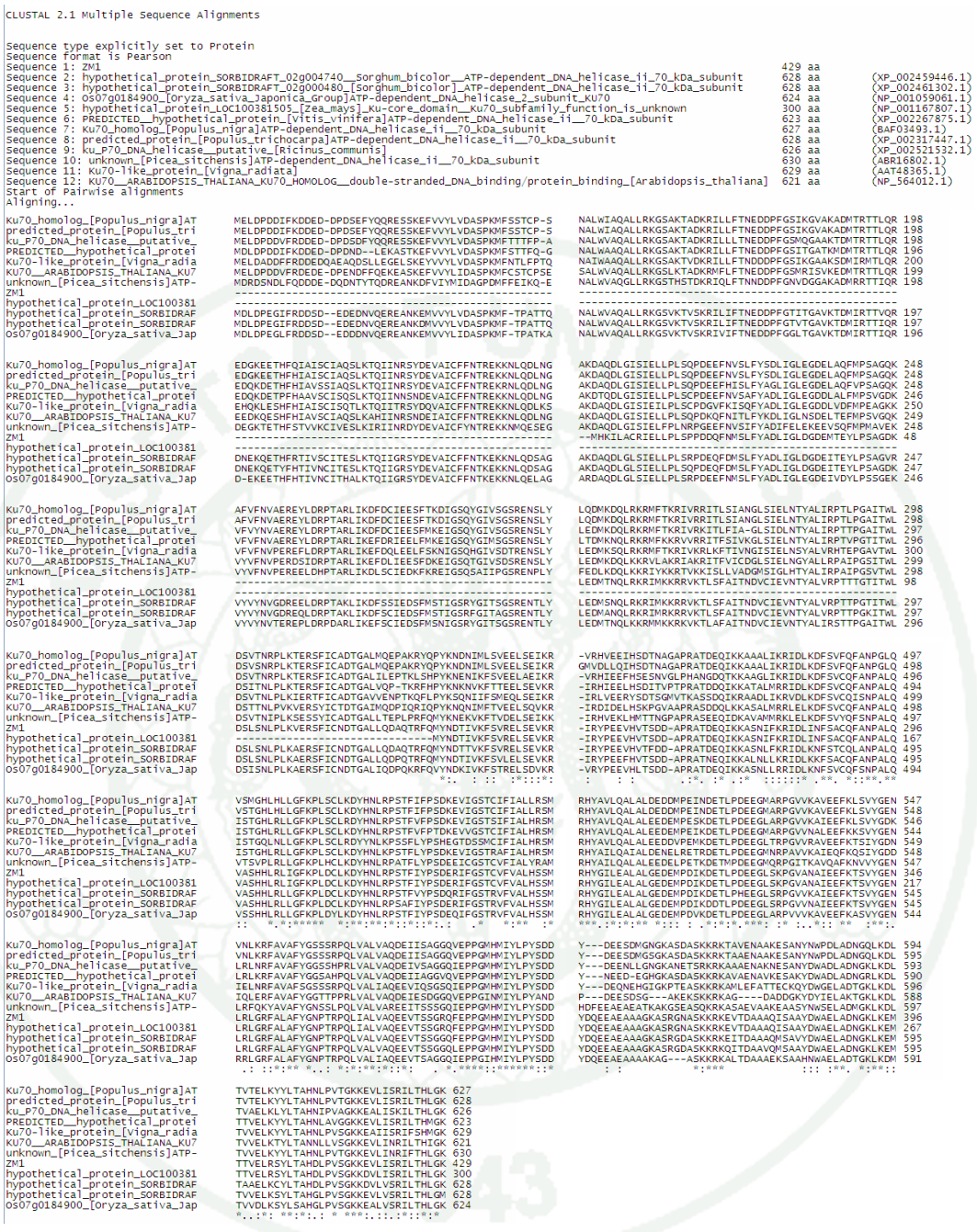
Appendix Figure 10 (Continued)



Appendix Figure 11 Graphical view of ZM1 fragment by using ORF Finder program from NCBI database.



Appendix Figure 12 Graphic view of ZM1 sequence alignment using BLASTP (2.2.24) program from NCBI database.



Appendix Figure 13 Alignment of the amino acid sequences of ZM1 with Ku70 homologs from GenBank database under the following accession no: XP_002459446.1, XP_002461302.1, NP_001059061.1, NP_001167807.1, XP_002267875.1, BAF03493.1, XP_002317447.1, XP_002521532.1, AAT48365.1, ABR16802.1 and NP_564012.1 by clustalW2 alignment of deduced amino acid sequences.

CURRICULUM VITAE

NAME : Ms. Phanatchakorn Banthaophit

BIRTH DATE : February 15, 1977

BIRTH PLACE : Loei, Thailand

EDUCATION :

YEAR	**INSTITUTE**	**DEGREE/DEPLOMA**
2000	Kasetsart Univ.	B.Sc. (General Science)