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IDENTIFICATION OF ETHYLENE RESPONSIVE GENES THAT CONTROL FLOWERING OF *GUZMANIA LINGULATA* 'ANITA'

A Dissertation Presented

by

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DEDICATION

To my dad

ABSTRACT

IDENTIFICATION OF ETHYLENE RESPONSIVE GENES THAT CONTROL FLOWERING OF *GUZMANIA LINGULATA* 'ANITA'

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It is well established that ethylene application induces flowering in bromeliads, and yet no ethylene inductive flowering pathway or ethylene responsive genes involved in flowering have been determined. Ethylene exposure time required to induce flowering of *Guzmania lingulata* 'Anita' was investigated by exposing plants to 100 $\mu l \cdot l^{-1}$ of ethylene for 4, 6, 8, 10, 12, 16, and 24 hours. Control plants were exposed to ethylene-free air for the same lengths of time. Plants exposed to ethylene for 4 hours did not flower, while exposure for 6 hours or longer resulted in 100% flowering. Suppression of endogenous ethylene synthesis, using ethylene synthesis inhibitor amino-ethoxyvinylglycine (AVG), resulted in the longer ethylene exposure time of 20 hours to obtain 100% flowering. This result indicates that endogenous ethylene production is involved in floral induction. Ethylene treatment on single young leaf induced flowering as well. Application of the protein synthesis inhibitor, cycloheximide, prevented flowering induced by ethylene, suggesting that activation of ethylene responsive genes is followed by synthesis of proteins involved in flowering.

The differential gene expression associated with ethylene induced flowering was investigated by 'differential display'. The RNA was isolated from the leaves of *Guzmania* plants exposed to ethylene for 4 or 6 hours and to ethylene-free air for 6 hours. Reverse transcriptase polymerase chain reactions (RT-PCR) were conducted and sixteen differentially expressed cDNAs were isolated, cloned and sequenced. Reverse Northern blotting technique, conducted to confirm differential expression, resulted in selection of 5 cDNAs expressed in flowering plants. The protein predicted from the cDNA number 5 is a putative sugar transporter belonging to the Major Facilitator Superfamily (MSF). Translated sequence of cDNA number 11 has homology to an *Arabidopsis* sucrose transporter and belongs to the ABC transporter suparfamily. Putative protein 9 had homology to an *Arabidopsis* ethylene-response element involved in plant defense. Two cDNA sequences (number 8 and number 10) did not have homology to any reported genes in the GenBank. The activation of sugar transporter genes in the leaves of ethylene-induced plants provides suggestive evidence that sucrose is a long distance signaling molecule.

Key words: Bromeliad, amino-ethoxyvinylglycine (AVG), cycloheximide, differential display, sucrose

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

LITERATURE REVIEW

Ethylene induced flowering

The gaseous hormone ethylene has a wide range of effects on plant growth and development, from seed germination to fruit ripening and senescence (Abeles, 1992). It is also acknowledged that ethylene plays a role in flowering, and is commonly used to induce flowering in bromeliads. The artificial induction of flowering in pineapple dates back to 1874 when it was accidentally discovered that wood smoke forced pineapple in the field to flower uniformly (De Greef et al., 1989). Rodriguez (1932) reported that ethylene was the constituent of the smoke that affected flowering and that application of ethylene gas alone results in flowering. The promotion of flowering in pineapple by ethylene had become an important horticultural practice for synchronizing flowering and harvesting (Bartholomew, 1985). In addition, ornamental bromeliads such as *Guzmania*, *Vriesea*, *Aechmea*, *Billbergia*, can be forced to flower by exogenous ethylene (Abeles, 1992).

Ethylene releasing chemicals

Since the application of ethylene gas on a large scale is inefficient, other methods have been developed to apply ethylene directly to the plant (De Greef at al., 1989). Ethylene releasing compound, 2-chloroethylphosphonic acid (Ethrel, ethephon) is commercially used as a flower forcing agent in the pineapple production. Ethephon is stable at pH 4.5 and below and is applied as an acidic solution. Once ethephon enters the alkaline environment of the plant cell it breaks down and release ethylene gas.

The use of ethephon to promote flowering of ornamental bromeliads has also been described (De Greef et al, 1989). A freshly prepared 3.5 mM solution of ethephon induced 100 % flowering of *Guzmania lingulata* 'Minor'. Ethylene release from the tested plant tissue was very high at the beginning of the treatment (2.9 µl per plant per hour) and lasted for 10 days after the onset of ethephon application.

De Greef et al. (1983) demonstrated that 1-aminocyclopropane-1-carboxylic acid (ACC) can also be used to promote flowering in bromeliads. Application of 0.2 mM ACC solution in the well of the plant induced 100% flowering in *Guzmania, Aechmea,* and *Vriesea.* Furthermore, it was reported in a separate study that ACC can promote flowering after natural flowering had been inhibited by aminooxyacetic acid (AOA) or aminoethoxy-vinylglycine (AVG) (Mekers et al., 1983).

A structural analogue of ethylene, acetylene gas, as well as calcium carbide, which release acetylene in contact with water, are used as floral forcing agents (De Greef et al., 1989). Calcium carbide treatment, commonly used as an inductive agent in pineapple production in Hawaii, is very effective but not as predictable as the treatment with ethylene or ethephon (Bartholomew, 1985).

Application of auxins, such as naphthalene acetic acid (NAA) or 2,4-D, caused flowering in bromeliads as well (Cooper and Reese, 1942). This response was due to the ability of auxins to promote ethylene synthesis by up-regulating the transcription of ACC synthase gene and thus enhancing the conversion of S-adenosyl-methionine to ACC (Taiz and Zeiger, 1998). Application of 0.5 mg NAA per pineapple plant resulted in flowering 6 weeks later (Zeevaart, 1978). Ethylene release from the plant was detected within one

day and lasted for at least 7 days. Plants evolved between 15 and 60 µl of ethylene per kg fresh weight per day. No ethylene was detected around non-treated plants.

Treatments that induce a burst of ethylene are effective in inducing flowering, for example shaking in *Guzmania lingulata* (De Greef et al., 1989) or anaerobiosis in *Iris hollandica* (Kinet, 1993).

Ethylene synthesis inhibitor, AVG, was used to investigate the role of endogenous ethylene in flowering of bromeliads (De Proft et al., 1984). AVG is a very potent inhibitor of spontaneous flowering of bromeliads and is commercially used to increase plant size of ornamental bromeliads. Application of AVG suppresses auxin-induced ethylene production as well. Concentrations of 10-20 $\mu l \cdot l^{-1}$ AVG fully inhibit flowering of *Guzmania* plants, and this inhibition can be nullified by subsequent ethylene treatment (100 $\mu l \cdot l^{-1}$ of ethylene for 24 hours).

Plant maturity and flowering

During a plant development the shoot apical meristem progresses through three developmental phases: juvenile, adult vegetative and adult reproductive (Taiz and Zeiger, 1998). While the transition from juvenile to adult vegetative phase is gradual, the phase change from adult vegetative to adult reproductive is very sudden. The duration of the juvenile phase varies from a few days or weeks in herbaceous plants to as long as 30 to 40 years in some trees (Vince-Prue, 1987). In the juvenile phase, flowering cannot be induced by any treatment. When a plant can respond to a specific developmental signal in the expected manner, it has reached the competent stage. The competent stage describes the capacity of the shoot apical meristem to develop reproductive organs.

To be able to respond to ethylene treatment, bromeliads need to reach a certain maturity level. Age, or perhaps more importantly the size of the plant, is a necessary internal factor controlling the switch to maturity. In *Aechmea victoriana,* plants weighing less than 22 grams were not able to respond to ethylene treatment (100 $\mu l \cdot l^{-1}$ for 24 hours) (De Greef et al., 1983). A full response was observed in plants weighing more than 23 grams. Furthermore, the number of flowers per inflorescence increased, from 10 to 33, as the plant weight increased. When *Guzmania lingulata* reaches 20 to 35 grams, it is considered 'ripen to respond' to ethylene treatment (De Greef at al., 1989). It has been shown that a minimum plant size of 1 kg is required for forcing pineapple to flower (Zimmer, 1985).

The size of the apex itself can be a possible cue for the transition to maturity; older apices are usually larger (Thomas and Vince-Prue, 1984). For example in ivy, mature apices have a much larger meristematic areas than juvenile ones.

Some authors explain the induction of flowering by ethylene exposure as a form of ethylene-enhanced aging (Abeles, 1992). It has been shown that ethylene treatment of the small size corms in *Triteleia laxa* reduces the duration of the juvenile phase and hastens ripeness to flower. Ethylene treatment resulted in a greater final size of the apical meristems in the corms (Han et al., 1990). Treatment of Mango (*Mangifera indica*) orchards with ethephon reduced the juvenile phase from 6 to 3.5 years, thus allowing the earlier production of fruits (Bernier et al., 1981).

The ability to convert ACC to ethylene has been used as an indicator of the physiological maturity of bromeliads. Juvenile *Aechmea* plants have a reduced capacity to convert exogenous ACC to ethylene, compared to mature plants (De Greef et al.,

1983). Treating juvenile *Guzmania* plants with naphthalene acetic acid (NAA), not ethylene, hastens ripeness of plants to flower by conferring on them the capacity to convert exogenous ACC into ethylene (De Greef et al., 1983). Pretreatments of bromeliads with auxins without addition of ACC afterward had no effect on the flowering response, while combined with a post-treatment of 50 $\mu l \cdot l^{-1}$ ACC at day seven had dramatic effect on flowering.

Following a 24-hr ethylene treatment, Traub (1940) studied the initiation and development of the pineapple inflorescence in longitudinal sections of the shoot apex. After 3 to 4 days, apex started to enlarge and, 10 to 14 days later, flower primordia appeared in axils of the bracts. Gifford (1969) studied early cytohistological changes in the shoot apical meristem following flower induction. Higher RNA levels and enlarged nuclei are determined in the cells of the apical zone three days after the ethylene treatment. The cells in the apical zone of induced apex were less vacuolated as compared to cells of non-induced apex.

Ethylene and photoperiod

It has been proposed that there is an interaction between ethylene and photoperiodic induction of flowering (Zeevaart, 1978). Some authors hypothesized that ethylene may function normally in the regulation of photoperiodic flower induction. In pineapple, a quantitative SD plant, photoperiod does not seem to be the main controlling factor for flower induction under natural Hawaiian conditions. Depending on the kind of plant material used and the time of planting, natural initiation of flowering can occur in any month of the year (Gowing, 1960).

Preliminary studies have shown that ethylene treated *Guzmania lingulata* 'Minor' plants flower regardless of photoperiodic conditions. Ethylene treated plants grown under long day conditions underwent flower transition significantly faster than plants grown under any other photoperiodic treatment (Dukovski et al., 2001). Plants grown under long day condition were exposed to approximately 2 ^{o}C higher temperature, due to the build up of heat under black cloth which might speed up the flower transition.

Ethylene application inhibited or delayed flowering in many SD plants (*Perilla, Xanthium*, *Lemna, Chenopodium, Chrysanthemum*) under inductive photoperiod (Zeevaart, 1978). In contrast, ethylene induced flowering in SD plant, *Plumbago indica,* under non-inductive photoperiod (Nitsch and Nitsch, 1969). In a separate study, exposure of the long day plant, *Spinacia oleracea,* to single long day increased the conversion of ACC to ethylene (Bernier, 1988). Exposure of *Chenopodium,* a short day plant, to long nights resulted in decrease of the ACC to ethylene conversion.

Changes in gene expression associated with floral induction

The flowering process is very complex and is composed of sequences of events that are temporally and spatially ordered (Kinet, 1993). The process involves floral induction, transduction of the induced state to the meristem, floral evocation of the meristem, and organogenesis. These different steps have their own specific requirements and are affected differently by environmental and chemical factors. Floral induction involves mainly leaves, and results in production and translocation of floral stimulus to the apex. This stimulus irreversibly commits the shoot apical meristem to produce an inflorescence. Floral evocation refers to the processes occurring in the shoot apical meristems, following signal transduction, that commits the apical meristem to floral

morphology. Organogenesis involves the development of the flower parts. Being highly affected by the environmental factors, floral induction and floral evocation are key steps for the regulation of the floral transition (O'Neill, 1993).

Several studies have examined protein changes associated with floral induction. Stiles and Davies (1976) reported the disappearance of a major polypeptide in photoperiodically induced leaves of *Pharbitis nil*. The disappearance was registered within one hour after the end of the treatment. The authors suggested that this finding supports the hypothesis that floral stimulus is synthesized constantly but is rapidly degraded by a specific enzyme (floral inhibitor). In photoperiodically induced cotyledons, the hypothetical enzyme may be reduced, allowing floral stimulus to accumulate and be translocated to the shoot apex. However, in the separate study on *Impatiens balsamina,* total protein content increased after the inductive photoperiod (Sawhney et al., 1976).

In 1986, two groups of scientists examined polypeptide composition in induced and non-induced cotyledons of *Pharbitis nil* (Bassett et al., 1986., Lay-Yee, 1986). They used two-dimensional polyacryamide gel electrophoresis (PAGE) and minimized differences between two samples by exposing plants to 16 hours of darkness or 16 hours of darkness with a 15-minute light break. Lay -Yee demonstrated a decrease in the abundance of four polypeptides which is in agreement with the floral inhibitor theory. In agreement with the floral inhibitor theory, the decrease in the abundance of some polypeptides was detected following floral induction of *Xanthium strumarium* (Kannangara et al., 1990). Using very sensitive silver staining technique, the authors

were able to demonstrate the disappearance of two polypeptides (15 and 16 kDa) from the leaf tissue after photoinduction.

Warm (1984) analyzed changes in the mRNA composition associated with floral induction using *in vitro* translation products of mRNA, isolated from the leaves of induced and non-induced long-day plant *Hyosciamus niger*. He was able to detect changes in the mRNA composition in florally induced plants. The level of several mRNA encoding for small polypeptides (4 - 48 kDa) increased. In a separate study, comparison of photoperiodically induced *Pharbitis nil* and non-induced (control) plants resulted in a single mRNA quantitatively increased in induced plants (Lay-Yee et al., 1987).

In maize, a gene *indeterminate* (*id*) has been isolated, which may regulate the synthesis of a flower promoting substance (Colasanti et al., 1998). A mutation of *id* limits plant's ability to make floral transition, and thus causes prolonged vegetative growth. This is the first gene in monocots to be implicated in floral signaling, while 3 genes have been found in *Arabidopsis* to have a putative role in controlling floral signals (*co, ld*, and *fca*).

Carbohydrates in the control of flowering

Carbohydrates play an important, but still not fully determined, role in floral transition (Bernier et al., 1993). Several hypotheses have been proposed on the role of carbohydrates in flower induction. One theory is in agreement with the nutrition concept of flowering which states that in floral evocation the level of soluble sugars, such as sucrose, glucose, fructose, glucose-6-phosphate, and fructose-6-phosphate, increases to provide energy required for cell divisions in shoot apex (Bernier, 1988). This theory suggests that flower initiation is the result of increased supply of assimilates to the apex

and it is supported by the evidence of increasing content of soluble sugars in the apex of *Cheiranthus cheiri* L. after inductive cold treatment (Diomaiuto, 1988).

Additional evidence that sucrose may function in a long distance signaling comes from the studies on a short day (SD) plant *Xanthium* (Houssa et al., 1991). Analysis of the phloem sap from a leaf located on a plant induced to flower by a single long night (16 hours) showed an increase of sucrose when compared to vegetative plants being kept at 18 hour long day. Despite the reduction of the light period, the increased sucrose content in the apex of induced plants indicates that sucrose is not a result of *de novo* synthesis of the carbohydrates. Bodson and Outlaw (1985) suggested that sucrose accumulates very early in the apical meristems of induced plants and this accumulation is followed by energy-requiring, intensive mitotic action in the apical meristem. This suggests that sucrose function as a messenger in the floral transition. Using $^{14}CO_2$, Bodson et al. (1977) proved that recently synthesized assimilates in the plant were not used for sucrose elevation in the meristem. The authors suggested that extra sucrose comes from the mobilization of starch stored in the leaves and the stems.

Ethylene perception and signal transduction

Ethylene action is similar in all plant responses it regulates: ethylene binding to a receptor is followed by one or more transduction pathways and results in an alteration of a pattern of gene expression (Abeles, 1992). Ethylene alters the expression of many genes, including genes that encode pathogenesis-related (PR) proteins, many ripening-related genes, and ethylene biosynthesis genes (Taiz and Zeiger, 1998). Some of these genes have been isolated, cloned and sequenced which led to a better understanding of their functions. Cellulase genes from bean abscission zone and avocado fruit,

polygalacturonase gene from tomato, β-1, 3-glucanase, and chitinase genes from beans and potato are among the first genes cloned in relation to abscission and ripening (Abeles, 1992). Great progress has been made in cloning of ACC synthase genes from various plant species. Their functional expression in different systems has revealed that ACC synthase is encoded by a multigene family. At least nine ACC synthase genes were determined in tomato, six in mung bean, two in zucchini, and eight in pineapple. They are expressed differentially in response to different stress factors. For example, in tomato *LE-ACS2* is induced in response to wounding, while *LE-ACS3* is expressed during flooding (Fluhr et al., 1993, Olson et al., 1995). In mung bean, two ACC genes are induced by auxins (Botella et al., 1992), while one of them is also induced by mechanical stress (Botella et al., 1995). In a study with pineapple, a cold shock-induced ACC synthase gene was isolated and characterized (Bottela et al, 2000).

There is very little information on a tissue distribution of ethylene binding sites. In pea epicotyls, the apex had the highest concentration of binding sites (Sanders et al., 1991). In *Phaseolus,* the highest concentration of binding sites was found in the abscission zones. Furthermore, using electron microscopy autoradiography of ^{14}C labeled ethylene, it was found that ethylene binding sites are located in the plasma membrane and the endoplasmic reticulum membranes (Evans et al., 1982).

Remarkable progress has been made in the understanding of ethylene perception and action using *Arabidopsis* mutants (Taiz and Zeiger, 1998). The triple response morphology of etiolated seedlings is used to isolate mutants affected in their response to ethylene. The triple response is described as reduced stem elongation, increased radial growth (swelling), and abnormal horizontal growth. Two classes of ethylene mutants

have been isolated: ethylene resistant and ethylene insensitive mutants (*etr1, ein2, ein3*) which do not show triple response in the presence of ethylene, and mutants that show triple response in the absence of exogenous ethylene, constitutive mutants (*ctr1*).

Through analysis of ethylene insensitive mutants, identified as tall seedlings among many short seedlings in the presence of ethylene, five ethylene receptors have been identified in *Arabidopsis* (ETR1, ETR2, EIN4, ERS1 and ERS2 (Hua et al., 1995, 1998). The *ETR1* gene has been cloned and sequenced and it exhibits significant sequence homology to the bacterial two-component sensing system (Chang et al., 1993). The *Arabidopsis* ETR1 protein functions as a dimer consisting of two transmembrane proteins linked by disulfide bonds and can be divided into three functional domains (Chang et al., 1993). The amino-terminal sensor domain contains three putative transmembrane segments within which all known mutations resulting in a loss of ethylene sensitivity are located. When expressed in yeast, this domain has been shown to bind ethylene, however *etr1* mutation abolished ethylene binding (Shaller et al., 1995). This finding was the evidence that amino-terminal of ETR1 is the ethylene binding site. The second domain, carboxy-terminal, exhibits homology to His kinases that are autophosphorylated in bacterial two-component sensing systems. The carboxy-terminal portion of the ETR1 protein in *Arabidopsis* has shown His kinase activity *in vitro* as well (Gamble et al., 1998). Bacterial two-component system consists of two proteins, a sensor, hystidine kinases, and a response regulator that often acts as a transcription factor mediating responses to a range of environmental stimuli. The third domain, the response regulator or receiver domain, may receive the phosphate group from the His of the His-kinase domain at an Asp residue (Chang et al., 1993).

The *ctr1* (constitutive triple response 1) recessive mutation in *Arabidopsis* results in a constitutive activation of ethylene responses. The fact that this mutation activates response suggests that the wild-type protein acts as a negative regulator of the ethylene response pathway (Taiz and Zeiger, 1998). The constitutive triple response of *ctr1* mutant is not reversed by inhibitor of ethylene biosynthesis and binding, suggesting that the plant is defective in ethylene signal transduction. The CTR1 gene product is present in a cytoplasm and acts downstream of ethylene receptor as a negative regulator of ethylene signaling (Kieber et al., 1993). More precisely, CTR1 acts to phosphorylate components in ethylene signal transduction pathway making them inactive. When CTR1 becomes phosphorylated by the receiver domain of the ethylene receptor it becomes deactivated which leads to dephosphorylation of downstream components and finally transduction of ethylene signal.

Analysis of other ethylene-insensitive (*ein*) mutants revealed a series of gene products in *Arabidopsis*, EIN2 through EIN7, that are involved in the central part of the ethylene signal transduction pathway and are regulated by CTR1. The *EIN2* encodes a protein that contains 12 membrane spanning domains, suggesting that it may act as a channel or pore (Taiz and Zeiger, 1998).

The *EIN3* encodes a protein that acts as a transcription factor. This nuclear gene product is made functional by post-transcriptional modifications involving dephosphorylation. The EIN3 is a direct regulator of transcription factors that control ethylene regulated genes. Ethylene regulated genes contain sequences called ethylene response elements (EREs) in the upstream regulatory region (Taiz and Zeiger, 1998). An AGCCGCC motif (GCC box) was identified and found to be necessary for ethylene-

induced gene expression. Ethylene response element binding proteins (EREBPs) bind to the GCC box to initiate transcription. It has been shown that the steady state level of these proteins increases dramatically in the presence of ethylene.

Genetic control of flowering: *Arabidopsis* as a model system

The transition from the vegetative rosette leaves to the reproductive inflorescence has been extensively studied in *Arabidopsis*. Many mutations of genes involved either in delaying or promoting floral transition have been identified (Levy and Dean, 1998). Studies of these mutations have revealed information about the genetic control of flowering (Weigel, 1995; Coupland, 1997, Pieiro and Coupland, 1998). Flowering involves the sequential action of two groups of genes: floral meristem identity genes, responsible for the transition of the shoot apical meristem from a vegetative to a reproductive state, and floral organ identity genes which direct the formation of the various floral organs. The third group, flowering time genes, control flowering time by interacting with floral meristem identity genes, which then turn-on the floral organ identity genes. If the activity of floral meristem identity genes is reduced, flowers that develop would have various shoot-like structures. The best characterized floral meristem identity genes in *Arabidopsis* are: *LEAFY* (*LFY*), *APETALA1* (*AP1*), *APETALA2* (*AP2*), *CAULIFLOWER* (*CAL*), and *UNUSUAL FLOWER ORGANS* (*UFO*) (Pineiro and Coupland, 1998). Most floral meristem identity genes encode transcription factors: *LFY* encodes a DNA binding protein that acts as a developmental switch (Weigel and Nilsson, 1995), *ap1* and *cal* encode transcription factors with MADS domain (MCM1-Agamous-Deficiens-SRF), and *ap2* encodes a protein carrying a novel DNA-binding motif with homology to ethylene-responsive element-binding proteins (Weigel, 1995). The

functions of floral meristem identity genes are partly redundant, thus plants with mutation in a single gene typically retain at least some flower-like structures (Weigel and Nilsson, 1995). Double mutants *lfy/ap1* show a more severe phenotype than either single mutant, with flower-like structures rarely observed.

In addition, floral mristem identity genes *AP1* and *AP2*, together with *APETALA3* (*AP3*), *PISTILA* (*PI*), and *AGAMOUS* (*AG*) play a role in the 'ABC' system of floral organ development, thus can be classified as floral organ identity genes as well (Taiz and Zeiger, 1998). In this system, each of the four whorls of floral organs is defined by the groups of genes expressed, giving each whorl its distinctive identity. The organ identity genes are grouped into three categories, designated A, B, and C. The group A includes *AP1* and *AP2*, group B includes *AP3* and *PI*, and group C consists of *AG*. While, group A genes are expressed in the sepals, groups A and B are expressed in the petals. In the stamens genes from groups B ad C are expressed, and in the pistil, group C genes are expressed. Furthermore, while the expression of C genes is masked by the activity of A genes in the sepals and petals, the expression of A genes in the stamen and pistil whorls is repressed by the activity of C genes.

The response of flowering time mutants to environmental factors, such as vernalization and photoperiod, together with genetic analyses have established the existence of at least four pathways that control flowering time in *Arabidopsis* (Pineiro and Coupland.,1998; Levy and Dean,1998). Two of these pathways, the floral repression pathway and the autonomous promotion pathway, control developmental process of the plant. The genes in floral repression pathway prevent flowering until the plant has reached a certain age or size, whereas genes in the autonomous promotion pathway

antagonize this repression as the plant develops. Mutation of the major repressor genes *EMBRYONIC FLOWER* (*EMF*) results in flowering without forming any rosette leaves (Sung et al., 1992). The *EMF* genes act as negative regulators of floral meristem identity genes. It has been shown that some gene products that promote flowering may act as repressors of *EMF* function (Levy and Dean, 1998). Another floral repressor, *TERMINAL FLOWER 1* (*TFL1*), functions to suppress flower formation at the apex by repressing the function of genes operating in autonomous promotion pathway. The last two genes from repression pathway, *CURLY LEAF* (*CLF*) and *WAVY LEAVES AND COTYLEDONES* (*WLC*) delay flowering by repressing floral meristem identity genes *AG* and *AP3*.

As the plant develops, the genes in the autonomous promotion pathway act to overcome the repression genes (Levy and Dean, 1998). Mutations of the genes in the autonomous promotion pathway result in a plant with late flowering phenotype. Two genes acting in the autonomous promotion pathway, *LUMINIDEPENDENCE* (*LD*) and *FCA*, encode regulatory proteins: a transcription factor and RNA-binding protein (Pineiro and Coupland, 1998). Analysis of the interaction of *FCA* and floral meristem identity genes indicates that *FCA* function is required for activation of *LFY* and *AP1* (Levy and Dean, 1998).

The last two pathways, the photoperiodic pathway and the vernalization pathway, mediate signals from the environment. In the photoperiodic pathway, light is perceived by the photoreceptors, which initiate signals that interact with a circadian clock, somehow measure day length, and when the length of the dark period decreases below a critical length, genes that promote flowering are activated (Levy and Dean, 1998). This

activation leads in turn to the activation of floral meristem identity genes, and results in flowering. Overexpression of circadian clock component, *LATE ELONGATED HYPOCOTYLS* (*LHY*) gene delays flowering time under long day conditions and causes many circadian clock-regulated processes to become arrhythmic. Even though the correlation between circadian clock and flowering time is established, the way of interaction is still unclear. Within the photoperiodic pathway, the gene *CONSTANCE* (*CO*) encodes a transcription factor that is required for the acceleration of flowering under long day conditions (Ma, 1997). It was found that the *CO* mRNA is present at a higher level under long days than short days, and overexpression of *CO* causes very early flowering that is insensitive to day length. This suggests that the low level of *CO* expression under short days is insufficient to promote flowering. Other genes, in particular, *SUPPRESSOR OF OVEREXPRESSION OF CO* (*SOC1*) and *FLOWERING LOCUS T* (*FT*) are required for *CO* to promote early flowering. This indicates that these genes act downstream of *CO* in flowering time regulation, and that *CO* activates the transcription of *SOC1* and *FT* (Samach et al., 2000).

Several genes which regulate floral induction in vernalization have been isolated in *Arabidopsis*. For example, *FLC* gene products prevent flowering in biennials unless they have experienced the cold winter (Levy and Dean, 1998). The other gene, *FRI* up-regulates *FLC* leading to delayed flowering and prolonged vegetative growth. The *FRI* is dominant over the effect of autonomous pathway genes (*LD*, *FCA*). In annuals, the late-flowering allele of *FRI* is absent thus the autonomous pathway down-regulates *FLC* expression. Vernalization promotes flowering in biennials by turning-off the *FLC* expression, thus alleviating the block to flowering. Following cold treatment, the *FLC*

transcript levels remain low for the remainder of plant's life, but return to high levels in the next generation.

So far, there is no study in the literature attempting to correlate ethylene induced flowering with already known pathways of floral induction. There is a possibility that ethylene-regulated gene products acts within already proposed flowering pathways, such as autonomous, repressive, photoperiodic or vernalization, or through completely separate flowering pathway. To address this question we conducted experiments to identify and characterize differentially expressed genes in the leaves of *Guzmania lingulata* 'Anita' induced to flowering by ethylene.

CHAPTER 2

FLOWERING INDUCTION OF *GUZMANIA LINGULATA* 'ANITA' BY ETHYLENE

Introduction

The plant hormone ethylene is known to regulate a variety of responses during plant growth and development. Some of these responses are seed germination, root hair formation, flowering, senescence, abscission, and fruit ripening (Abeles, 1992). Depending on the species, ethylene has different effects on flower induction. Flower induction of bromeliad species such as *Guzmania*, *Aechmea*, *Vriesea, Billbergia*, *Neoregelia,* and *Ananas* can be achieved by exogenous ethylene application (De Greef et al., 1989). Besides bromeliads, other species such as *Pumbago indica* L., *Mangifera indica* L., and *Chicorium intybus* L. can be induced to flower by ethylene treatment (Abeles, 1992). On the oher hand, flowering of *Xanthium, Lemna*, *Pharbitis, Chrysanthemum*, and *Chenopodium* is inhibited or delayed by ethylene when grown under inductive photoperiods.

In cultivation of bromeliads, flowering inducing agents such as acetylene gas, calcium carbide which releases acetylene in contact with water, and ethylene releasing compound, ethephon (2-chloroethylphosphonic acid), have been used (De Proft et al., 1986). De Greef et al. (1989) demonstrated that the ethylene synthesis precursor, ACC, could also be used as a floral inducing agent. While small amount of ethylene induces flowering, higher levels released from ethephon can cause a decrease in growth and development of the inflorescence. Compared to ethephon, ACC application results in a low amount of ethylene produced by the plant itself, sufficient for flower induction with

no adverse effect on inflorescence development. The size of the *Guzmania monostachya* L. R. inflorescence was not affected by the ACC treatment (De Proft et al., 1986).

Different species of bromeliads, even different varieties within the same species, have different threshold levels of ethylene exposure required for flowering. Studies of ethylene-induced flowering in pineapple (*Ananas comosus* L. Merr.) using different concentrations of ethylene and exposure times showed that a longer exposure time is needed when plants are exposed to lower concentrations of ethylene (Cooper and Reese, 1942). Application of ethylene at 100 $\mu l \cdot l^{-1}$ for 12 hours or at 1000 $\mu l \cdot l^{-1}$ for 6 hours resulted in a 100% flowering response. Furthermore, it was shown that the sites of ethylene perception and primary action are in the leaves, as ethylene did not induce flowering if all leaves were removed. However, if one leaf remained on the plant or if all leaves were removed 24 hours after the ethylene treatment, the plants would flower.

The intermediary processes which transduce flower inductive signals (i.e. ethylene, light) perceived in leaves to the apical meristem where flower transition takes place remain unknown. The biochemical processes resulting in the production of floral stimulus are most likely regulated at the level of gene transcription or posttranscriptional processes, such as RNA processing and stabilization, initiation of translation or post-translational modifications (O'Neill, 1993). Application of protein synthesis inhibitor, cycloheximide, to the leaves completely inhibits floral induction of *Xanthium strumarium* L. under inductive short day, suggesting that synthesis of new proteins is necessary for the floral induction (Ross, 1970). Synthesis of new proteins in the ethylene induced flowering in bromeliads and the mechanism by which ethylene triggers protein synthesis are still unexplored.

Endogenous ethylene production is positively correlated with flowering induction of *G. lingulata* 'Minor' L. B. Sm. (DeProft et al., 1984). Application of 0.1 mM solution of AVG, an inhibitor of endogenous ethylene production, fully suppressed spontaneous flowering by reducing endogenous ethylene production by 50% (from $3nl \cdot h^{-1}$ in control plants to $1.5nl \cdot h^{-1}$ in AVG treated plants). If AVG is applied at least one week before the ethylene treatment, it prevents flowering without damaging the leaves and disturbing the inflorescence development (Mekers et al., 1983). The inhibitory effect of AVG on flowering of *G. lingulata* 'Minor' can be counteracted by a subsequent application of either ethylene ($100 \mu l \cdot l^{-1}$ for 24 hours), ACC ($50 \mu l \cdot l^{-1}$), or ethephon ($500 \mu l \cdot l^{-1}$), resulting in 100% flowering (De Greef et al., 1989).

A series of experiments were conducted to determine the minimum ethylene exposure time for flowering of *Guzmania lingulata* 'Anita'; the role of endogenous ethylene production in flower induction; the flowering response to single-leaf ethylene application at different stages of development; and the role of protein synthesis in floral induction.

Material and Methods

Plant Material

The bromeliad, *Guzmania lingulata* 'Anita', was used as a model plant (Figure 1). The plants, supplied by Deroose Plants, Inc. (Apopka, Florida), were grown in a greenhouse under ambient light conditions and supplemented with 40-50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from high pressure sodium lamps daily between 4:00 PM and 10:00 PM. Temperature was maintained in a range of 21 °C to 27 °C. Plants were fertilized once a week using

110 mg·l^{-1} N-P-K 20-10-20 fertilizer. Once exposed to ethylene, plants were not fertilized until the end of experiment.

Figure 1: *Guzmania lingulata* 'Anita'

Ethylene treatment

Plants of uniform size and weight, at least 20 grams, were selected for each experiment. During the treatment, plants were exposed to continuous light (average of 7.3 µmol·m^{-2}·s^{-1}) and temperature (19 °C – 26 °C) condition. Six plants from each treatment were placed in three 36-l glass tanks (Figure 2), with two plants per tank. An initial volume (360 ml) of 10,000 µl·l^{-1} ethylene was injected into each of three 36-l sealed glass tanks to bring the starting concentration up to 100 µl·l^{-1}. Steady stream of 100 µl·l^{-1} ethylene was provided by a flow-through system at a flow rate of one air exchange per hour. The concentration of ethylene was monitored by collecting gas samples from the outlet of each tank and injecting into a Shimadzu GC-9A (Kyoto,

Japan) gas chromatograph fitted with a flame ionization detector. The exposure times were calculated from the time of the initial ethylene injection until the plants were removed from the tanks. For ethylene treatment on one leaf, 8-ml silicon chambers were constructed with an inlet and an outlet to allow a constant air flow (Figure 3). A section of the leaf (≈ 4.5 cm^2) was placed in the chamber and exposed to 100 $\mu l \cdot l^{-1}$ethylene. After the ethylene treatment, plants were returned to the greenhouse and monitored for changes.

Figure 2 : Flow-through system for ethylene treatment. For each treatment plants were placed in three 36-l sealed tanks. Ethylene was provided at a flow rate of one air exchange per hour and at a concentration of 100 $\mu l \cdot l^{-1}$.

Data were collected 6 weeks after the treatments, when the commitment to flowering, expressed by the formation of a central rosette containing at least 10 overlapping leaves (each less than 8 cm long), can be determined. The number of plants committed to

flowering was expressed as percentage of flowering. All experiments were conducted three times, with six replicate plants per treatment.

Figure 3 : Ethylene treatment on one leaf, flow-through system. Eight-ml silicon chamber with an inlet and outlet is shown. A section of the leaf (~ 4.5 cm^2) was placed in the chamber and exposed to constant flow of 100 $\mu l \cdot l^{-1}$ ethylene.

Minimum ethylene exposure time for flower induction

Ethylene exposure time required to induce flowering of *Guzmania lingulata* 'Anita' was investigated by exposing plants to 100 $\mu l \cdot l^{-1}$ ethylene for 0, 4, 6, 8, 10, 12, 16, and 24 hours. Control plants were exposed to ethylene-free air for the same lengths of time.

Role of endogenous ethylene production in flower induction

Following the same experimental design, the minimum ethylene exposure time for flower induction of AVG-treated *Guzmania* plants was determined. Plants were sprayed with a 10 mM AVG solution 14 days prior to the ethylene treatment, as suggested by Mekers et al. (1983). A period of two weeks was allowed between AVG application and ethylene treatment to ensure normal development of inflorescence. Plants were exposed to 100 $\mu l \cdot l^{-1}$ ethylene for 6, 12, 18, 20, and 24 hours. Plants not sprayed with AVG and exposed to ethylene for the same lengths of time served as the positive control. Plants sprayed with AVG and exposed to ethylene-free air for the same lengths of time served as the negative control.

Ethylene perception and developmental stages of leaves

An experiment was conducted to determine the variation in sensitivity of leaves of different stages to ethylene. The developmental stages of leaves were defined based on their length: 'young' leaves as those <10 cm and 'mature' leaves as those >10 cm. Six treatments were conducted: exposure of one mature or young leaf to 100 $\mu l \cdot l^{-1}$ ethylene for 24 hours, exposure of one mature or young leaf to ethylene-free air for 24 hours (negative control), and exposure of the whole plant to ethylene or ethylene-free air for 24 hours.

Role of protein synthesis in ethylene induced flowering

To study the role of protein synthesis in floral induction, protein synthesis inhibitor, cycloheximide (Sigma, St. Louis, MO), was used. A 0.1 mM solution of cycloheximide combined with Tween-20 (Polyoxyethylene-20-Sorbitan Monolaurate,

Fisher Scientific, Pittsburgh, PA), two drops per 100 ml, was poured into the 'center'of the plants, and leaves of each plant were brushed with the inhibitor at the beginning and at the end of a 24-hour ethylene or ethylene-free air exposure, as described by Ross (1970). Twenty-four hours after the end of the treatment, cycloheximide was poured out from the center and replaced with water. Water was used in place of the inhibitor in the control plants. Two additional controls were included: (1) exposure of the whole plant to 100 $\mu l \cdot l^{-1}$ of ethylene for 24 hours and (2) exposure to ethylene-free air for 24 hours.

Statistical analysis

Data were analyzed using the SAS General Linear Model procedure (SAS Institute Inc., 1999, Cary, NC). An arcsin transformation was performed on percentage data prior to analysis. ANOVA test were conducted and differences among treatments were further analyzed using Duncan's New Multiple Range Test.

Results and Discussion

Minimum ethylene exposure time for flower induction

Plants exposed to 100 $\mu l \cdot l^{-1}$ of ethylene for ≤ 4 hours did not flower, while ≥ 6 hours exposure induced 100% flowering (Figure 4). Every replication in this experiment showed a clean delineation between 0% flowering at 4 hours of ethylene exposure and 100% flowering at ≥ 6 hours of ethylene exposure. None of the plants exposed to ethylene-free air flowered. These results agree with those from studies conducted on pineapple and different genera of ornamental bromeliads (Cooper and Reese, 1942.; De Greef et al., 1989). Treatment of *Ananas comosus* plants with 1000 $\mu l \cdot l^{-1}$ of ethylene for 6 hours or longer resulted in 100% flowering (Cooper and Reese, 1942). Longer treatment time of 12 hours was required for 100% flowering when plants were exposed to

a lower ethylene concentration of 100 $\mu l \cdot l^{-1}$. De Greef et al. (1989) treated *G. lingulata* 'Minor' with 100$\mu l \cdot l^{-1}$ of ethylene for variable lengths of time and demonstrated that exposure to ethylene for 7 hours was enough to force plants to flower.

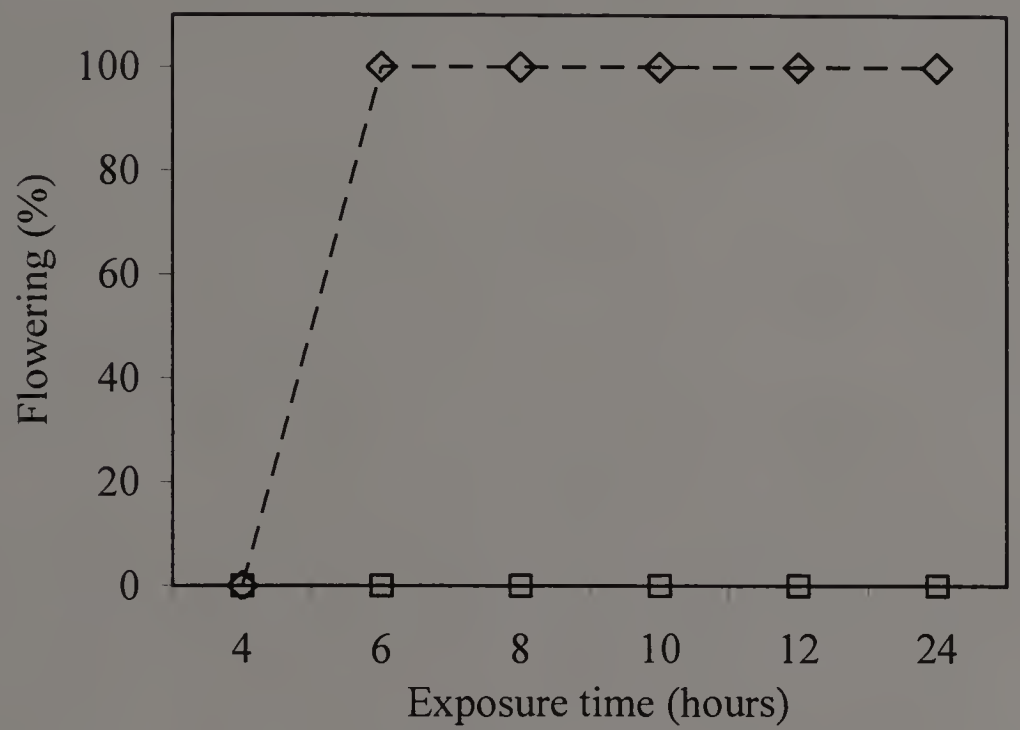


Figure 4: Minimum ethylene exposure time for flowering of *G. lingulata* 'Anita'. Plants were treated with 100 $\mu l \cdot l^{-1}$ of ethylene for 4, 6, 8, 10, 12, 16, and 24 hours (diamonds) or ethylene-free air for the same lengths of time (squares). Symbols represent the average of 3 replications, with 6 plants per treatment. SE are smaller than the symbols.

These results suggest that in order for bromeliads to go through the process of floral transition, they must surpass a threshold, which is reached after exposure to ethylene for a certain length of time. In the case of *G. lingulata* 'Anita', the threshold occurs after 6 hours of exposure to 100 $\mu l \cdot l^{-1}$ of ethylene.

The sharp difference in flowering between plants treated with ethylene for 4 hours (no flowering) and 6 hours (100% flowering) could be a result of difference in gene expression. It is possible that the products of ethylene-responsive genes accumulate in

the leaves until reaching a threshold level (6 hours of ethylene exposure) which then results in a signal sent to the apex where genes involved in floral evocation are activated. In the case of spontaneous flowering, there might be a slow accumulation of message or ethylene-responsive gene products during plant development and maturation. This explanation is consistent with the study of Colasanti et al. (1998) suggesting that a certain number of leaves is needed in order to produce the quantity of message needed for floral transition. In the case of maize, a minimum of eight leaves is required to achieve flower transition, as flower promoting message, a protein encoded by *indetermined (id)* gene, is expressed specifically in leaves. Mutation of *id* caused prolonged vegetative growth and inability to make the floral transition.

The role of endogenous ethylene production in flower induction

Ethylene biosynthesis is affected by several factors, including developmental stages of the plant, environmental conditions, physical and chemical injury, and other plant hormones (Taiz and Zeiger, 1998). Exogenous ethylene application induces production of autocatalytic ethylene in some plant species by activating ACC synthase genes (Abeles, 1992). ACC synthase is encoded by members of a multigene family in which each gene is differentially regulated by various environmental and developmental factors. Tomato, for example, has at least 9 ACC synthase gene members, which are differentially regulated by inducers such as auxin, fruit ripening, and wounding (Olson et al., 1991). To determine if endogenous ethylene production contributes to floral induction, *Guzmania* plants were sprayed with AVG, an ACC synthase inhibitor, 2 weeks before exposure to ethylene.

All AVG-treated plants exposed to ethylene for 20 hours or longer flowered, whereas none of the AVG-treated plants exposed to ethylene for 12 hours or less or to ethylene-free air flowered (Figure 5). Exposure to ethylene for 18 hours resulted in a partial flowering response (61.1%). Six hours or longer ethylene exposure induced 100% flowering in control plants not treated with AVG.

There was an obvious shift of the ethylene threshold from 6 hour in non-treated plants to 20 hours in AVG-treated plants. This result suggests that endogenous ethylene production greatly contributes to the floral induction by shortening the minimum exposure time from 20 to 6 hours.

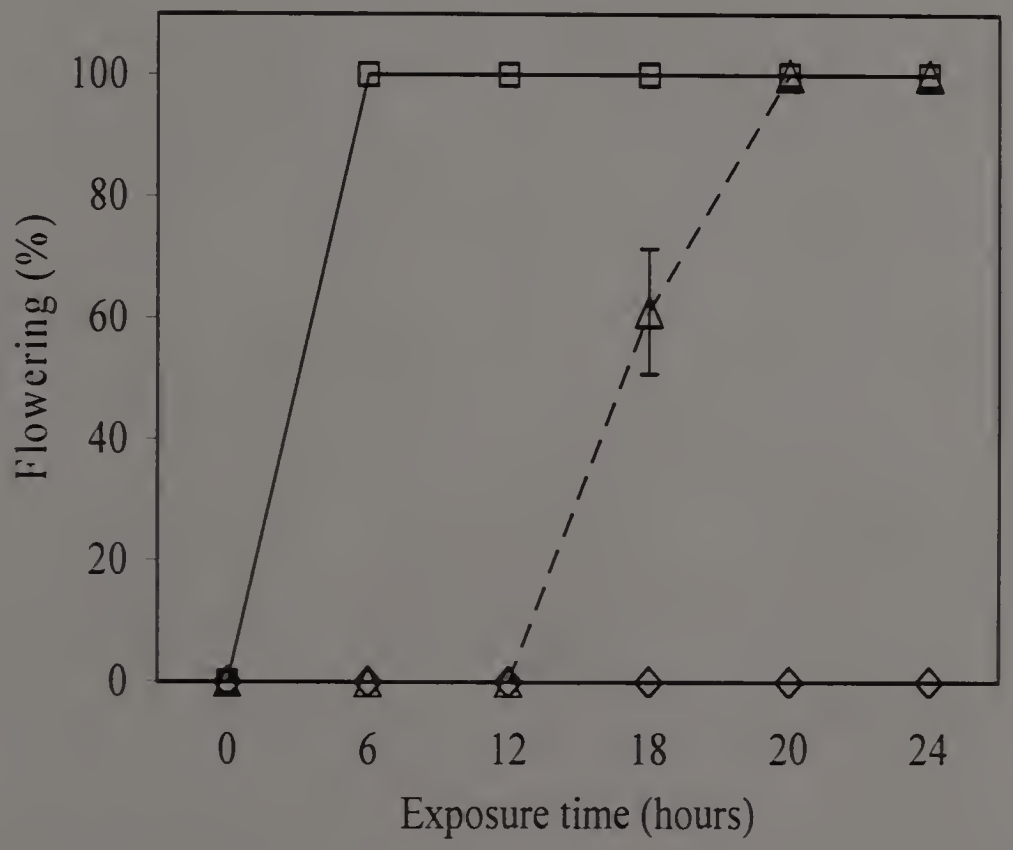


Figure 5: Minimum ethylene exposure time of AVG-treated *G. lingulata* 'Anita'. Plants were treated with AVG and 100 $\mu l \cdot l^{-1}$ of ethylene for 6, 12, 18, 20, and 24 hours (triangles) or with ethylene-free air for the same lengths of time (diamonds) or with ethylene only (squares). Symbols represent the average of three replications, with 6 plants per treatment (mean $\pm$ SE). Bars smaller than the symbols are not shown.

In agreement with previous results, a critical threshold level of ethylene-responsive gene products is needed to achieve floral transition. This level is reached after a specific amount of ethylene is perceived by a plant. Reduction in the endogenous ethylene level, due to inhibition of the autocatalytic synthesis might explain the increase in the length of ethylene exposure time needed for flowering.

Ethylene perception and developmental stages of leaves

To determine the sensitivity of leaves at different developmental stages to ethylene, one leaf per plant, 'mature' or 'young', were exposed to 100 $\mu l \cdot l^{-1}$ of ethylene or ethylene-free air for 24 hours. No difference is detected in flowering responses between plants exposed to ethylene on the single young leaf and those on the whole plant (Figure 6).

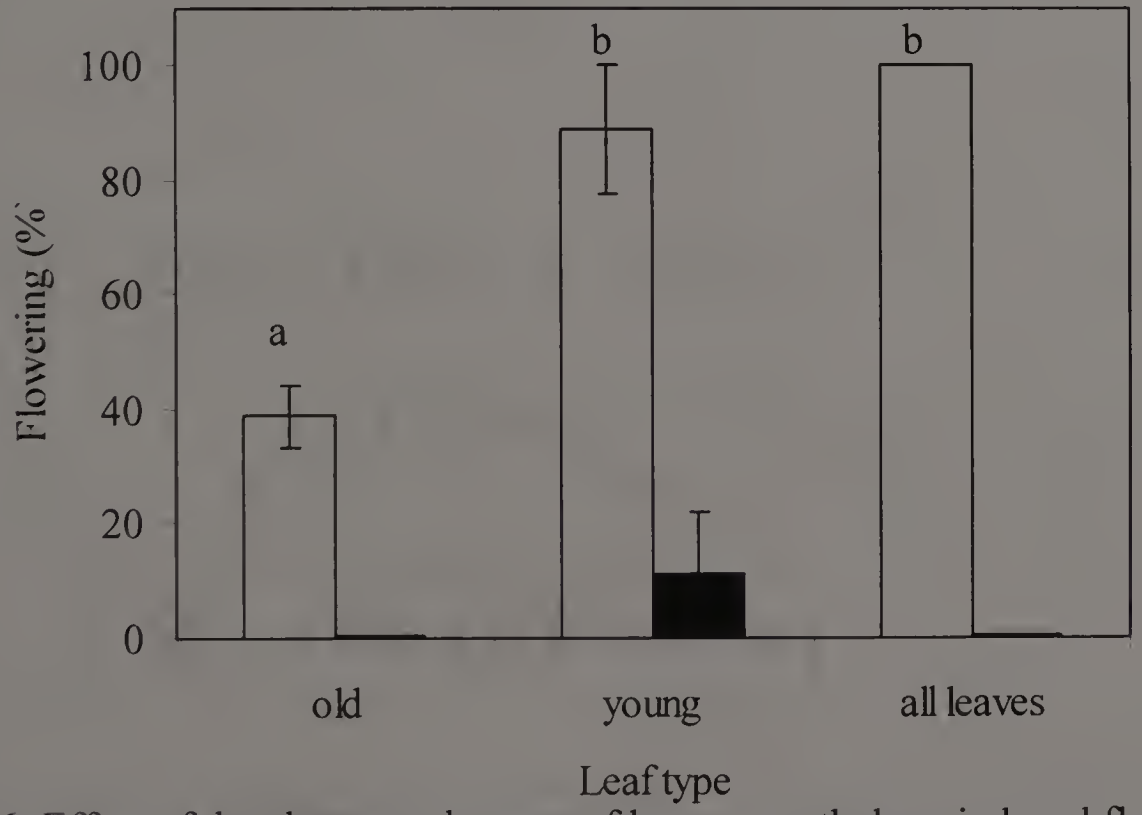


Figure 6: Effect of developmental stages of leaves on ethylene-induced flowering of *G. lingulata* 'Anita'. A single mature leaf (>10cm in length), a single young leaf (<10cm in lrngth), or whole plants were exposed to 100 $\mu l \cdot l^{-1}$ of ethylene (open bars) or ethylene-free air (solid bars) for 24 hours. Values for treatments (mean $\pm$ SE, $\underline{n} = 18$) sharing the same letter are not significantly different by Duncan New Multiple Range Test (DNMRT) at $\underline{P} = 0.05$.

The flowering response of plants where ethylene was applied to single mature leaf was significantly lower (38.9%).

Application of ethylene-free air to single mature leaf did not induce flowering, whereas a low percentage of plants (11.1%) where the young leaf was exposed to ethylene-free air flowered. This might be a result of stress-induced ethylene production caused by chamber installation. De Greef et al. (1989) reported that mechanical stress caused by handling or shaking *Guzmania* plants for 15 seconds may induce endogenous ethylene production (up to 110 nl ethylene· plant^{-1} ·hour^{-1}) and a consequent spontaneous flowering. Our results show that ethylene is perceived mainly by the young leaves. There is some ethylene sensitivity in mature leaves as well. Longer ethylene exposure to mature leaves might result in higher percentage of flowering.

Role of protein synthesis in ethylene induced flowering

Ethylene treatment on cycloheximide-treated plants resulted in 11.1% flowering. The response was not significantly different from control plants treated with cycloheximide and ethylene-free air or from plants treated with ethylene-free air only (Figure 7). Ethylene treatment alone, without cycloheximide, resulted in 94.4% flowering. The results suggest that *de novo* protein synthesis is needed for floral induction. It is possible that ethylene-responsive genes are activated by ethylene exposure and new proteins involved in floral transition are made. Since cycloheximide was applied to both, leaves and shoot apical meristem, the site for synthesis of new proteins involved in floral transition could not be determined.

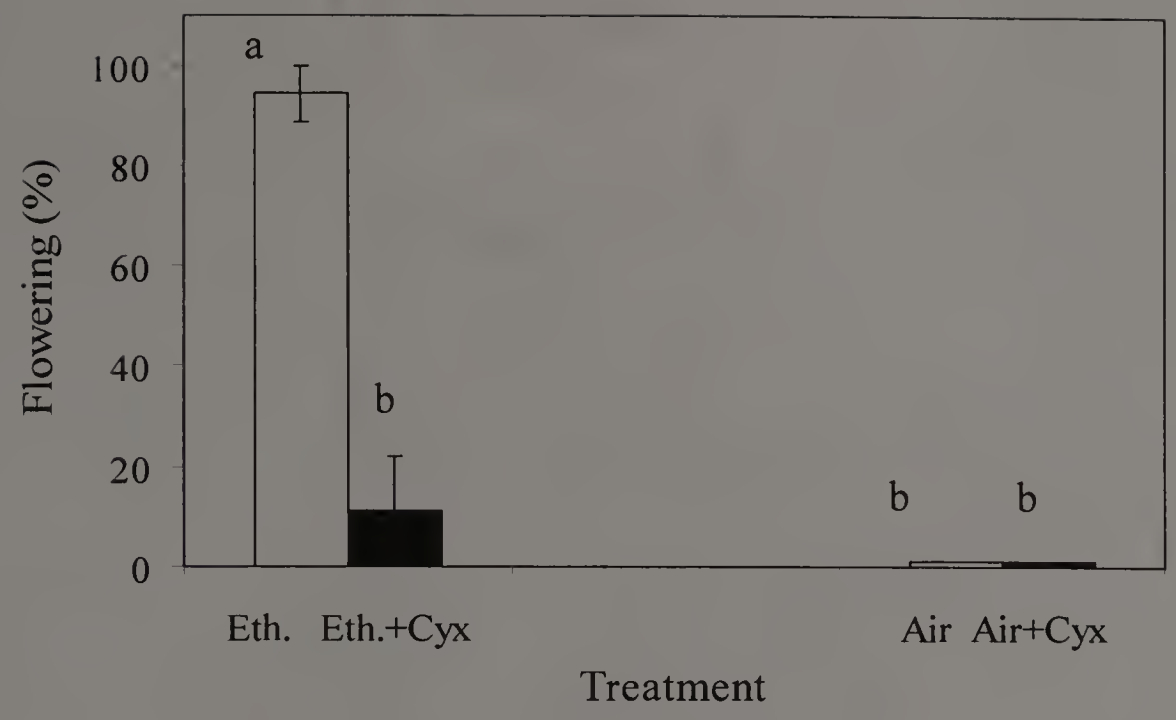


Figure 7: Effect of cycloheximide on flowering of *G. lingulata* 'Anita'. Plants were treated with cycloheximide (Cyx)(solid bars) or water (open bars) and exposed to 100 $\mu l \cdot l^{-1}$ of ethylene or ethylene-free air for 24 hours. Values for treatments (mean $\pm$ SE, $\underline{n} = 18$) sharing the same letter are not significantly different by Duncan New Multiple Range Test (DNMRT) at $\underline{P} = 0.05$.

Ross (1970) reported that flower initiation of short-day plant, *Xanthium strumarium* L., by inductive short days is inhibited by application of cycloheximide to the leaf or shoot-tip, although the effect was much greater when applied to the leaf.

Conclusions

Our study indicates that exposure of *Guzmania lingulata* 'Anita' to ethylene for 6 hours or longer can induce 100% flowering. Inhibition of endogenous ethylene production by AVG results in shifting of the threshold of ethylene exposure to achieve 100% flowering, from 6 to 20 hours. Exogenous ethylene application most likely induces autocatalytic ethylene production resulting in longer ethylene presence in the leaf tissue. Ethylene exposure on ≈ 4.5 cm^2 of a young leaf is sufficient to induce flowering. Once exposed to ethylene, plants synthesize new proteins needed for floral transition, as

inhibition of protein synthesis prevented flowering in ethylene treated plants. Further studies are needed to characterize proteins involved in ethylene induced flowering, their localization and function, as well as the mechanism by which ethylene activates their synthesis.

CHAPTER 3

ISOLATION OF DIFFERENTIALLY EXPRESSED GENES IN THE LEAVES OF *GUZMANIA* PLANTS EXPOSED TO ETHYLENE

Introduction

Flowering in plants is a result of the transition of the shoot apex from vegetative to reproductive development so that it gives rise to flowers rather than leaves (Bernier et al., 1993). The timing of this transition is determined by the age of the plant and by environmental conditions. A majority of plants use environmental cues, such as photoperiod and temperature, to regulate the transition to flowering. However, the biochemical mechanisms by which plants sense and respond to environmental variables are unknown. Environmental factors are perceived by different parts of the plant. Photoperiod and irradiance are perceived by mature leaves, while temperature is perceived by all plant parts, although low temperature is detected mainly by the shoot apex. Once perceived, signals are transported to the apical meristem and the fate of the apical meristem, remaining vegetative or becoming reproductive, is controlled by arrays of these long-distance signals (Taiz and Zeiger, 1998).

Studies on *Arabidopsis thaliana* have led to the identification of many components within individual signaling pathways that affect flowering. Flowering of *Arabidopsis* occurs rapidly under long days of 16 hours light, but is dramatically delayed under short days of 10 hours light. Genes acting in the long-day promoting pathway, *CONSTANCE*(*CO*), *CRYPTOCHROME2/FHA* (*CRY2*), *GIGANTEA* (*GI*), *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *FWA* are very well characterized (Koornneef et al., 1997). The *CO* acts downstream from *CRY2*

and *GI* and upstream from *SOC1*, *FT* and *FWA*. Mutations of *CO* delay flowering under long but not short days, suggesting that this gene is required to promote flowering only under long days. The *CO* encodes a nuclear protein that acts as a transcription factor.

Exposure to a low temperature for 4 to 8 weeks speeds up the flower transition in some *Arabidopsis* varieties (Michaels and Amasino, 2000). Two key genes acting in vernalization flowering pathway are *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*). The *FLC* gene encodes a repressor of flowering, a MADS box transcription factor, which is expressed at high level in vernalization-requiring winter annual varieties of *Arabidopsis*. Vernalization promotes flowering by reducing *FLC* expression and thus alleviating the block to flowering. The product of *FRI* gene up-regulates *FLC* expression, leading to delayed flowering.

Although separate, photoperiodic and vernalization pathways eventually converge to regulate the expression of the same floral meristem identity genes, which in turns initiate floral transition and activate floral organ identity genes (Pineiro and Coupland, 1998). However, some environmental effects on flowering have not been placed within the pathway structure. Furthermore, it has been widely observed that plants under stress flower prematurely. When plants are under stress, there is an increase in endogenous ethylene biosynthesis, due to the upregulation of the ACC synthase gene (Johnson and Ecker, 1998). Thus, the premature flowering observed in stressed plants can be correlated to an increased endogenous ethylene production in the affected plant. This is in agreement with the study on *Guzmania lingulata* showing that mechanical stress caused by shaking the plants can produce high ethylene level which leads to spontaneous flowering (De Greef et al., 1989). Since many plant species exhibit stress-induced

premature flowering, there is a possibility that ethylene acts as a universal flowering signal (Abeles, 1992).

So far, there is no study in the literature that correlates ethylene-induced flowering with already known pathways of floral induction. One possibility is that ethylene acts within already proposed flowering pathways, or that it acts through a completely separate pathway. Furthermore, the nature of the signaling molecules that carry a message from the ethylene perception site, leaves, to the shoot apical meristem, where flower initiation takes place, is still not fully understood.

The objective of this study was to identify the genes involved in ethylene-induced flowering. Early ethylene-regulated genes in the leaves of ethylene-induced flowering plants were screened using the RT-PCR differential display approach.

Material and Methods

Differential Display and RT-PCR

Differential gene expression associated with ethylene induced flowering of *Guzmania lingulata* 'Anita' was investigated by 'differential display', using RNA ectracted from the leaves. Plants were exposed to 100 $\mu l \cdot l^{-1}$ of ethylene for 4 and 6 hours, and control plants were exposed to ethylene-free air for 6 hours. The 4 -hour non-inductive treatment was used to identify all ethylene-induced genes not related to floral transition. The experiment was repeated twice, with two replica plants per treatment. After the treatments, leaves were excised, frozen in liquid nitrogen, and stored in a -80°C freezer. Total RNA from the leaves was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) (Appendix A). Isolated RNA was separated from DNA contamination using Message Clean Kit (GenHunter Corporation, Nashville, TN)

(Appendix B). The integrity of the RNA samples was determined by separation on a 2% formaldehyde gel (Appendix C). Quantities of RNA were determined using spectrophotometer (Spectronic 1201, Milton Roy Company) and diluted to 0.1 μg/μl, the final concentration used in reverse transcription reaction. Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted using RNA Image kits from GeneHunter Corporation (Nashville, TN) (Appendix D). The resulting single stranded cDNAs from each RT step were used as templates for synthesis of double stranded cDNAs in PCR reactions, using one of 72 combinations of fixed and arbitrary primers (Image Kits1, 2, and 3, GenHunter Corporation, Nashville, TN). These PCR products were labeled with ^{33}P deoxyadenosine triphosphate (dATP) and separated on 0.4mm thick, 6% polyacrylamide gels (Gel-Mix 6, Invitrogen Corporation, Carlsbad, CA) (Appendix E). Six lanes were run per primer combination: replicate samples from each treatment were run side by side and there were three treatments: 0, 4, and 6 hours of ethylene exposure. Gels were vacuum-dried for 40 minutes at 80 °C (Bio-Rad Gel Drier, model 583) and exposed to X-ray film for one to several days.

The autoradiograms were analyzed for the presence or absence of differential cDNA banding patterns between the different treatment samples amplified with the same primer combination. Only those primer combinations that displayed consistent patterns in the replicated samples were selected for the repeated run of RT-PCR reactions. Bands of interest were extracted from the gel, cleaned, and reamplified (Appendix F). Amplified cDNAs were resolved on 1.5% agarose gels and compared with 100 bp ladder (Promega, Madison, WI) to estimate the size of the fragments. Fragments were extracted and cleaned from agarose gels using Qiaex II Kit (Qiagen, Valencia, CA) following

manufacturer's instructions (Appendix G). The cDNA fragments were stored at -20°C until needed for cloning.

Cloning of cDNAs isolated from differential display gels

Approximately 50 ng of cDNA was ligated into the pGEM-T Easy plasmid cloning vector (Promega, Madison, WI) (Appendix H). The Taq Polymerase used in the PCR reaction leaves an 'A' overhang on each cDNA fragment and this plasmid has a corresponding 'T' overhang to allow an efficient ligation of the cDNA fragments into the cloning vector. The positive control reaction had 2 µl control insert DNA instead of cDNA. The background control had the same ingredients, except DNA (water was used to substitute DNA). The ligation reaction was incubated for 16 hours at 4 °C. After ligation, 2 µl of each product was used for transformation of *E.coli* JM 109 competent cells (Promega, Madison, WI) (Appendix I). Transformants were selected using the blue/white screening process: white colonies contained recombinant plasmid while blue colonies contained non-recombinant plasmids.

Colony screening for the multiple cDNA species of the same size

Knowing that multiple cDNA species of the same size can be amplified from the same differential display band, we screened colonies from each ligation reaction against two individual selected colonies that produced fragments with sizes expected from the display gel. Recombinant (white) colonies, picked at random from each ligation reaction were transferred to a new LB/Amp/IPTG/X-Gal plates and grown for 16 hours. These colonies were blotted onto a Hybond-N^+ membranes (Amersham Pharmacia Biotech, Ltd. Buckinghamshire, England) using the procedure for colony blotting (Appendix J). Blots were stored at 4 °C until used for probing with radiolabeled fragments of correct size

isolated from two individual plasmids. Hybridization reactions were conducted overnight at 68 °C (Appendix M). The blot was exposed to film for one day. If all colonies lighted up, we assumed that they contained the same fragment sequence and that there is only one species of cDNA of certain size.

Sample preparation for sequencing

Two white colonies from each ligation reaction were streaked onto LB/Amp plates and grown overnight. A single colony from each plate was inoculated into 5 ml liquid LB/Amp medium and incubated on a shaker (200 rpm) overnight at 37 °C. Plasmids were extracted from bacterial cells using standard plasmid-mini-prep procedure (Appendix K). 5 µl of plasmids was digested with *Eco*RI (Appendix L) and separated on 0.9% agarose gel, to confirm the presence of the fragment and estimate the size and the quantity of the fragment. Approximately 500 ng of recombinant plasmid DNA (dissolved in water) was sent for sequencing to the University of Maine DNA Sequencing Facility (Orono, ME) using ABI373 Sequencer (Applied Biosystems, Foster City, CA). Sequencing was done from both ends with T7 and Sp6 sequencing primers.

Reverse Northern blotting verification of differential expression

This experiment was conducted to confirm the presence of cloned cDNA sequences in the total RNA extracted from bromeliads leaves. Digested plasmids containing cDNAs of interest were run in duplicate, on separate halves of the same 0.9 % agarose gel to allow identical transfer onto a Hybond-N^+ membranes (Amersham Pharmacia Biotech, Ltd. Buckinghamshire, England) (Appendix N). One half of the membrane was hybridized overnight to a total cDNA probe from bromeliads leaves treated with ethylene for six hours and the other to a total cDNA probe from bromeliads

leaves exposed to ethylene for 4 hours. The probes were synthesized from cDNA obtained from reverse transcription of 5 μg of total RNA from each sample using 0.5 μg of oligo(dT)15 (Promega) (Appendix O). The whole reactions (25 μL) were radiolabeled using the previously described procedure (Appendix M). After hybridization, the blots were washed twice in 2X SSC 0.1% SDS and once in 1X SSC 0.1% SDS, covered with plastic wrap and exposed to film for several days.

Sequence analysis

Obtained sequences were cleaned from vector contamination using VecScreen tool at NCBI web site (http://www.ncbi.nml.nih.gov). Nucleotide sequences were translated to protein sequences using Ex-PaSy (Expert Protein Analysis System) bioinformatics tools at proteomics server (www.expasy.org) of the Swiss Institute of Bioinformatics (SIB) (Geneva, Switzerland). Sequence analysis included homology searches and alignments, superfamily or family classification, prediction of subcellular localization and structure-function analysis, including motif fingerprints, domain architecture, and examination of secondary structure.

BLAST search for homologs

cDNAs partial sequences were compared to sequences of previously reported genes using BLAST (Basic local alignment search tool) (Altschul et al., 1997) at the NCBI web server (http://www.ncbi.nml.nih.gov). The BLASTn was used to find homologous nucleotide sequences from the International Nucleotide Sequence Database (INSD), a collaboration of the GenBank, EMBL (European Molecular Biology Laboratories) and DDBJ (DNA Databank of Japan) databases. The protein sequences were queried with BLASTp and BLASTpsi using the SWISS-PORT

(www.ebi.ac.uk/swissport) (Bairoch and Apweiler, 2000), TrEMBL (Translated European Molecular Biology Laboratories) (www.ebi.ac.uk/trembl/index.html) (O'Donovan et al., 2002), and PIR (Protein Information Resource) (www.pir.georgetown.edu/pir) (Wu et al., 2002) protein databases.

Families and Superfamilies

A family or superfamily contains all proteins for which there is structural evidence of a common evolutionary ancestor. Affiliation of derived proteins with family and superfamily was determined using InterProScan bioinformatics tool from EMBL-EBI web site (www.ebi.ac.uk/interpro/scan.html), FingerPrintScan from EBI (www.ebi.ac.uk/printscan/index.html) and Superfamily (http://supfam.mrc-lmb.cam.ac.uk/Superfamily) (Gough et al., 2001). All these search algorithms scan and align the query sequence against homologous sequences from the InterPro protein databases and provide integrated information about coserved motif patterns (fingerprints), functional domains, domain architecture and protein family of the query sequence.

Topology, transmembrane regions, and localization site in the cell

Topology and the prediction of transmembrane regions were performed using TopPred II web-based program (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), (Claros and von Heijne, 1994). TopPred II program uses different algorithms to analyze hydrophobicity and polarity of the amino acid residues as well as secondary structure data to determine whether or not a given protein is an integral membrane protein. Localization of predicted proteins in the cell was predicted using PSORT web-based program (http://psort.nibb.ac.jp) (Nakai and Horton, 1999).

Secondary structure

Secondary structures of predicted proteins were determined using PSIPRED program (http://bioinf.cs.ucl.ac.uk/cgi-bin/psipred). The position of helical structures, strands, and coils can be visualized with the graphics created by PSIPRED.

Results and Discussion

Reverse transcriptase polymerase chain reaction (RT-PCR) and differential display

The differential display step was conducted as previously described (Liang and Pardee, 1992) using total RNA extracted from leaves of *Guzmania* plants exposed to 100µl·l^{-1} of ethylene for 4 (non-inductive) and 6 (inductive) hours and from control plants exposed to ethylene-free air for 6 hours (Figure 8).

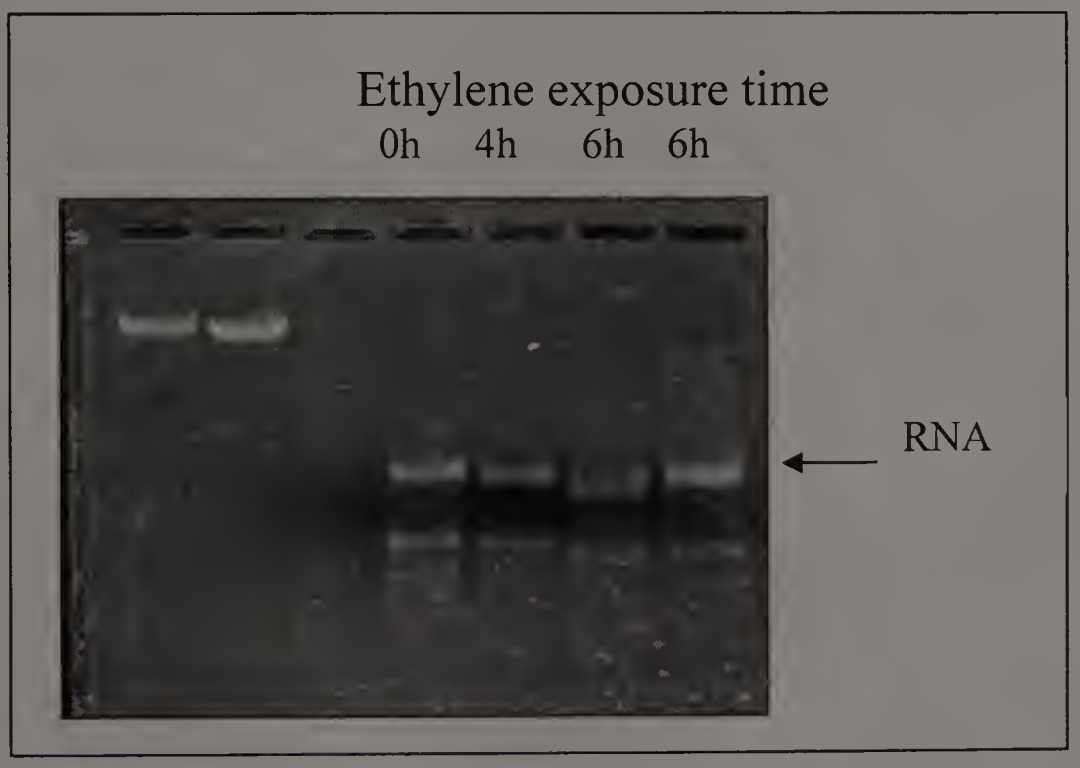


Figure 8: RNA extracted from *Guzmania* leaves. Plants were exposed to 100 µl·l^{-1} ethylene for 0, 4, and 6 h. Leaves were excised, than frozen in liquid nitrogen and RNA was extracted. RNA samples were separated on a 2 % formaldehyde gel.

A typical differential display autoradiogram is presented in Figure 9. The autograms were analyzed for the presence or absence of differential cDNA banding patterns in three treatments described earlier.

Figure 9: Example of typical differential display autoradiogram. The lane labels represent different treatments (0, 4, and 6 h of ethylene exposure). The same primer combination was used to amplify the fragments in all six samples. Bands were selected based on the three criteria described in the text (1. present in 6-h ethylene treatment, 2. present in 4- and 6-h ethylene treatment, and 3. present in 0- and 4-h ethylene treatment.

Sixteen cDNA fragments were isolated (Table 1) based on three different criteria. The first criteria was to identify bands that were present in the 6 hour ethylene treatment (plants induced to flower) but absent in the 0 and 4 hour ethylene treatment. Based on the

first criteria, seven fragments were isolated from the differential display gels. This pattern may be indicative of genes with floral expressed promoters.

Based on the second criteria which was to identify bands exclusive to both 4 and 6 hour-ethylene treatment, three fragments were isolated. There is a possibility that 4 hours of ethylene exposure activates synthesis of the floral signal, but it takes 6 hours of exogenous ethylene exposure for the floral signal to be transcribed, translated, and translocated from leaves to the meristems. This would suggest that the message for flower induction has been made throughout the ethylene treatment, but a threshold amount of the signal must reach the apex before the other genes in the flower inductive pathway are turned on. In other words, exposure to ethylene for 4 hours produces a flowering signal but the level has not reached the theashold need for flowering. In *Arabidopsis,* the gene *CO* (*CONSTANCE*) is involved in inductive photoperiodic pathway but, under non-inductive SD condition is expressed in leaves and stems at a level too low for its function (Ma, 2000). This suggests that during vegetative stage, the *CO* mRNA accumulates gradually, and when it reaches a threshold level or when the plants are grown under inductive long day (LD) conditions, the downstream genes are activated. The role of *CO* is to act as a timer that measures the age of the plant, but also to respond to the environment by flower induction.

Table 1: Differentially expressed eDNAs seleeted from differential display gel

Fragment #	Label*	Description
1	4G	present in 6 h treated
2	4G	present in 4 and 6 h treated
3	6G	present in 0 and 4 h treated
4	6G	present in 0 and 4 h treated
5	6G	present in 6 h treated
6	6G	present in 0 and 4 h treated
7	6G	present in 6 h treated
8	8C	present in 6 h treated
9	8C	present in 6 h treated
10	8C	present in 6 h treated
11	17C	present in 6 h treated
12	17C	present in 4 and 6 h treated
13	17C	present in 4 and 6 h treated
14	22C	present in 0 h treated
15	10G	present in 0 h treated
16	10G	present in 0 h treated

* The first number represents random primer, letter represents fixed primer

The third criteria was to identify bands present in 0 and 4 hour ethylene treatment (plants remained vegetative) but not in 6 hour ethylene treatment. Six fragments matching this criterion were isolated: three were present only in control plants, while the other three were present both, in control and 4 hour-treated plants. This pattern could be indicative of a floral inhibitor that exists in the plant throughout its vegetative lifetime. When the plant perceives an inductive signal, synthesis of the inhibitor turns off and floral transition can take place. An example is the *Arabidopsis* floral repressor gene *EMF* (*EMBRYONIC FLOWER*) (Sung et al., 1992). The *emf* mutants flower immediately after seed germination, without forming any rosette leaves, suggesting that the *EMF* genes negatively regulate the transition from vegetative to reproductive development. The extreme early flowering of *emf* mutants suggests that *EMF* genes are central repressors of flowering with activities that decline during plant development. When their activity drops below a certain threshold plants undergo the transition to flowering.

Cloning of cDNAs isolated from differential display gels

Nine cDNAs out of sixteen selected for differential expression were successfully cloned. The average size of the fragment inserts was approximately 300 base pairs, except fragment # 10 and # 11, which was approximately 600 bp, and the concentration was approximately 50 ng/µl (Figure 10).

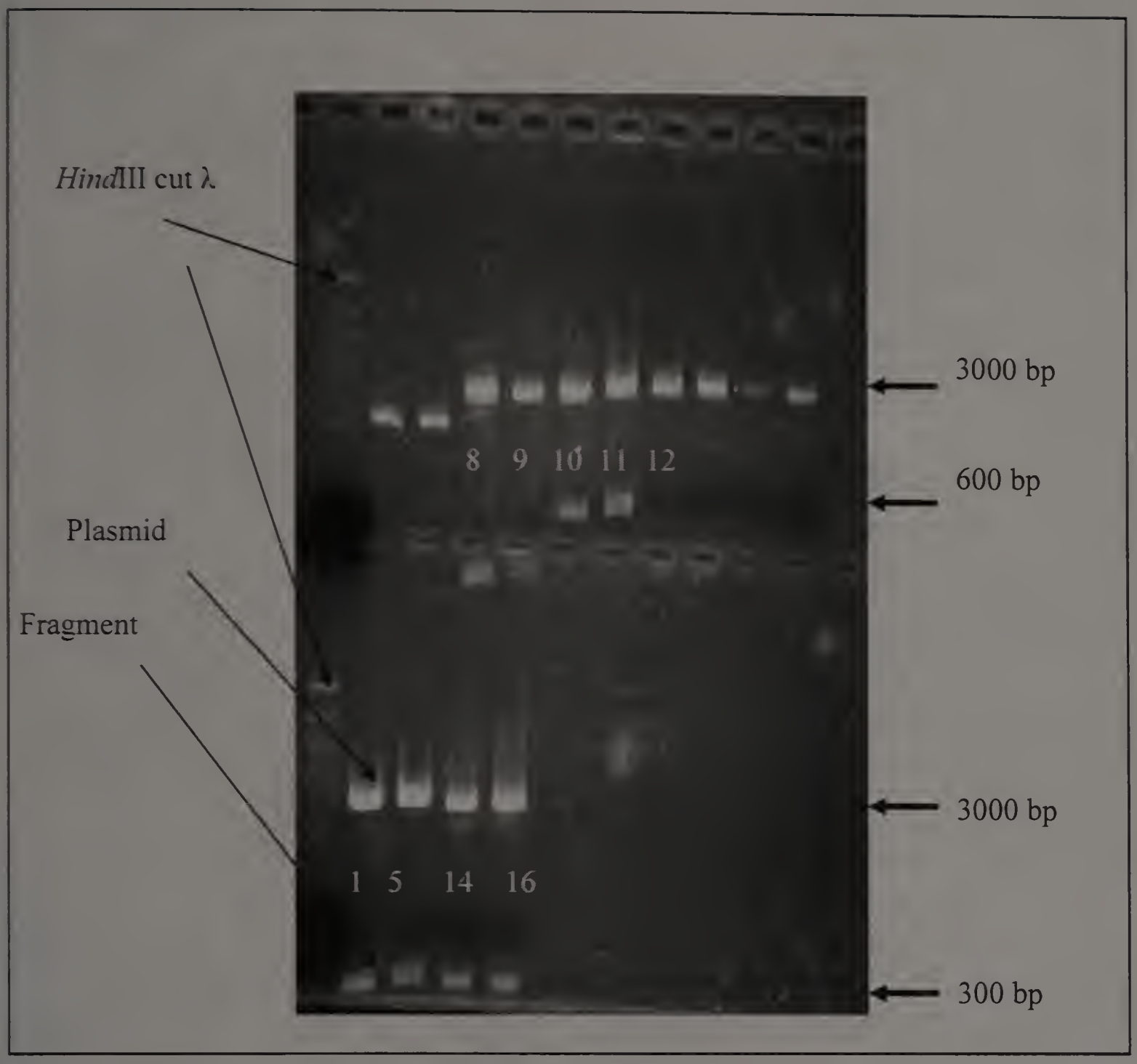


Figure 10: Size estimation of cDNAs inserted into plasmids. Plasmids extracted from the transformed *E. coli* cells were digested and separated on a 0.9% agarose gel. *Hind*III cut λ was run in the first lane. The numbers (8, 9, 10, 11, 12, 1, 5, 14, and 16) represent the labels of cDNAs reffered to in the text. The average size of the fragment inserts is 300 bp, except fragments 10 and 11, which is 600 bp.

Colony screening for multiple cDNA species of the same size

Screening colonies from each ligation reaction for the presence of multiple cDNA species of the same size revealed the likely presence of the single species of cDNA in each ligation reaction (Figure 11).

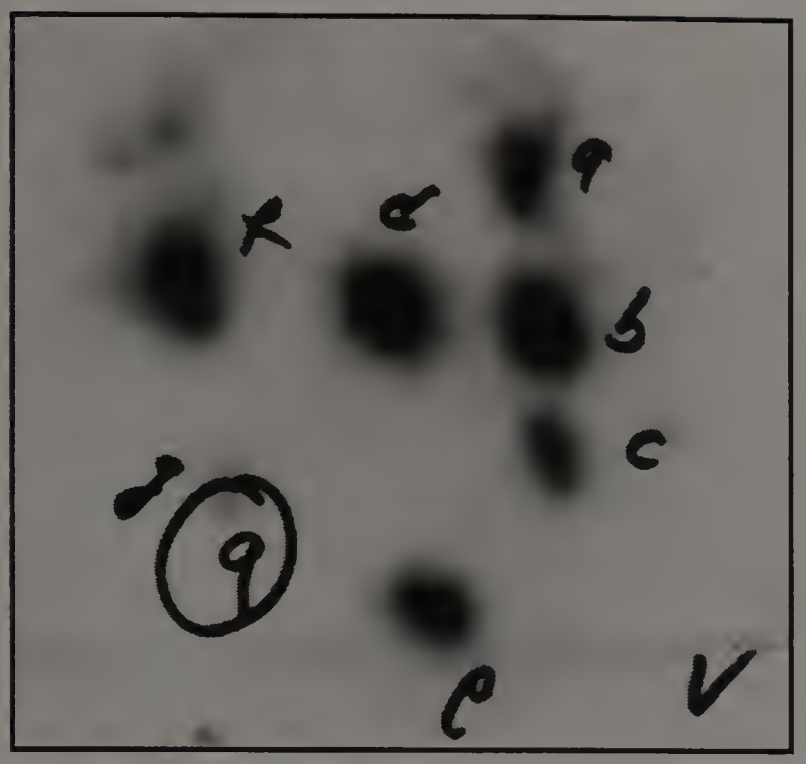
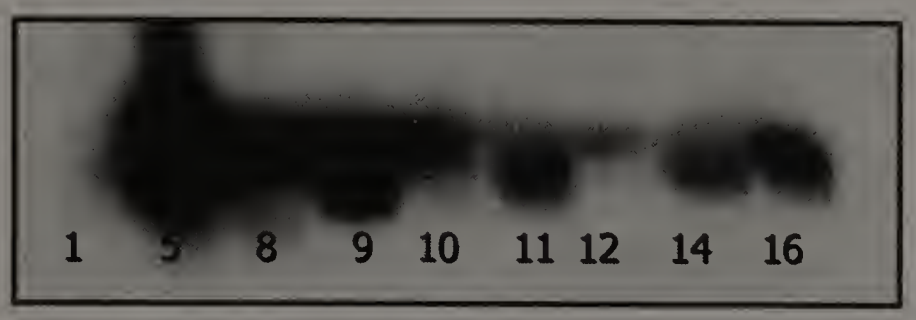


Figure 11: Screening of bacterial colonies for different cDNA species using colony blotting method. Transformed bacterial colonies were transferred onto Hybdon-N+ membrane and used in hybridization reaction with radiolabeled fragments of correct size isolated from the single colonies.

Verification of differential expression, reverse Northern blotting

To discriminate between true and false positives prior to further sequence analysis and to verify differential expression, a reverse Northern blotting procedure was used. When the hybridization patterns corresponding to inductive 6 hour-ethylene treatment and non-inductive 4 hour-ethylene treatment were compared (Figure 12), only 5 fragments displayed differential expression. Fragments # 5 (obtained from RT-PCR using combination of arbitrary primer 6 and fixed primer G), # 8 (using arbitrary primer 8 and fixed primer C), # 9 (using the same primer combination as for fragment 8), # 10 (the same primer combination as for fragment 8), and # 11 (arbitrary primer 17 and fixed primer C) were expressed only in plants induced to flower. This is in agreement with previously described results (Table 1), as these fragments were initially isolated from the 6-h treatment lane in the differential display gel. Three other fragments, #12 (arbitrary

primer #17 and fixed primer C), #14 (arbitrary primer 22 and fixed primer C), and #16 (arbitrary primer 10 and fixed primer G) were expressed both in 6-hour and 4-hour ethylene treated plants. There is a possibility that these cDNA fragments are not involved in the transition to flowering since 4-hour ethylene treated plants did not flower. The other possibility is that these messages might be involved in the process of flowering, but at 4-hour ethylene treatment did not reach the threshold for flowering.



Hybridization to cDNA from 6 h treated plants

Hybridization to cDNA from 4 h treated plants

Figure 12: Elimination of false positive bands prior sequence analysis using reverse Northern screening. Digested lasmids containing cDNAs of interest were run in duplicate on 0.9% agarose gel and than transferred onto Hybdon-N+ membranes. Membranes were hybridized overnight with a total cDNA probe from either 4h or 6h ethylene treatment. Five fragments were present only in cDNA from 6h ethylene treated plants, numbered: 5, 8, 9, 10, and 11, while the other three were present in cDNAs of both 4h and 6h ethylene treated plants (fragments 12, 14, and 16).

Fragment # 1 (arbitrary primer 4 and fixed primer G) isolated from the 6-hour ethylene treatment differential display lane was not detected in any pool of total cDNAs suggesting that it might be a false positive from differential display, thus was not further analyzed. Based on these findings, further sequence analysis was performed on the five fragments expressed in flowering plants only (fragments #5, #8, #9, #10, and #11).

Sequence analysis

Characterization of cDNA # 5

The cDNA # 5, 316 base pair long, and its putative protein sequence (Appendix P) were submitted to the Genbank database (http://www.ncbi.nlm.nih.gov) under accession number AY263359. The translated cDNA # 5, using ExPasy translation tool described earlier, is 104 amino acid-long. The amino acid sequence at the position 59 to 88 is affiliated with Major Facilitator Superfamily (MSF), also called the uniporter -symporter - antiporter family (Figure 13). There is 23.8% identity and 40.5% similarity to the bacterial protein, accession number PD090791 in protein database.

MSF is one of the two largest families of primary and secondary membrane transporters and it is present ubiquitously in bacteria, archea, and eukarya (Pao et al., 1998). MSF transporters are single-polypeptide secondary carriers capable only of transporting small solutes in response to chemiosmotic ion gradients (solute uniport, solute/cation symport, solute/cation antiport and/or solute/solute antiport with inwardly and/or outwardly directed polarity). Members of MSF superfamily are grouped in 17 families, based on phylogenic analysis, and each family generally transports a single class of compounds: simple sugars, oligosaccharides, inositols, Krebs cycle metabolites, amino acids, nucleotides, organophosphate esters, and a variety of organic and inorganic anions

and cations. The amino acid sequence at the N terminus of putative polypeptide #5 (10 aa, position 39-49) has 27.% identity and 72.7% similarity to the prtein with accession number PD478297, and it is associated with the family of Rhabdovirus spike glycoprotein, frequently abbreviated as G protein (Protein families database at the web site of Sanger Institute: www.sanger.ac.uk). The glycoprotein spike is made up of a trimer of G proteins and the channel they make is thought to function in a similar manner to influenza virus M2 protein channel, thus allowing a signal to pass across the viral membrane to signal for viral uncoating.

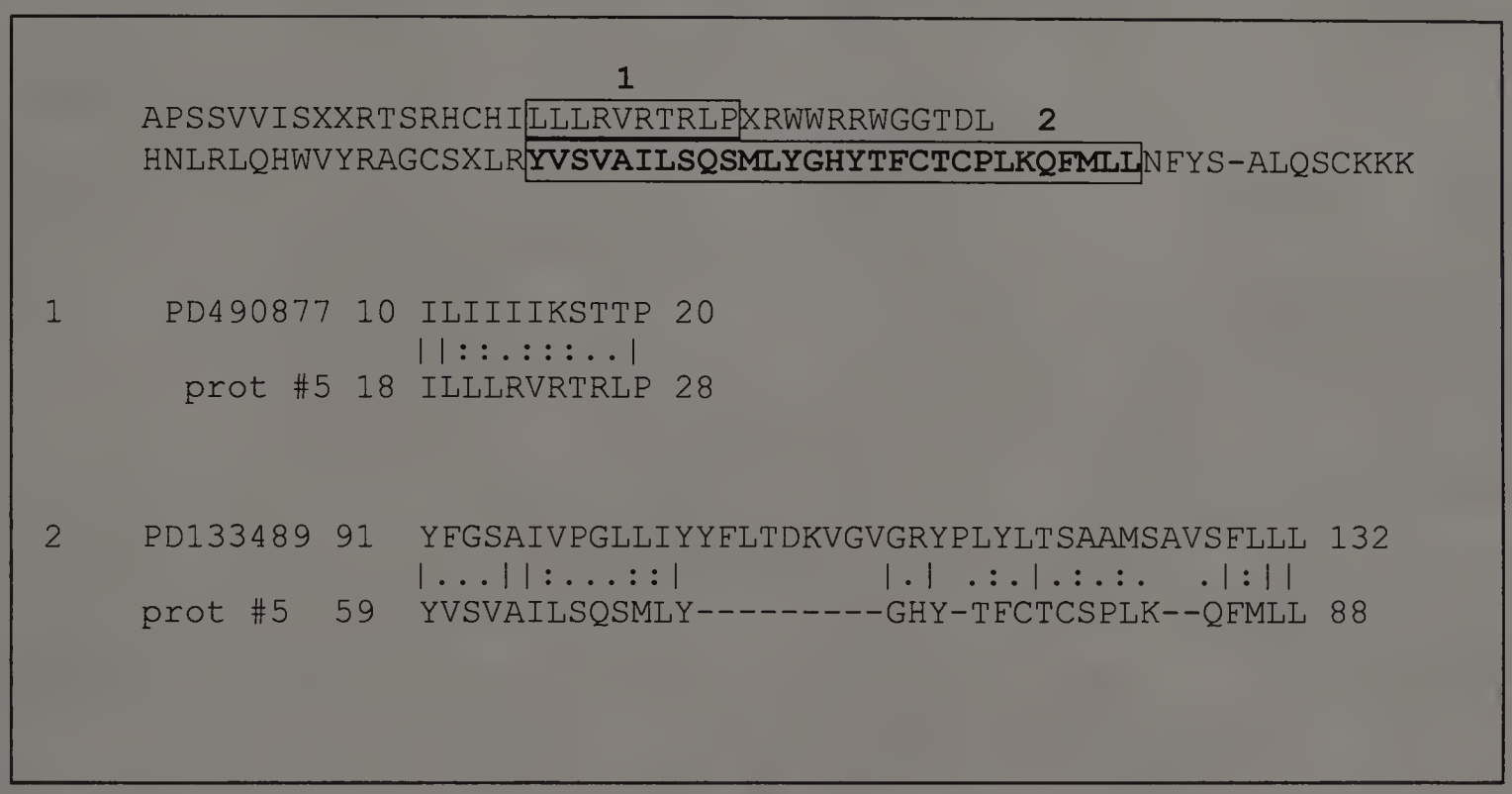


Figure 13: Alignment of putative protein # 5 to its homologue sequences. 1: Alignment to a glycoprotein, accession # PD478297, 27.3% identity, 72.7% similarity, 2: Alignment of local sequence (AA59-88) to a protein associated with MSF superfamily of sugar transporters, accession #PD090791, 23.8% identity, 40.5% similarity.

The putative secondary structure of polypeptide #5 is presented in Figure 14 as an output from PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred). The major part of the polypeptide is helical, approximately 65 %, 27.5 % is coil, and 7.5 % is strand.

Topological analysis, taking into account polarity and hydrophobicity of the amino acid residues and secondary structure data, showed one transmembrane region (helical region from amino acid 70 to 90) (Figure 15).

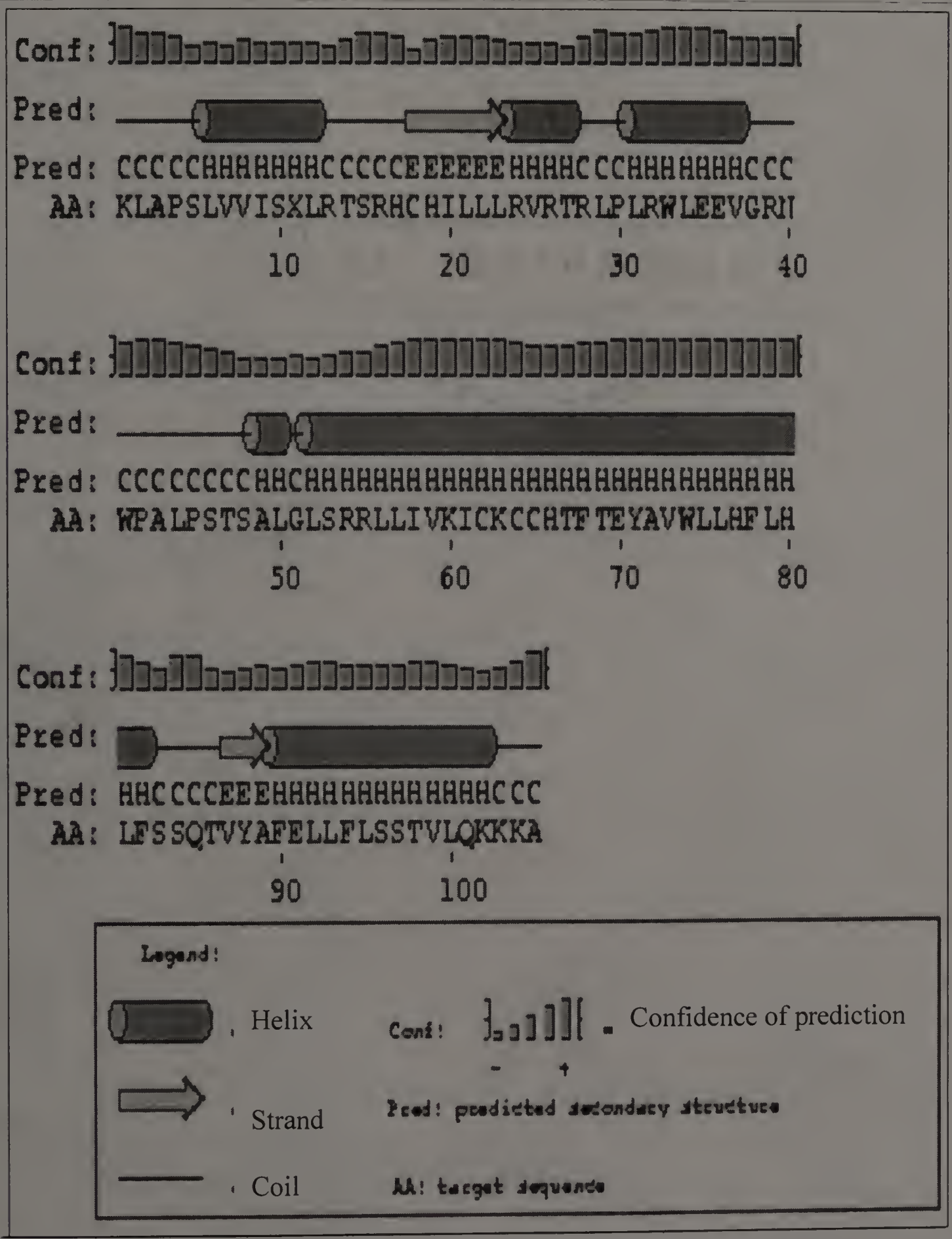


Figure 14: Secondary structure of the polypeptide #5, predicted by PSIRED. Approximately 65 % of the protein is helical, 27.5 % is coil, and 7.5 % is strand.

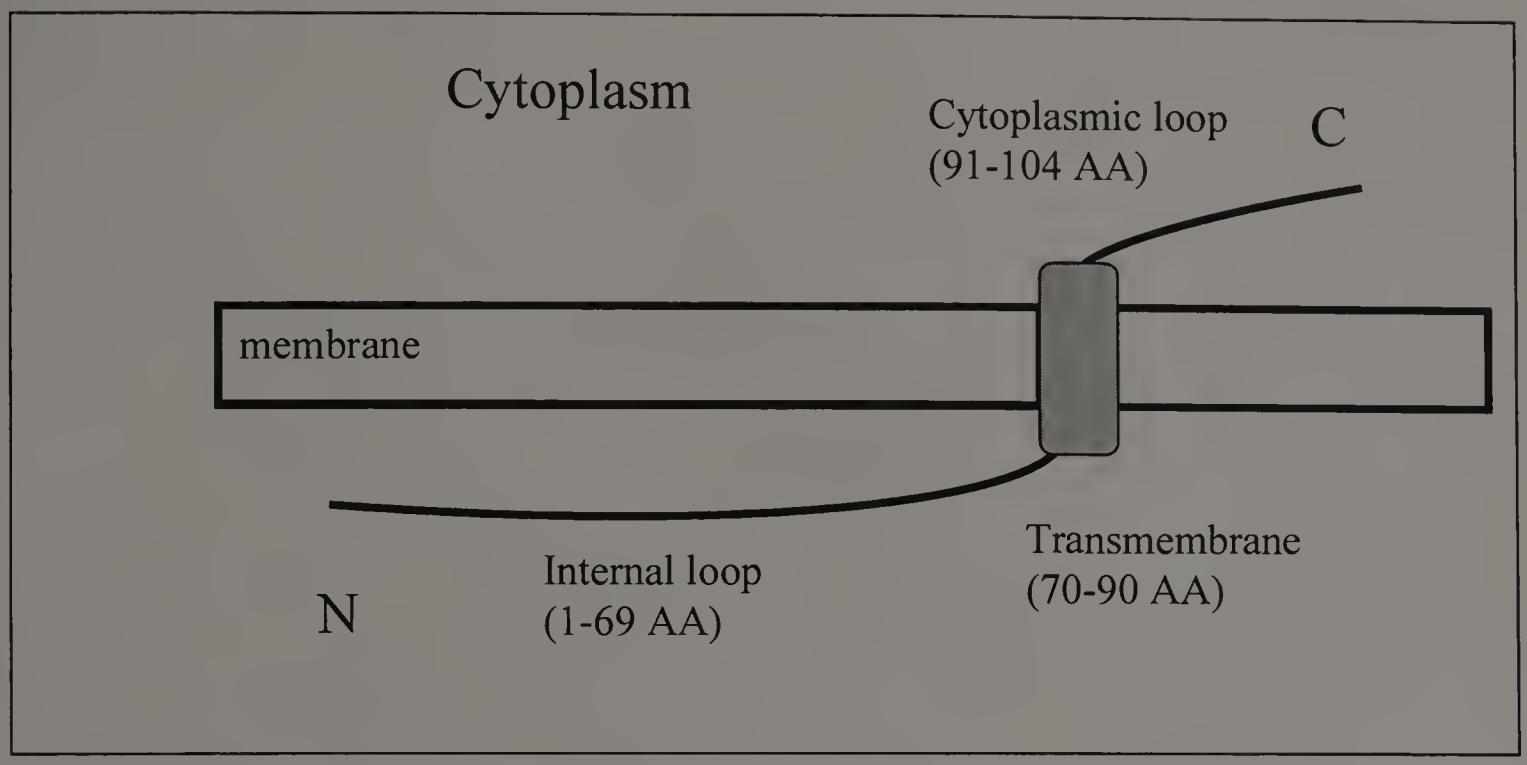


Figure 15: Topology and localization of the polypeptide #5 determined by TopPred II and PSORT. Protein 5 has one transmembrane domain (20 amino acids long) and 69 aa long internal loop at N terminus.

The peptide (partial sequence derived from partial cDNA sequence) had one internal loop, 69 amino acids-long and one cytoplasmic loop (13 amino acid-long).

PSORT analysis revealed that the polypeptide # 5 might be localized in the mitochondrial or endoplasmic reticulum membrane. Predicted amino acid sequence is probably a section of a large protein with many transmembrane domains.

Characterization of cDNA # 11

Partial sequence of cDNA # 11, 698 base pair long, and its predicted protein sequence (Appendix P) were submitted to the Genbank database (http://www.ncbi.nlm.nih.gov) under accession number AY294285. Based on the amino acid sequence at the position 142 to 192, the predicted protein of 236 amino acid residues was determined to be a member of the ABC transporters family (sequence in bold in Figure 16 A). All ATP-binding transport proteins (ABC transporters) are integral

membrane proteins involved in a variety of transport systems (EMBL-EBI web site). Members of this family include multi-drug resistance proteins, bacterial leukotoxin secretion ATP-binding protein, the cystic fibrosis transmembrane conductance regulator (CFTR), the mammalian sulfonylurea receptor, and antigen peptide transporter 2.

A search for homologous amino acid sequence using BLASTp tool implemented in TAIR database (*Arabidopsis* database) identified that an *Arabidopsis* sucrose transporter protein (sucrose/proton symporter), accession number At5g06170.1, has 35 % identity and 53 % similarity to predicted 11 protein (Figure16).

A

A Y Q V K I N V R Y V Y F L S V S S P L K L C L R L P N I L R F
Q Q N L E I D P Q V P T Y K G D T L I R L D T L C G N T S S W F
F N Q V Y L T L A S Q L F K F I Y H S K L T F T L N Q E N A C N
F D X W V M I X S F L X S X W I C E K G V L G Q N C Y S Y S H
L Y C S F P F S F C **A N F H X F S N L I S L V F L F I L T V S L Q**
I X D L V I T S S K I L L E V Y L S A C C L L V S I L L N Q I P
I C S A T S L A N Q E L R I E A V L S F Y A C X T C F L
C X G - F A P F H L V S

B

#11	121	VLGQNCYSYSHLYCSFPFS---------- FCANFHXFSNLISLVFLFILTV	161
		VLG SY++L+ FPF+ + CAN +IS+ L +LT+	
At5	193	VLGYAAGSYTNLHKIFPFTVTKACDIYCANLKS—CFI ISITLLIVLTI	239

Figure 16: Alignment of putative protein #11 to its homologue. A: Amino acid sequence of putative protein # 11. The underlined sequence represent sequence with a homology to an *Arabidopsis* protein, accession number At5g06170.1. Sequence represented with bold is associated with ABC family of transporters. B: Alignment of homologue sequences # 11 and At5g06170.1.

The putative secondary structure of the polypeptide # 11 is presented in Figure 17, as an output from PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred). The major part of the polypeptide is helical, approximately 51.33 %, 32.00 % is coil, and 16.66 % is strand.

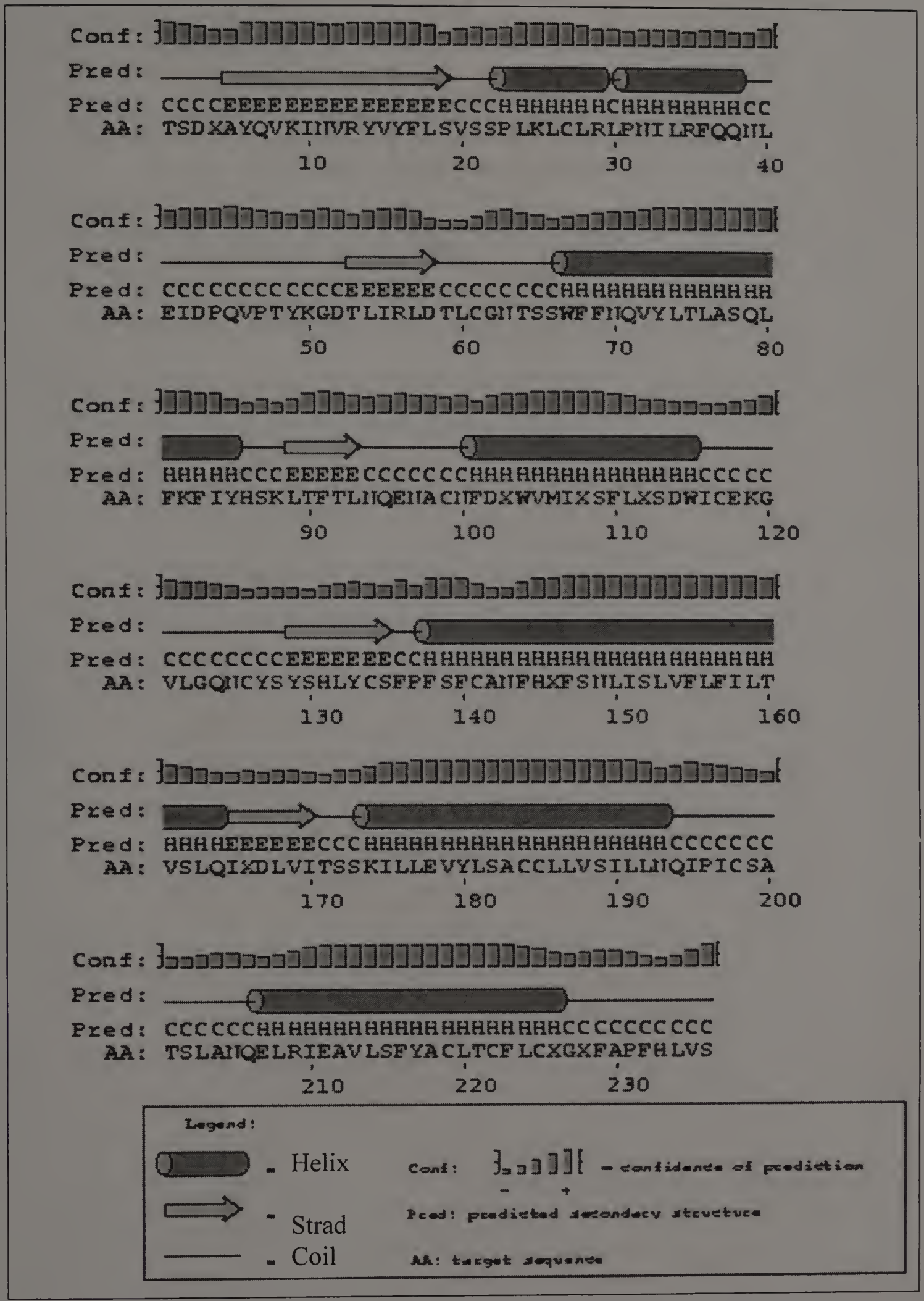


Figure 17: Secondary structure of the polypeptide # 11 predicted by PSIPRED. Approximately 51.33 % is helical, 32 % is coil, and 16.6 % is strand.

Topological analysis of the polypeptide # 11, taking into account polarity and hydrophobicity of the amino acid residues and secondary structure data, showed 4 transmembrane helical regions (Figure 18).

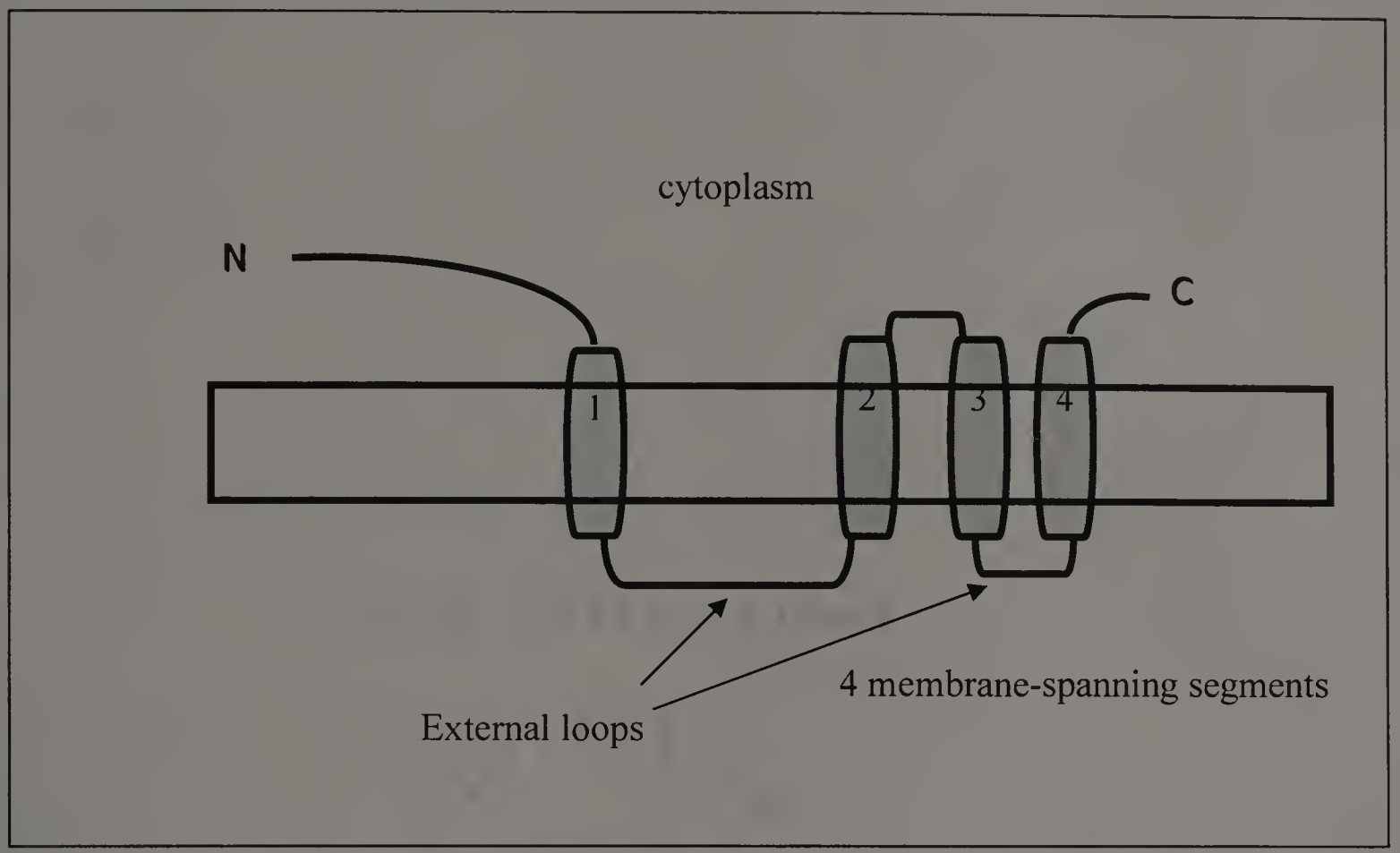


Figure 18: Topology of the polypeptide 11predicted by TopPred II and PSORT. Protein has four transmembrane domains and its presence is predicted in plasma membrane.

It has been reported that ABC transporters usually have six transmembrane regions (EMBL-EBI web site). Results of PSORT program for the prediction of protein localization site suggested that protein # 11 is localized in the plasmamembrane.

Characterization of cDNA # 9

Protein # 9, 103 amino acid residues long, derived from partial cDNA # 9 (314 base pairs) (Appendix P), showed homology to an *Arabidopsis* disease resistance protein EDS1 (enhanced disease susceptibility). Local alignment of these two nucleotide sequences is presented in Figure 19. EDS1 is a component of R-gene-mediated disease

resistance in *Arabidopsis*, with homology to eukaryotic lipases (Falk et al., 1999). Thus, anticipated function for EDS1 is hydrolase activity on a lipid-based substrate. Two plant defense pathways have been defined previously that depend on salicylic acid, a phenolic compound, or jasmonic acid (JA), a lipid derivative. Expression studies of *EDS1* mRNA in wild types and *eds1* mutants showed that *EDS1* functions upstream of salicylic acid-dependent pathogen related (PR) mRNA accumulation and is not required for JA-induced PR mRNA expression. Ethylene seems to play an important role in various plant disease resistance pathways as well (Wang et al., 2002).

A

CGACGTCGCATGTNCCCAGACGCCATGTTCNCCACCCGGAATTCGA
TTACCCCNTTTACCGCAGAGGTCTCATTGCTGGGGTCNTAGAGGAC
AAGCCCATAAAAATGGATTTCTATAACCAAATTAACACAACAAAG
GTTTAACTATGCTTGCAAACTNTTCATTTAGTGTCAAAAATTACAA
CCTAGCGTAATGTAACGAAATTCAGTTCCCCACTGAGTTTGAAGTC
CTATAAATGCAAATGCACCACTATAGTCACTCTAACAGGTGAGTTT
TTTGCAGCAAGTAAATCATACTCGAAAAAAAAAAAAGCTT

B

```
92        CAAGCCCATAAAAATGG  108
          || |||||||||||||||
At.1240 CAAGCCCATAAAAATGG  1256
```

Figure 19: Alignment of cDNA #9 to its homologue. A: cDNA #9 nucleotide sequence. The underlined sequence present the sequence with the homology to *EDS1*. B: Local alignment of cDNA #9 and At3G48090.1 with 100% homology.

The ethylene signal transduction pathway interacts with JA pathway to co-regulate expression of subset of defense-related PR genes, for example *PDF1*, involved in plant

disease resistance. There are considerable interactions between JA/ethylene- and SA-dependent pathways in systemic acquired resistance. In *edr1* mutant, ethylene potentiates SA-mediated PR-1 gene expression. Therefore, ethylene-induced production of mRNA '9'might be related to pathogenesis response rather than the process of flowering. Differential expression of gene # 9 might be a result of longer ethylene exposure time needed for mRNA-9 accumulation. It is possible that 4 hours ethylene exposure does not activate gene # 9, but 6 hours of ethylene exposure is sufficient for its expression.

Characterization of cDNAs # 8 and #10

Partial cDNA sequences # 8 (319 base pairs) and # 10 (213 base pairs) (Appendix P), as well as their predicted protein sequences did not have significant homology to any reported gene from the GenBank database.

Summary of sequence analysis

Differential expression of mRNAs # 5 and # 11 in the leaves of flowering *Guzmania* plants, encoding proteins with the homology to the carbohydrates transporters, suggests that carbohydrates may be involved in the long distance signaling: from the ethylene perception sites in the leaves, to the reaction sites in the apical meristems. Evidence that sucrose, the major sugar in both leaves and apical exudates, may function in long-distance signaling during floral induction comes from studies of *Sinapis alba*, a long-day plant (Havelange and Bernier, 1983). The concentration of sucrose in the phloem reaching the apex increases rapidly and transiently after a single inductive long day. As a result, sucrose accumulates very early in the apical meristem of induced plants. Bodson and Outlaw (1985) suggested that the increased sucrose supply to the meristem precedes the activation of energy-consuming processes such as mitosis and thus it is not

the result from a higher demand from the meristem. This suggests a message-like role for sucrose. The sucrose reaching the apex is derived from the mobilization of stored carbohydrates, most likely from starch in the leaves and stems (Lejeune et al., 1991).

Where exactly sucrose fits in the flowering pathways scheme is not clear yet. It is reported that the sucrose supply to *Arabidopsis* late flowering ecotypes bypasses the inhibition of flowering normally conferred by the existence of dominant alleles at *FRI* and *FLC* (Roldan et al., 1997). Sucrose supply also accelerates the flowering of *fve*, *fpa*, *fca*, *co*, and *gi* but not *ft* and *fwa* mutants. Based on these results, *FVE, FPA, FCA, CO,* and *GI* function in processes that are either upstream or separate from the control of sucrose availability to the vegetative apex, while *FT* and *FWA* function in processes that are downstream of sucrose control point. The position of sucrose availability in the scheme of flower induction pathway is presented in Figure 20.

The results presented here should provide basic information needed for further analysis of the role of sucrose in floral induction, and for the determination of the genes that integrate sugar signals in floral transition.

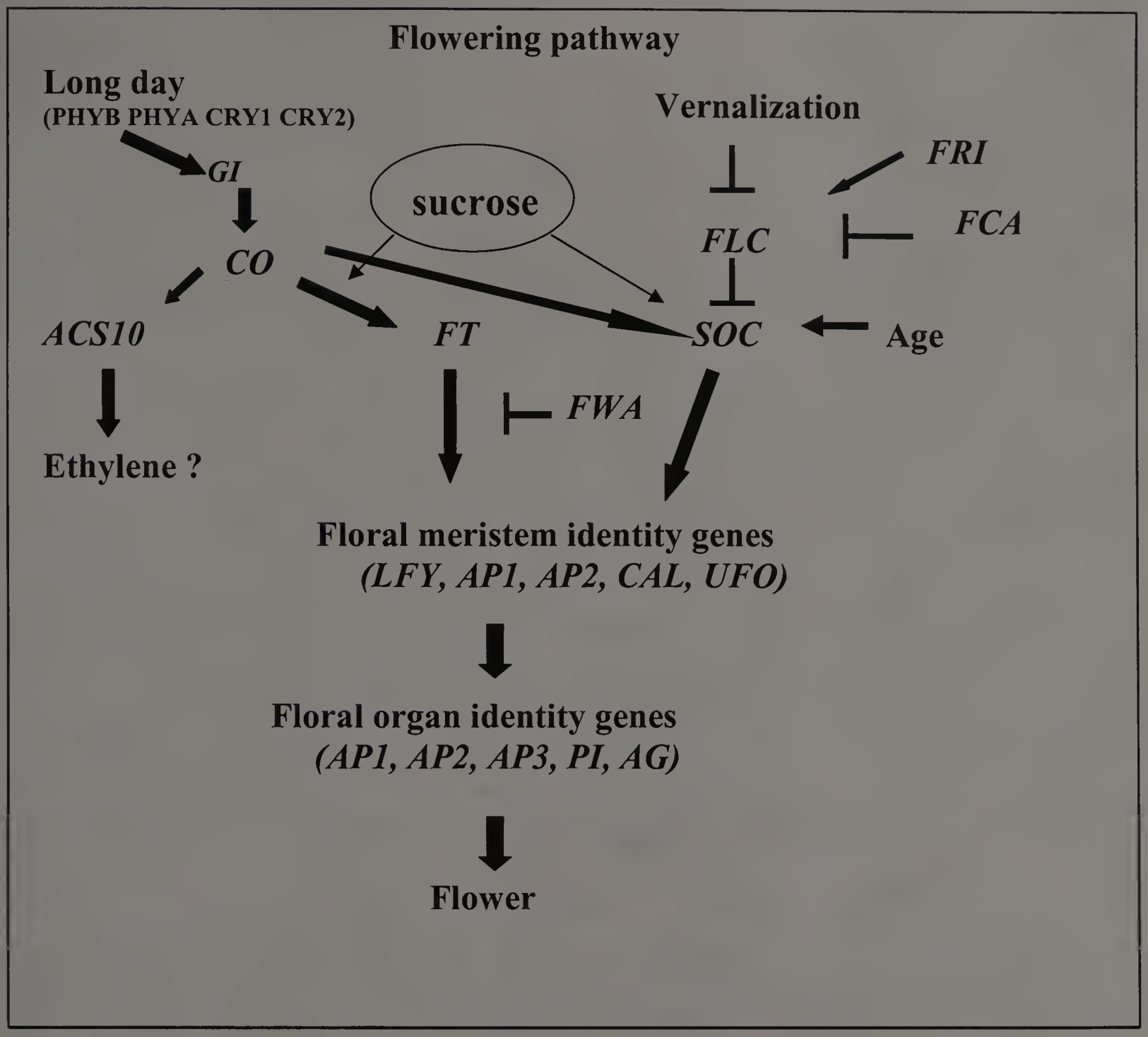


Figure 20: The position of sucrose availability in the scheme of flower induction pathway, adapted from M.A. Blazquez, Cell Sci. 2000 (113, p3547-3548) with some modifications: The position of sucrose was predicted based on the results described in Roldan et al., 1997. and added to the original figure. Long day inductive pathway,vernalization, and autonomous promotive pathway (age) converge at the level of flowering time genes (*FT* and *SOC*). Flowering time genes promote the transition to flowering by activating floral meristem identity genes which in turns initiate floral transition and activate floral organ identity genes. Floral organ identity genes regulate the formation of floral organs.

Conclusion

Differential display was conducted to isolate ethylene-responsive genes from *G. lingulata* 'Anita' leaves to obtain a broader understanding of the molecular basis by which ethylene induces flowering in bromeliads. From five ethylene-responsive cDNA clones, isolated from induced plants, two sequences, cDNA # 5 and cDNA # 11, and their predicted proteins have a role in carbohydrate transporting. Genes coding for sucrose and other carbohydrates transporters could fit into the flowering scheme as sucrose is involved in the long distance signaling during floral induction.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

1. Results of the experiments conducted to determine ethylene threshold for flowering show that minimum ethylene exposure time for 100 % flowering of *Guzmania lingulata* 'Anita' is 6 hours.
2. Data from experiments testing the effect of endogenous ethylene production on flowering, by the application of ethylene synthesis inhibitor AVG, show that endogenous ethylene production is involved in ethylene-induced flowering of *Guzmania lingulata* 'Anita'.
3. Results from experiments testing flowering response of plants if single leaf is exposed to ethylene show that exposure of single young leaf to ethylene induces flowering.
4. Data from experiments conducted to determine the role of protein synthesis in flower induction, by using protein synthesis inhibitor, cycloheximide, show that *de novo* protein synthesis is needed for floral induction by ethylene.
5. Results from differential display experiments, conducted to determine differentially expressed genes in flower induction by ethylene show that genes coding sugar transporters are activated in the leaves of *Guzmania* plants induced to flower by ethylene.
6. One type of transporter (clone # 11) is a member of the ABC transporter superfamily, and it is probably localized in the plasma membrane.
7. The other type of sugar transporter (clone # 5) is a member of the MFS superfamily, and it is localized in endoplasmic reticulum and mitochondrial membranes.
8. Results described in this study suggest that carbohydrates might be the long distance flowering signals between ethylene perception site and the apex.

These results should contribute to a better understanding of ethylene-induced flowering and to the understanding of the role of sucrose as a signaling molecule in floral induction. Further analysis should determine the full-length cDNA sequences using partial cDNAs in primer extension procedure. It would be of interest to determine if a GCC box is present in the regulatory region of these genes, which would indicate that they are turned on by an ethylene response element binding proteins (EREBP). These results might answer the question how ethylene activates these genes and thus provide a definite connection between ethylene and floral transition. Furthermore, the functions of the cloned genes can further be analyzed by knocking out the gene function through the RNA interference (RNAi). The transformation experiments, using isolated genes, might help elucidate other effective ways of controlling flowering time of many agriculturally important species.

APPENDICES

APPENDIX A

RNA EXTRACTION FROM PLANT TISSUE

(Protocol provided by the manufacturer of TRI reagent, Molecular Research Center Inc., Cincinnati, OH)

1. Grind 300 μg of plant tissue in liquid nitrogen using mortar and pestle.
2. Transfer ground plant tissue to microcentrifuge tube.
3. Mix tissue with 0.5 ml TRI-reagent for 5 minutes. Vortex and let set on ice until all samples are prepared. Let samples sit at room temperature for 5 minutes. (TRI reagent contains phenol and 4 M guanidine thiocyanate pH 4.0, which makes phenol aqueous. It also contains sarcosyl to assist in disruption of cell membranes).
4. Add 100 μl chloroform. Vortex and let set at room temperature for 2-3 minutes. (Chloroform separates bottom-organic phenol/chloroform phase with proteins and small DNA from top aqueous phase containing guanidne thiocyanate with RNA. Interface contains large DNA molecules).
5. Centrifuge for 15 minutes at 12,000 g at 4 °C.
6. Transfer clear upper phase to a new tube.

Removal of polysaccharides and RNA precipitation:

7. Add 125 μl isopropanol, mix by inversion.
8. Add 125 μl 0.8 M sodium citrate/1.2 M sodium chloride salt solution. Mix by inversion and let set at room temperature for 5-10 minutes.
9. Centrifuge for 10 minutes at 12,000 g at 4 °C.
10. Discard supernatant using pasteur pipette and let pellet air dry.

Removal of excess salt:

11. Add 0.5 ml of 75% ethyl alcohol, vortex and centrifuge for 5 minutes at 12,000 g at 4 °C. Discard alcohol and let the pellet air dry.

12. Resuspend RNA by adding 50 μl diethyl pyrocarbonate (DEPC)-treated water.

APPENDIX B

REMOVAL OF DNA CONTAMINATION FROM RNA

(Message Clean Kit, GenHunter Corporation, Nashville, TN)

1. **DNase I digestion**:

   Add in order: 50 μl total RNA (10 – 50 μg), 5.7 μl 10X reaction buffer (provided in the kit), and 1.0 μl DNase I (10 units / μl). Mix well and incubate for 30 minutes at 37 °C.

2. **Phenol/Chloroform (3:1) extraction**: complete removal of protein contamination and the DNase I from the RNA sample.

   Add 40 μl of phenol/chloroform to each sample and vortex for 30 seconds. Let samples sit for 10 minutes on ice. Centrifuge at 4 °C for 5 minutes (12,000 g). Collect and save the upper phase.

3. **Ethanol precipitation:**

   Add 5 μl of 3M sodium acetate and 200 μl 100% ethanol to the collected upper phase from step 2. Mix well and let it sit for 1 hour at -80 °C. Centrifuge for 10 minutes at 12,000 g at 4 °C to pellet the RNA. Remove the supernatant and rinse the RNA pellet with 0.5 ml of 70% ethanol (in DEPC-treated water). Do not disturb the RNA pellet.

   Spin for 5 minutes at 12,000 g, remove ethanol, spin again briefly and remove residual liquid. Dissolve RNA in 10 to 20 μl DEPC-treated water.

4. Store samples as 1-2 μg aliquots at -80 °C.

APPENDIX C

RNA GEL ELECTROPHORESIS

2% Mini Formaldehyde/Agarose gel

Combine 26 ml distilled water with 0.8 g agarose and melt it in the microwave. Cool to 65 °C, add 13 ml 3X formaldehyde stock. Pour the gel mix in the hood.

Running buffer: 40 mM MOPS, pH 7.0. Use 300 ml for mini gel (30 ml 10X stock + 270 ml dH_2O).

Sample preparation: To 10μl RNA sample add 20 μl RNA loading buffer (GenHunter). Incubate samples for 10 minutes at 65 °C, spin them down, than set them on ice for 5 minutes. Mix and load samples on gel. Load known quantity of uncut lambda to quantify the RNA samples.

Running conditions: 75V, 4W, 35mA, run for 3 hours.

Stock Solutions:

A. 0.4M MOPS stock pH 7.0 (10X)

20.93 grams of MOPS (FW = 209.3) per 250 ml. Adjust pH to 7.0 with sodium hydroxide. Bring volume to 250 ml.

B. 3X Formaldehyde stock

50 ml formaldehyde (37%)

30 ml 0.4M MOPS

20 ml dH_2O

Store 3X formaldehyde stock solution in 13 ml aliquots in freezer.

APPENDIX D

REVERSE TRANSCRIPTION AND PCR

(Instructions for Image Kit, GenHunter Corporation, Nashville, TN)

1. Reverse transcription of mRNA

Set up three reverse transcription reactions for each RNA sample in three PCR tubes. Each reaction should contain one of the three different one-base-anchored H-$T_{11}M$ primers (where M may be G, A, or C).

Thaw the following components and set them on ice:

per reaction

dH_20 9.4 μl

5X RT buffer 4 μl

dNTP (250μM) 1.6 μl

H-$T_{11}M$ (2μM) 2 μl

Total RNA (DNA-free) 2 μl (0.1 μg/ μl, freshly diluted)

Total 19 μl

If two or more RNA samples are used, it is recommended to make a core mix without an RNA template for each anchored oligo-dT primer.

Program thermocycler to 65 °C for 5 minutes → 37 °C for 60 minutes→ 75 °C for 5 minutes→ 4 °C. After the tube has been at 37 °C for 10 minutes, pause the thermocycler and add 1μl MMLV reverse transcriptase to each tube and quickly mix by finger tipping before continuing incubation. At the end of reverse transcription,

spin the tubes down briefly to collect condensation. Set the tubes on ice for PCR reaction or store them at -20 °C.

II. PCR

Combine the following components on ice:

dH_2O 10 μl

10X PCR buffer 2 μl

dNTP (25μM) 1.6 μl

H-AP primer (2μM) 2 μl

H-T_{11}M (2μM) 2 μl

RT-mix from step I (contains the same H-T_{11}M used for PCR) 2 μl

α-[^{33}P]dATP (3000 Ci/mM, New England Nuclear, Boston, MA) 0.2 μl

Taq DNA polymerase (Qiagen) 0.2μl

Total 20 μl

Set up the PCR reaction in thin-walled 200 μl reaction tubes (GenHunter, Cat.No. T101). Thin wall allows even heat transfer and the mineral oil is not needed to prevent evaporation due to the new design of the tubes. Make core mix as much as possible to avoid pipetting errors.

Program the thermocycler at 94 °C for 30 seconds→ 40°C for 2 minutes→ 72 °C for 30 seconds for 40 cycles followed by 72 °C for 5 minutes→ 4 °C.

APPENDIX E

6 % DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

1. Clean 2 glass plates with 80% ethanol and kimwipes. Coat plates with Sigmacote (Sigma) in hood and allow to dry. Clean spacers and flat-tooth gel comb (GenHunter, Cat# CM1). Place spacers on a large glass plate, let them hang off slightly and keep foam tabs facing up. Place small glass plate over large glass plate (the siliconized sides will touch the gel). Cut a piece of blotting paper to seal up the bottom (3-4 cm wide). Place the glass sandwich in gel mold.
2. Pour 37.5 ml Gel Mix 6 (GibcoBRL) into empty gel mix container and add 225μl 10% ammonium persulfate. Mix well for 30 seconds, hold gel mold at an angle and pour gel mix continuously down the side of a spacer. Tap out bubbles. Place comb in with teeth facing upwards. Allow gel to polymerize for 2 hours.
3. Prepare the gel: remove comb and rinse large well with 1X TBE buffer. Place the gel in running unit (with foam pads against the unit). Fill the large well and chambers with 1X TBE. Put comb in so that teeth are just touching the gel. Flush out wells with 1X TBE to eliminate air bubbles.

   Pre-run gel for 30 minutes to flush out urea. Running conditions: 1500V, 40W, 30mA.
5. Prepare samples by combining 3.5 μl of RT-PCR sample with 2 μl dye (provided in the RNA Image kit). Heat samples at 80°C for 2 minutes. Keep comb in the gel when loading samples. Run gel at 1500V, 40W, 30mA for 2 hours (until second dye front gets to the bottom of the gel).

6. Take the gel down: separate the glass plates using metal spatula. Gel will remain attached to the large plate. Place a piece of blotting paper over the gel, press down to make sure the gel attaches than lift the paper with the gel on it. Place saran wrap over the gel and label first lane on the paper.
7. Drying the gel: Place gel on the surface of gel dryer with gel facing up. Fill liquid collector with liquid nitrogen. Dry gel for 40 minutes at 80°C. Take the gel out, orient the gel on film using needle punches and place them in film cassette. Keep the cassette overnight to 72 hours at -80°C.

TBE buffer:

Make 10X TBE (pH 8.0) running buffer stock:

54 g Trizma base

27.5 g Boric acid

40 ml EDTA (0.25M, pH 8.0)

Add dH_2O up to 1 l.

Use 700 ml of 1X TBE to run a gel.

APPENDIX F

REAMPLIFICATION OF cDNA PROBES

(Image Kit, GenHunter Corporation, Nashville, TN)

1. After developing film, orient the autoradiogram with the gel. Locate bands of interest by punching through the film with a needle at the four corners of each band of interest. Cut out the located bands with a clean razor blade. (Handle the dried gel with gloves and save it between two sheets of clean paper).
2. Soak the gel slice along with 3M paper in 100 μl dH_20 for 10 minutes.
3. Boil the tube with cap tightly closed (covered with parafilm) for 15 minutes.
4. Spin for two minutes to collect condensation, and pellet the gel and paper debris.
5. Transfer supernatant to a new tube. Add 10 μl 3M sodium acetate, 5 μl glycogen (10 mg/ml) and 450μl 100% ethanol. Centrifuge for 10 minutes at 12,000 g to remove the residual ethanol.
6. Dissolve the pellet in 10 μl of dH_2O and use 4μl for reamplification.
7. Reamplification is done using the same primer set and PCR conditions except the dNTP concentration should be at 20μM (use 250 μM dNTP stock). No isotopes are added.

   dH_2O 20.4 μl

   10X PCR buffer 4 μl

   dNTP (250μM) 3.2 μl

   H-AP primer (2 μM) 4 μl

   H-T_{11}M (2 μM) 4 μl

   cDNA template from step 6. 4 μl

Taq DNA polymerase (Qiagen) 0.4 μl

Program the thermocycler at 94°C for 30 seconds→ 40°C for 2 minutes→ 72°C for 30 seconds for 40 cycles followed by 72°C for 5 minutes→ 4°C.

8. Thirty microliters of the PCR samples are run on a 1.5% agarose gel and stained with ethidium bromide. Use 100bp ladder to determine the size of fragments.
9. Extract the reamplified cDNA from the agarose gel using QIAEX kit from Qiagen.

APPENDIX G

QIAEX II AGAROSE GEL EXTRACTION PROTOCOL

(Protocol provided by Qiage, Valencia, CA)

1. Excise the DNA band from the agarose gel with a clean scalpel.
2. Weigh the gel slice in a microcentrifuge tube. Add 3 volumes of buffer QX1 (provided in kit) to 1 volume of gel for DNA fragments 100 bp-4000 bp. For example, add 300μl of buffer QX1 to each 100 mg of gel. Buffer QX1 contains solubilization and binding buffer with pH indicator.
3. Resuspend QIAEX II suspension (provided in kit) by vortexing for 30 seconds. For less than 2 μg DNA add 10μl of QIAEX II and mix.
4. Incubate at 50°C for 10 minutes to solubilize the agarose and bind the DNA. Mix by vortexing every 2 minutes to keep QIAEX II in suspension. The color of mixture should be yellow. (The adsorption of DNA to QIAEX II particles is only efficient at pH $\leq$7.5.
5. Centrifuge the sample for 30 seconds and carefully remove supernatant with a pipette.
6. Wash the pellet with 500μl of Buffer QX1. Re-suspend the pellet by vortexing then spin it for 30 seconds and carefully remove supernatant.
7. Wash the pellet twice with 500 μl of Buffer PE (wash buffer provided in kit).
8. Air-dry the pellet for 10-15 minutes.
9. To elute DNA, add 20 μl of 10mM Tris-Cl, pH 8.5 or H_2O and resuspend the pellet by vortexing. Incubate sample at room temperature for 5 minutes.
10. Centrifuge for 30 seconds and carefully pipette the supernatant into a clean tube.

APPENDIX H

LIGATION USING pGEM-T EASY VECTOR

(Protocol provided by Promega, Madison, WI)

1. Briefly centrifuge the pGEM-T Easy vector and control insert DNA (provided in Promega vector system, cat. # A1380).
2. Set up ligation reaction:

	Standard reaction	Positive control
2X Rapid ligation buffer, T4 DNA ligase*	5μl	5μl
pGEM-T Easy (50ng)	1μl	1μl
PCR product	3μl	—
Control insert	—	2μl
T4 DNA Ligase (3Weiss units/μl)	1μl	1μl
Deionized water	—	1μl

*Vortex 2X Rapid Ligation Buffer vigorously before each use.

3. Mix the reactions by pipetting. Incubate the reactions overnight at 4°C.

APPENDIX I

TRANSFORMATION USING pGEM-T EASY VECTOR - LIGATION REACTIONS

(Protocol provided by Promega, Madison, WI)

1. Prepare 2 LB / ampicilin/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency. Equilibrate the plates to room temperature prior plating.
2. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom of the tube. Add 2 μl of each ligation reaction to a sterile 1.5 ml microcentrifuge tube on ice. Set up another tube on ice with 0.1 ng uncut plasmid for determination of the transformation efficiency of the competent cells.
3. Remove tubes of frozen JM 109 High Efficeincy Competent Cells (GenHunter) from -70°C storage and place them on ice until just thawed (about 5 minutes). Mix the cells by gently flicking the tube.
4. Transfer 50 μl of cells into each tube prepared in step 2 (100 μl cells for determination of transformation efficiency).
5. Gently flick the tubes to mix and place them on ice for 20 minutes.
6. Heat shock the cells for 45-50 seconds in a water bath at 42°C (Do not shake samples).
7. Immediately return the tubes to ice for 2 minutes.

8. Add 950 μl SOC medium (room temperature) to the tubes containing cells transformed with ligation reactions and 900 μl to the tube containing cells transformed with uncut plasmid.
9. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
10. Plate 100 μl of each transformation culture onto duplicate LB/ampicilin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. Incubate the plates overnight at 37°C.

Buffers and Solutions:

A. IPTG stock solution (0.1 M): 1.2 g IPTG (Promega, Cat# V3951) in 50 ml water. Filter-sterilize and store at 4°C.

B. X-Gal (2 ml): 100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Promega, Cat#V3941) dissolved in 2 ml N, N'-dimethyl-formamide. Cover with aluminum foil and store at -20°C.

C. LB medium (per liter)

   10 g Bacto-tryptone

   5 g Bacto-yeast extract

   5 g NaCl

   Adjust pH to 7.0 with NaOH and add water to 1 liter.

D. LB plates with ampicillin

   Add 15 g agar to 1 liter of LB medium. Autoclave and allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100 μg / ml. Pour 30-35 ml of medium into 85 mm Petri dishes. Let the agar harden and store them at 4°C for up to 1 month.

E. LB plates with ampicillin/IPTG/X-GAL: Combine 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-Gal and spread it over LB/ampicillin plates from step D. Allow 30 minutes at 37°C prior to use for absorption.

F. SOC medium (100 ml)

2 g Bacto-tryptone

0.5 g Bacto-yeast extract

1 ml 1M NaCl

0.25 ml 1M KCl

1ml 2M Mg^{2+} stock, filter sterilized (20.33 g $MgCl_2 \cdot 6H_2O$, 24.65 g $MgSO4 \cdot 7H_2O$, and 100 ml distilled water).

1 ml 2M glucose, filter sterilized

Combine the first four ingredients in 97ml water, stir to dissolve and autoclave. Let it cool down before adding the last two components, bring to 100 ml with sterile distilled water. Filter the complete medium through a 0.2 μm filter unit. The final pH is 7.0.

APPENDIX J

COLONY BLOTTING

(Modified protocol provided by Amersham Pharmacia Biotech Inc., England)

1. Select 10 white colonies from original plate and transfer them to a new LB/amp/IPTG/X-Gal plate. Let them grow overnight at 37 ºC.
2. Select correct membrane size and pre-wet in water.
3. Carefully place membrane on the agar surface. Mark the membrane and agar using sterile needle to ensure correct orientation of colonies.
4. Remove membrane after 1 minute and place, colony side up, on the fresh agar. Place plate with the membrane at 37 ºC overnight.
5. Remove the membrane and place it on a filter paper soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 7 minutes.
6. Transfer the membrane to the filter paper soaked in neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH7.2, 1 mM EDTA). After three minutes place the membrane on a new filter paper soaked in neutralizing solution.
7. Wash filter in 2X SSC (made from 20X SSC stock, 3M NaCl, 0.3M sodium citrate, pH 7.0, adjusted with HCl) transfer to dry filter paper and air dry, colony side up.
8. Fix DNA blots using alkaline fixation protocol: Place the membrane on a pad of 2-3 filter papers soaked in 0.4M NaOH for 20 minutes.
9. Rinse the membrane by immersion in 5X SSC with gentle agitation for no more than 1 minute.

10. Air dry the membrane, wrap it in Saran Wrap, and store at 4 °C until needed for hybridization.

APPENDIX K

RAPID MINI PLASMID PREPARATION

1. Turn heat block on to 100°C. Prepare 1ml of 10mg/ml lysosyme, keep it on ice.
2. Transfer 1.4 ml of liquid culture grown overnight in shaking (150 rpm) at 37 °C to microcentrifuge tube and centrifuge at high speed for 30 seconds. Remove all of supernatant and transfer another 1.4 ml to same tube and repeat the procedure.
3. Resuspend pellet by vortexing in 200 μl LiCl buffer. Clumps of bacteria should be completely dispersed. Add 10 μl lysosyme solution and 1μl RNAse solution. Mix well and incubate at room temperature for 5 minutes.
4. Heat tubes for 2 minutes in heat block set at 100°C. Cool on ice for 5 minutes.
5. Centrifuge 25minutes at 12,000 g at 4 °C.
6. Transfer 150 μl of supernatant to a microcentrifuge tube being careful not to transfer any of the precipitate. Add 300 μl 95% ethanol and mix well.
7. Centrifuge at 12,000 g for 5 minutes at 4 °C.
8. Discard supernatant, drain tube by inverting on paper towel, and rinse pellet with 500 μl 70% ethanol. Pour ethanol off and rinse pellet with 500 μl 95% ethanol. Drain pellet well and allow to air dry for 10 minutes.
9. Resuspend pellet in 50 μl TE. Place tube in 60°Cwater bath for 5 minutes to allow pellet to solubilize.

Buffers and solutions:

LiCl buffer:

50 mM Tris pH7.5

62.5mM EDTA

0.4% Triton X-100

2.5 M LiCl

Lysosyme (made fresh): 10 mg/ml (water)

RNAse: 10 mg/ml (10mM Tris pH 7.5, 15mM NaCl)

Process it in boiling water bath for 10 minutes. Freeze in 100 μl aliquots.

TE buffer: 10mM Tris pH8.0, 1mM EDTA

APPENDIX L

PLASMID RESTRICTION ENZYME DIGEST

1. Combine 7.5μl water and 1.5μl 10X enzyme buffer on ice.
2. Add 5μl plasmid DNA (from mini-prep, appendix K).
3. Add 1.0μl restriction enzyme (10U). We used *Eco*RI.
4. Mix gently and place at 37 °C for 2 hours.
5. Run all samples on 0.9% agarose gel with 1X TBE buffer (appendix E) to determine the presence of the fragment in the plasmid, the size and the approximate concentration of the fragments.

APENDIX M

RADIOLABELING PROBES AND BLOT HYBRIDIZATION

1. Combine the following components:

   DNA 10 μl

   dH_2O 18 μl

   Random primers (6bp, 30 ng/μl) 2.4 μl

3. Place tubes on a heat block (100°C) for 5 minutes then add:

   10X Hepes 5μl

   dNTP (6mM each, pH 7.0, without dCTP) 6μl

   Klenow enzyme 1 μl (1unit)

   Stock Klenow from BRL comes as 500 units in 84µl. Dilute to 1 unit by adding 420 µl of klenow buffer to the 500 unit/84 µl stock.

   Store at – 20 °C.

   ^{32}PdCTP 7 μl (3000 Ci/mmole)

4. Keep tubes in the plexiglass box at 37 °C for 1 hour.

5. Run probe over column to make sure it was labeled and clean from free nucleotides. Use a 1ml plastic syringe, cut the cap end at the 1.0 ml mark. Push cotton into bottom of syringe to hold the Sephadex. Add 1ml Sephadex- G50 slurry. Centrifuge at low speed in a clean tube until column is white and all liquid is out.

6. Add 3 μl stop dye to the probe (1:5). Bromophenol blue stays with unincorporated nucleotides. Blue dextran stays with DNA probe.

7. Put syringe in a clean tube and add probe to column. Add 200 μl column buffer and centrifuge at low speed for 30 seconds.
8. Collect the probe in microcentrifuge tube and discard the column.
9. Estimate the activity using Geiger counter.
10. Place blots into hybridization tubes and prehybridize them at 65 °C for 30 minutes. Add dextran sulfate (5%) and blocking DNA (2%) to the hybridization buffer just before using.
11. Add radiolabeled λ (the same procedure for labeling) to the probe.
12. Punch the hole in microcentrifuge tube cap with needle.
13. Place samples at 100 °C for 5 minutes.
14. Add labeled probe to 15ml hybridization buffer in the hybridization tube.
15. Add blot and incubate at 65 °C overnight rotating.
16. Pour hybridization buffer with labeled probe into 50ml tube and freeze for later use.
17. Heat 500ml 2X SSC, 0.1% SDS to below 68 °C, rinse the blot.
18. Heat 500ml 2X SSC, 0.1% SDS to below 68 °C and wash blot for 15 minutes.
19. Repeat step 18.
20. Heat 500ml 1X SSC, 0.1% SDS to below 68 °C and wash blot for 10 minutes.
21. Heat 500ml 0.5X SSC, 0.1% SDS to below 68 °C and wash blot for 10 minutes.
22. Place the blot onto filter paper, wipe with kimwipe, wrap in saran wrap and put on film.

    Keep the film at -80 °C for one or few days.

Buffers and solutions:

Sephadex G 50-80:

5ml 20% SDS

10ml 0.25M EDTA

85ml dH_2O

add 5g dry Sephadex and swell overnight

Denhardt's solution 50X:

5 g Ficoll (Type 400 Pharmacia)

5 g Polyvinylpyrrolidone

5 g BSA

Add dH_2O to 500 ml and filter.

Hybridization buffer:

250 ml 20X SSC pH 7.0 (43.8g NaCl and 36.8g citric acid)

490 ml dH_2O

30 ml 20% SDS

50 ml 1M $NaPO_4$ buffer (pH 7.4)

50 ml 100X Denhardt's

10 ml 0.25M EDTA

APPENDIX N

SOUTHERN BLOTTING

1. Soak the gel in 300ml of 0.25 M HCl for 10 minutes with gentle shaking. The tracking dye if present will turn yellow. This step is not necessary for fragments smaller than 1Kb.
2. Pour off the acid, rinse the gel with water and soak the gel in 300 ml of 0.4 M NaOH for 25 minutes.
3. Cut a piece of blotting membrane to the dimension of the gel, write in one corner with pencil in order to orient and identify the blot.
4. Cut three sheets of 3 MM paper to the dimension of the gel.
5. While the gel is being treated with acid and base, soak 2 of three filter papers and a membrane in 0.4 M NaOH.
6. Prepare a stack of paper towels about 4 cm high. After treatment of the gel, place it on an acetate sheet. Briefly blot the surface of the gel with the third dry sheet of 3 MM filter paper to remove excess buffer. Lay the blotting membrane on the gel without trapping any bubbles between the blot and the gel. Place 2 wet filter papers on the membrane, then the stack of paper towels and a light weight plexiglass sheet on top.
7. Leave the set-up on the bench overnight.
8. Wash the membrane after blotting in a solution of 2X SSC, 01% SDS at room temperature for 5 minutes, followed by a second wash in 0.5X SSC, 0.1% SDS at room temperature for 5 minutes. Air-dry the membrane on 3 MM filter paper and store them at room temperature under low humidity.

APPENDIX O

FIRST STRAND SYNTHESIS OF cDNA

1. Combine 5 μg of RNA and 0.5μg of the oligo-(dT)15 primer (Promega) in total volume of 15 μl in a sterile, RNAse-free microcentrifuge tube. Heat the tube to 70°C for 5 minutes to melt secondary structure within the template. Cool the tube immediately on ice to prevent secondary structure from reforming. Spin briefly to collect the solution at the bottom of the tube.
2. Add the following components to the annealed primer/template:

   M-MLV 5X Reaction buffer 5μl (Promega)

   dATP, 10 mM 1.25 μl

   dCTP, 10 mM 1.25 μl

   dGTP, 10 mM 1.25 μl

   dTTP, 10 mM 1.25 μl

   rRNasin®Ribonuclease inhibitor 25 units (Invitrogen)

   M-MLV RT(200 U/μl) 1μl (Promega)

   DEPC treated water to final volume of 25μl
3. Mix gently by flicking the tube and incubate at 42°C for 60 minutes following with 2 minutes at 70°C.

APPENDIX P

NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE FROM CLONED cDNAs

cDNA 5

```
gcaccatcatcagtggtgatttcttnctngcgcacgagcagacactgccatatattactc
 A  P  S  S  V  V  I  S  X  X  R  T  S  R  H  C  H  I  L  L
ctccgggttcgtacacgattaccatnaagatggtggaggaggtggggaggaactgacctg
 L  R  V  R  T  R  L  P  X  R  W  W  R  R  W  G  G  T  D  L
cataaccttcgacttcagcattgggtttatcgcgccggttgctcatngttaagatatgta
 H  N  L  R  L  Q  H  W  V  Y  R  A  G  C  S  X  L  R  Y  V
agtgttgccatactttcacagagtatgctgtatggtcattacactttctgcacttgttct
 S  V  A  I  L  S  Q  S  M  L  Y  G  H  Y  T  F  C  T  C  S
cctctcaaacagtttatgcttttgaacttttattcttaagctctacagtcctgcaaaaaa
 P  L  K  Q  F  M  L  L  N  F  Y  S  -  A  L  Q  S  C  K  K
aaa
 K
```

cDNA 8

```
tatgtatatggcagatttattttgatgacaggagtaaaagtcgatgttttaaattaanat
 Y  V  Y  G  R  F  I  L  M  T  G  V  K  V  D  V  L  N  -  X
nacaatgaagaaaaaaaagtgaacttatttttaaccacttgacgaactcgcaagatttt
 X  N  E  E  K  K  V  N  L  F  F  N  H  L  T  N  S  Q  D  F
tcttaacttcaactaagaaagttttcatgncagcgtaagtctcttgacaaacgccaggag
 S  -  L  Q  L  R  K  F  S  X  Q  R  K  S  L  D  K  R  Q  E
caagantaantgggaaaacaaaancanccgaatcgctttgtgtacgtggaannttaatcg
 Q  X  -  X  G  K  Q  X  X  P  N  R  F  V  Y  V  E  X  -  S
aattnccgcggncgccatggnggncgggancatgcgangtcgggnccaattcgccctata
 N  X  R  X  R  H  X  X  R  X  H  A  X  S  X  P  I  R  P  I
ntgagncgnattacaattc
 X  X  X  I  T  I
```

cDNA 9

```
cgacgtcgcatgtncccagacgccatgttcnccacccggaattcgattaccccntttaccgc
   T  S  H  X  P  R  R  H  V  X  H  P  E  F  D  Y  P  X  Y  R
Agaggtctcattgctggggtcntagaggacaagcccataaaaatggatttctataaccaa
 R  G  L  I  A  G  V  X  E  D  K  P  I  K  M  D  F  Y  N  Q
attaacacaacaaaggtttaactatgcttgcaaactnttcatttagtgtcaaaaattaca
 I  N  T  T  K  V  -  L  C  L  Q  T  X  H  L  V  S  K  I  T
acctagcgtaatgtaacgaaattcagttccccactgagtttgaagtcctataaatgcaaa
 T  -  R  N  V  T  K  F  S  S  P  L  S  L  K  S  Y  K  C  K
tgcaccactatagtcactctaacaggtgagtttttgcagcaagtaaatcatactcgaaa
 C  T  T  I  V  T  L  T  G  E  F  F  A  A  S  K  S  Y  S  K
aaaaaaaagctt
 K  K  K  L
```

cDNA 10

```
gatgtttctgaatattgattttaatcgtttgcatcatcttgtttaacataaaagaactgtt
  M  F  L  N  I  D  F  N  R  L  H  H  L  V  -  H  K  R  T  V
acaaaacacactcacacagatatacatatactaataattgatctctgtatatataaatat
 T  K  H  T  H  T  D  I  H  I  L  I  I  D  L  C  I  Y  K  Y
atatcgaaaaaagaaattctcaataaagcgatcatgttaatgaaatactactaacatcag
 I  S  K  K  E  I  L  N  K  A  I  M  L  M  K  Y  Y  -  H  Q
ctaagttcttcatta
 L  S  S  S  L
```

cDNA 11

```
gcttaccaggtaaagattaatgtcaggtatgtttatttttgtctgtttcatcccctctt
 A  Y  Q  V  K  I  N  V  R  Y  V  Y  F  L  S  V  S  S  P  L
aagctttgtttaaggctacccaatattttgcgctttcagcaaaatttggaaattgatcct
 K  L  C  L  R  L  P  N  I  L  R  F  Q  Q  N  L  E  I  D  P
caagtacctacctataagggtgatacacttattagattggatactctttgcggtaataca
 Q  V  P  T  Y  K  G  D  T  L  I  R  L  D  T  L  C  G  N  T
tcgtcctggtttttcaatcaggtttacctgacattggccagtcaattgttcaaatttata
 S  S  W  F  F  N  Q  V  Y  L  T  L  A  S  Q  L  F  K  F  I
taccattctaagcttaccttcactttgaaccaagaaaatgcctgtaatttcgattnatgg
 Y  H  S  K  L  T  F  T  L  N  Q  E  N  A  C  N  F  D  X  W
gtaatgatctnaagtttcctatnatctnattggatttgtgaaaagggcgtattgggccaa
 V  M  I  X  S  F  L  X  S  X  W  I  C  E  K  G  V  L  G  Q
aattgctactcttactcacatctatactgttctttcccatttttctttctgtgcgaacttc
 N  C  Y  S  Y  S  H  L  Y  C  S  F  P  F  S  F  C  A  N  F
cactnattttctaatctcatttctttagtttttctttttatccttaccgtttctctacaa
 H  X  F  S  N  L  I  S  L  V  F  L  F  I  L  T  V  S  L  Q
atttnagatcttgtgataacctcttcaaaaattctccttgaagtttaccttagtgcatgt
 I  X  D  L  V  I  T  S  S  K  I  L  L  E  V  Y  L  S  A  C
tgtctacttgtttcaatactgctgaaccaaattcctatttgctctgccacctccttggcc
 C  L  L  V  S  I  L  L  N  Q  I  P  I  C  S  A  T  S  L  A
aaccaggaactgcgtatagaggccgtgttgagttttatgcttgtttnacttgttttctc
 N  Q  E  L  R  I  E  A  V  L  S  F  Y  A  C  X  T  C  F  L
tgttnaggataattcgccccttttccacctggtaagctt
 C  X  G  -  F  A  P  F  H  L  V  S
```

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