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Expression of *EIN2* Gene in Petunia Flowers is Down-regulated During Glucose Treatment

Amin Mirshamsi Kakhki[1], Faraj Shahriari[1], Ahmad Reza Bahrami[2*], and Julie Gray[3]

[1]*Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, P. O. Box 9177948978, Mashhad, Iran*

[2]*Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*

[3]*Department of Molecular Biology and Biotechnology, University of Sheffield, P. O. Box 594, Sheffield, S10 2UH, UK*

Abstract. Glucose is a global regulator of growth and metabolism that is evolutionarily conserved from unicellular microorganisms to multi-cellular animals and plants. In photosynthetic plants, glucose shows hormone-like activities and modulates many essential processes, including embryogenesis, germination, seedling development, vegetative growth, reproduction and senescence. Expression analysis was carried out to evaluate the effect of glucose on ethylene biosynthesis and signaling in petunia flower. Flowers at the anthesis stage were treated with glucose solution and changes in *ACS2*, *EIL-1 and EIN2* genes were monitored over time. While control flowers displayed a typical peak of ethylene production, flowers treated with glucose did not. Glucose appeared to exert its effect on ethylene biosynthesis by suppressing *ACS2* expression. Interestingly, exposure to glucose treatment resulted in a rapid decrease in *EIN2* expression in petals. However, *EIN2* mRNA maintained to notably higher levels in petals of control flowers and decrease in mRNA level was slower than glucose-treated petals. Also, changes in *EIL-1* mRNA levels in glucose-treated flowers showed that glucose delays increase of *EIL-1* mRNA accumulation in petals. These results indicate that glucose may play an important role in ethylene-associated regulation of flower senescence.

Additional key words: *EIN2*, ethylene, flower senescence, glucose, starvation, petunia

Introduction

The plant hormone ethylene regulates a variety of processes, such as the 'triple response' of germinating seedlings, root hair formation, leaf expansion, fruit ripening, and petal senescence after fertilization. In addition, ethylene is known to induce defense responses in reaction to biotic and abiotic stresses (Abeles et al., 1992). Initiation of these processes involves complex regulation of ethylene biosynthesis and depends on the ability of cells to respond in appropriate manner. Flower senescence is one of the developmental processes in which ethylene plays a key role (Reid and Wu, 1992). In many commercially valuable crops, flower senescence is associated with increased production of ethylene. The climacteric rise of endogenous ethylene in these flowers has been shown to play a regulatory role in the events leading to death of some of the floral organs. Ethylene perception is an essential requirement for the initiation and progression of the senescence program (Van Doorn and Woltering, 2008). It is thought that the increase in ethylene responsiveness during petal development culminates in the ethylene climacteric. Therefore changes in ethylene responsiveness during development, most likely mediated by changes in ethylene signaling, which are critical parts in the developmental sequence of events lead to flower senescence (Woodson et al., 1992).

A number of ethylene signaling components have been identified, including five ethylene receptors, such as *ETR1*, a negative regulator of ethylene response gene *CTR1*, and downstream components *EIN2* and *EIN3* (Alonso and Stepanova, 2004; Alonso et al., 1999; Chao et al., 1997; Fujimoto et al., 2000; Guo and Ecker, 2004). *EIN2* acts downstream of *CTR1* and upstream of *EIN3* (Alonso and Stepanova, 2004), having a single copy gene in Arabidopsis and petunia (Alonso and Stepanova, 2004; Shibuya et al., 2004). *EIN2* encodes an Nramp family protein and is considered a central component in ethylene signaling pathway because it is the only gene whose null function mutations result in complete ethylene insensitivity in Arabidopsis (Alonso et al., 1999; Shibuya et al., 2004). Analysis of loss of function mutations indicated that *EIN2* may act as node mediating crosstalk of multiple hormone signaling pathways and responses to pathogens and pests (Alonso et al., 1999; Bent et al., 1992; Cary et al., 1995; Ghasemeian et al., 2000; Lorenzo et al., 2003; Penninckx et al., 1998; Tang et al., 2005).

Recent numerous studies have indicated crosstalk between hormone and sugar-mediated events. Sugars have been known for decades to influence plant growth and de-

*Corresponding author: ar-bahrami@ferdowsi.um.ac.ir

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velopment profoundly, and simply most likely providing a source of energy and carbon. Significant changes in carbohydrate levels can be observed throughout the development of petals and corollas. In most petals and corollas, starch (Tirosh and Mayak, 1988), sucrose and number of other sugars decline after anthesis in both cut and uncut flowers (Tirosh and Mayak, 1988; Van Doorn, 2004). However, high levels of sugars are relatively maintained through senescence in several species (Van Doorn, 2004). Exogenously applied sugars, specially sucrose and glucose, have long been shown to delay flower senescence (Van Doorn and Woltering, 2008). It was widely thought that sugar treatments prolonged vase life by increasing respiratory substrate to the levels that allow for prolonged flower maintenance (Van Doorn, 2004; Van Doorn and Woltering, 2008). However, the fact that a significant amount of sugars remain in floral tissues at the end of senescence in the several species seems to contradict this hypothesis (Ichimura et al., 2000; Van Doorn, 2004).

Glucose has profound effects on gene expression, metabolism, and development in microorganisms, animals, and plants (Koch, 1996). In plants, glucose is known to affect processes such as respiration and photosynthesis (Van Oosten and Besford, 1996), cell wall biosynthesis (Mitchum et al., 1991), and glucose-6-phosphate impacts membrane transport systems (Ramos et al., 1994). At the molecular level, the expression of board spectrum of genes is either repressed or induced by glucose. Recently, hexokinase (*HXK*), the enzyme that catalyzes the phosphorylation of hexose sugars at the first step of the glycolytic pathway, has been shown to be the glucose sensor in plants (Jang et al., 1997). Also, these studies provide supporting evidence that *HXK* is a bifunctional enzyme with catalytic and regulatory activities, and glucose may be uncoupled from glucose metabolism in petals (Jang and Sheen, 1994; Jang et al., 1997). Although the glucose signal transduction pathway are well characterized in unicellular microorganisms, relatively little is known about molecular basis of glucose responses in multi cellular eukaryotes. In higher plants, glucose has been implicated to be the primary sugar signal that controls many aspects of plant development, including germination, hypo-cotyl elongation, cotyledon greening and expansion, primary and lateral root growth, true leaf development, floral transition and the onset senescence (Koch, 2004). Extensive genetic analysis of sugar signaling mutants showing *gin* and *glo* phenotypes have identified unexpectedly intimate connection between the glucose and plant hormonal signaling pathways in *Arabidopsis* (Cheng et al., 2002; Rolland et al., 2002; Smeekens, 2000; Zhou et al., 1998). Of these findings, an antagonistic interaction between glucose and plant ethylene has been the least expected. Furthermore, there is a growing evidence to suggest a regulatory role for glucose, and the effects of glucose treatment have been related to alternations in ethylene biosynthesis and signaling (Zhou et al., 1998). However, the downstream components in the glucose-signaling pathway are mostly unknown. Although interaction between sugar and hormonal signaling pathways, such as ethylene has been suggested (Zhou et al., 1998), the mechanism underlying the crosstalk between glucose and other signaling pathways remain obscure. In this paper we examine the effect of glucose on ethylene biosynthesis and signaling in petals of petunia flower. Our results show that glucose can affect on ethylene responsiveness and production.

Materials and Methods

Plant material and treatment

Petunia×hybrida were grown from seed under standard greenhouse conditions (24/16℃ day/night temperature, with a 16/8 h photoperiod, and 60-80% relative humidity) in a plastic pots containing fertilized soil. Petunia flowers were harvested at anthesis and placed immediately in 1.5 ml micro tubes containing distilled water or 5% glucose solution and held in controlled environmental condition (25/18℃ day/ night temperature, 100 $\mu E \cdot m^{-2} \cdot s^{-1}$). Solutions were changed everyday. Following treatment, petals were collected separately at 0, 24, 48 and 96 h and then frozen in liquid nitrogen for later RNA extractions.

RNA extraction and gene expression analysis

Total RNA was extracted using RNeasy plant mini kit (Qiagen, Valencia, CA) and to avoid amplification of *EIN2* genomic DNA in RT-PCR analysis, isolated RNA was treated with RNase free DNase I (Fermentas) at 37℃ for 1 h. The reaction mixture was extracted with phenol:chloroform: isoamyl alcohol (25:24:1, v/v) followed by ethanol precipitation. *EIN2*, *ACS2*, and *EIL-1* genes were not amplified by PCR with their specific primers when the DNase I-treated RNA was used as template, confirming that the RNA samples were apparently free from genomic DNA contamination. Each RNA sample was quantified with a spectrophotometeric method. The *A*260 /*A*280 ratios of the RNA samples were all greater than 1.7. To ensure the quality of RNA for RT- PCR analysis, the RNA samples were also visualized on agarose gels following ethidium bromide staining. For first -strand cDNA synthesized, 2 μg of total RNA was reverse -transcribed in volume 25 μl containing 10 ng oligo (dT) 12-18 primer, 2.5 mM dNTPs, and 200 units of M-Muiv reverse transcriptase (Fermentas) in reaction buffer. PCR amplification was performed with initial denaturation at 94℃ for 2 min followed by 28

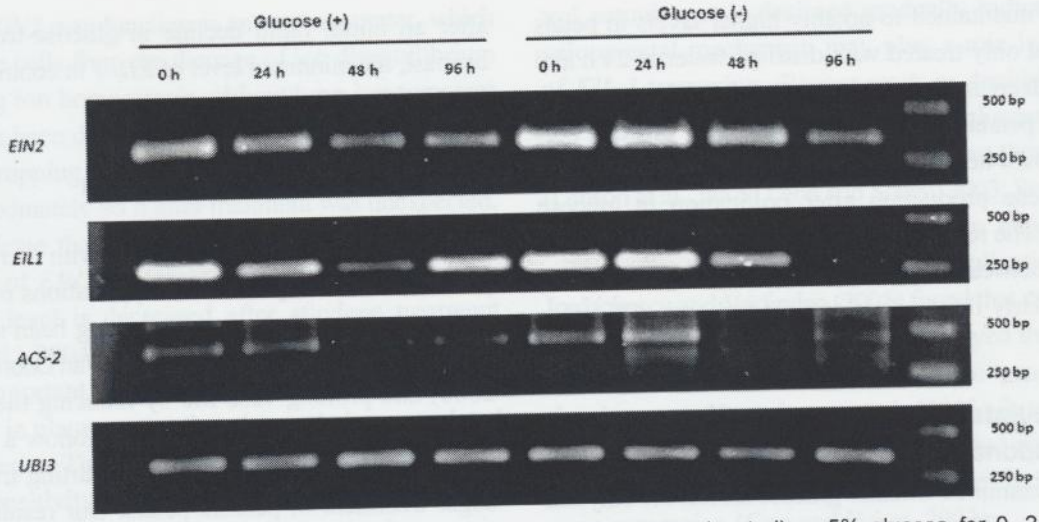

Fig. 1. *EIN2*, *ACS2* and *EIL-1*expressions in petals of flowers treated with water (control) or 5% glucose for 0, 24, 48 and 96 h.

cycles of incubations at 94℃ for 45 s, 50℃ for 60 s, and 72℃ for 75 s, and a final extension at 72℃ for 10 min. To find the suitable cycle for linear amplification, we used 18, 21, 24, 27, and 30 of cycles in PCR reactions, and linear increase of RT-PCR products was observed in reactions with 27 cycles. Therefore, we used 27 cycle for RT-PCR analysis in all of the experiments. The primers used for amplification were as follows: for *Ubi3*:5'-CAG GAC AAG GAA GGG AT-3' and 5'-ACC GCA CTC AGC ATT AG-3'; *EIN2* sense transcripts, 5'-ATT AGC AGG TCT TGG TCG-3'; *EIN2* antisense transcripts, 5'-GCT CCT TCG GGA CTC TAT-3'; *EIL*-1 transcripts, 5'-AGG AAA TGG GGT TCT GTG-3' and 5'-GGT AGG ACC AAC TGG ATT AGA-3'; *ACS2* transcript, 5'-TAA CCC AAA AGC CTC CAT-3' and 5'-AAG ACC GTA GCA GCA TAA AT-3'. *Ubi3* gene severed as an internal control for RNA quantity in RT-PCR. For a negative control, RT reaction mix without reverse transcriptase was used as template in the reaction. Semi-quantification of the RT-PCR products was performed by analyzing the digitized images of the DNA bands resolved on agarose gels using computer software (Total Lab Software, Phoretix ver. 1.10).

Results

EIN2 expression is reduced upon glucose treatment

We examined *EIN2* expression upon glucose treatment condition. The mRNA abundance of *EIN2* was measured in petals at anthesis stage treated with 5% glucose. The expression pattern of *EIN2* gene were analyzed using reverse transcriptase mediated PCR in petals following glucose treatment (Fig. 1). Exposure to glucose resulted in a rapid decrease in *EIN2* expression starting from 24 h of treatment time (Fig. 2A). The *EIN2* transcript reached minimum level at approximate 48 h after treatment, and the expression level remained at low level throughout the longer treatment time. However,

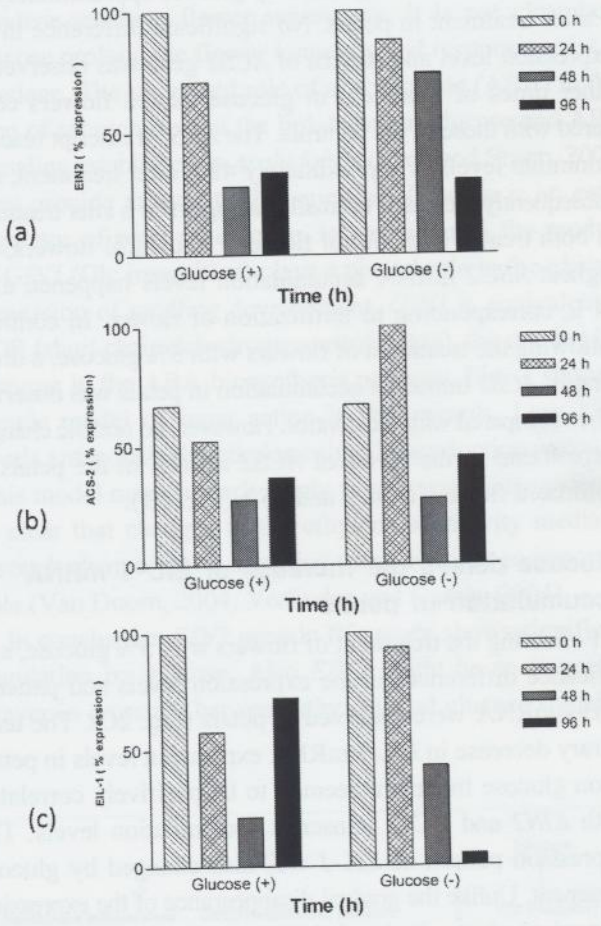

Fig. 2. Time courses of the accumulation of *EIN2*, *ACS2* and *EIL-1* mRNAs in petals of flowers treated with water (control) or 5% glucose. Relative expression levels in percentage for *EIN2* (2A), Relative expression levels in percentage for *ACS2* (2B), Relative expression levels in percentage for *EIL-1* (2C) for 0, 24, 48 and 96 h, respectively.

EIN2 mRNA maintained to notably higher levels in petals of flowers that only treated with distilled water at 24 h and 48 h and the decrease in mRNA levels of *EIN2* was slower in the control petals than the treated petals. A positive correlation is shown here between *EIN2* mRNA level and decreased ethylene production after pollination in glucose treated petals. The results indicate an important role of *EIN2* in a crosstalk between ethylene hormone and sugar-mediated events particularly flower development and post pollination events.

Glucose regulates gene expression pattern of ACS2 in response to glucose during pollination

We further examined whether glucose affects the ethylene regulatory gene, *ACS2* in petunia flowers. As shown in Fig. 1 *ACS2* gene is down-regulated by glucose approximately 24 h after treatment in petals. No significant difference in the expression level and pattern of *ACS2* gene was observed in other times of treatment in glucose treated flowers compared with those in the controls. The *ACS2* transcript reached minimum levels at approximately 48 h after treatment, and subsequently increased to small peak again 96 h after treatment in both treated and control flowers. In control flowers, the highest *ASC2* mRNA accumulation levels happened after 24 h, corresponding to fertilization of flower. In contrast, following the treatment of flowers with 5% glucose, a dropping in *ACS2* transcript accumulation in petals was observed when compared with the control. However, no notable changes were found in the levels of *ACS2* mRNA in the petals of pollinated flowers at 48 h and 96 h (Fig. 2B).

Glucose delays the increase of EIL-1 mRNA accumulation in petals

Following the treatment of flowers with 5% glucose, significance differences in the expression levels and patterns *EIL-1* mRNA were observed in petals (Fig. 2C). The temporary decrease in *EIL-1* mRNA expression levels in petals upon glucose treatment seemed to be positively correlated with *EIN2* and *ACS2* transcript accumulation levels. The expression pattern of *EIL-1* was also changed by glucose treatment. Unlike the gradual disappearance of the expression in control plants, its level drop more rapidly in the treatment up to 48 h and start to accumulate 96 h after glucose treatment. The subsequently rapid increase in *EIL-1* accumulation shifted its peak mRNA level to 96 h after treatment. Although, *EIL-1* mRNA levels in control petals decreased over time and its pattern was similar to glucose treated petals up to 48 h; however, the decrease in mRNA levels was slower in controls than glucose-treated. In another word, *EIL-1* transcript reached to its minimum level at 48 h after an initial rapid decline in glucose-treated petals. In contrast, the minimum level of *EIL-1* in control was observed at 96 h (Fig. 2C).

Discussion

Flower senescence is associated with increased ethylene production in many flowers. Applications of sugars, especially sucrose and glucose, have long been shown to delay flower senescence (Van Doorn, 2004; Van Doorn and Woltering 2008) and prolong vase life by reducing the ethylene sensitivity. This process is expected to follow a significant decrease in *EIN2* mRNA expression, starting from 24 h of the sugar treatment in petunia petals. Our results suggest that glucose may overcome some senescence-enhancing effects of detachment and senescence in petunia, as an ethylene -sensitive flower. Part of such effect might be due to the suppression of ethylene synthesis and decrease in ethylene sensitivity by petals, following glucose treatment.

During the petal senescence in petunia there is a progressive loss of membrane integrity, which results in loss of sub-cellular compartmentalization. The increase in membrane permeability during the petal senescence is characterized by the increased leakage of solutes like electrolytes, anthocyanins from tissue and occurrence of the cellular ion disequilibrium. The large increase in leakiness apparently reflects cell death (Van Doorn, 2004). This increase in membrane permeability is also stimulated by ethylene action and starvation. Thus, low sugar levels may trigger mobilization and petal cell death in cut flowers. In both senescing and starving tissues, sugars repress the expression of several genes involved in mobilization. However, it is not clear whether these similarities simply reflect a similar syndrome or point to a causal relationship between carbohydrate starvation and senescence. Low sugar levels may induce premature senescence through starvation and increased ethylene sensitivity. Possible sites of the interaction are supposed to be *EIN2* and other regulators of proteasomal degradation (Van Doorn and Woltering, 2008).

Recently, Cao et al. (2006) suggested an important physiological role for *EIN2* in plant adaptation to osmotic stresses. In our study, early reduction of *EIN2* gene expression in petunia petals after glucose treatment also supports the important role of *EIN2* in plant adaptation, and it is likely that initial rapid reduction in *EIN2* results in an increase in ABA levels. This may in turn reduce the ethylene production, leading to decreased sensitivity to ethylene to maintain cell function during petal senescence. Several cellular processes, such as changes in ion channels, might lead to the initial, rapid response. Based on the unique structure of *EIN2*, it is

plausible that *EIN2* may function as an ion transporter, which may protect the cells from the damage of ion disequilibrium by maintaining ion homeostasis, although no ion transport activity has yet been detected (Alonso et al., 1999).

However, dropping levels of *EIN2* mRNA in the control petals at approximately 96 h after treatment was unexpected. This may indicate that feedback inhibition is involved in the regulation of *EIN2* expression. In petal limb and tube, *EIN2* mRNA level is decreased after ethylene treatment (Shybuya et al., 2004). The flower senescence process is particularly important for successful pollination and subsequent seed set in plant species that show ethylene-regulated flower senescence. Thus, there may be a regulation system of ethylene sensitivity in flowers through control of *EIN2* expression. However, it is also possible that the decrease in *EIN2* mRNA level in a later time course of ethylene treatment is a consequence of ethylene-induced senescence process in petals (Shybuya et al., 2004), and suggest that *EIN2* expression in petunia flowers is responsible for ethylene sensitivity during senescence.

In petals, a decrease in *ACS2* mRNA levels was observed after 24 h of glucose treatment. However, there was no clear difference in mRNA levels and no notable changes in pattern of *ACS2* were found in other time courses of glucose treatment. Germination of the pollen in petunia was shown to begin as early as one hour post pollination (PP). It is followed by an initial burst of ethylene, 2-4 h after pollination, reaching to a peak at 12 h, then a higher peak at 24 h after pollination. Our results suggest that glucose inhibits ethylene production after pollination. We suggest that dropping the levels of *ACS2* might be a consequential effect of glucose on ethylene biosynthesis after pollination *per se*. These results are in line with other reports. 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase transcript accumulations are lower when pre-senescent sucrose-treated flowers are exposed to ethylene (Van Doorn, 2004; Verlinden and Garcia, 2004). Hong et al. (2004) showed that exogenous glucose could delay the ethylene climacteric in ripening tomato fruit. Also they found that decrease in glucose content of tomato locular tissue is an important factor in signaling the ripening initiation and it was hypothesized that glucose may affect ripening by impacting ethylene biosynthesis. At the higher concentrations of glucose, development of color was also retarded. Treatment of mature-green fruit with glucose increased the area of green-shoulder and inhibited color development.

As expected glucose treatment delayed the increase of *EIL-1* mRNA level in the petals of our experiment. However, the gradual decrease in *EIL-1* mRNA levels in the control flowers was unexpected. The fact that mRNA levels in control petunia petals declined gradually indicates that a developmental mechanism may play a role in accumulation of *EIL-1* transcripts. Recent work in *Arabidopsis thaliana* showed a close interaction between glucose, sucrose and ethylene signaling pathways that support these observations (Gibson et al., 2001; Leon and Sheen, 2003; Smeekens, 2000), and some studies showed that certain ethylene-signaling components are regulated by sugar applications to flowers. Iordachescu and Verlinden (2005) found that carnation flowers treated continuously with sucrose delayed the transcript accumulation of at least one *EIN3*-like gene during petal development. Yanagisawa et al. (2003) found that glucose enhances the degradation of *EIN3*, and that ethylene represses constitutive *EIN3* degradation, hence activating downstream gene expression (Guo and Ecker, 2003).

Although these results may explain a role for glucose in ethylene-sensitive flower senescence, it is not clear how glucose prolong the flower longevity and responsiveness to ethylene. The suggested role of abscisic acid (ABA) in this type of senescence and the link between glucose and ABA signaling established in *Arabidopsis* (Leon and Sheen, 2003) may provide answer to this question. Glucose is an early regulator of petal senescence. In *Arabidopsis*, the product of *GIN1* (Glc insensitive) plays a pivotal role in the glucose repression of seedling development. *GIN1* is equivalent to SDR (short chain dehydrogenase/reductase), the one but last enzyme in the ABA biosynthesis pathway. Fig. 3 shows a simple model of sugar action in this regards. Low ABA levels antagonize the ethylene signal transduction pathway. This model may similarly apply to senescence. However, it is clear that changes in the ethylene sensitivity mediated through changes in the signaling pathways play an important role (Van Doorn, 2004; Verlinden and Garcia, 2004).

In conclusion, *EIN2* gene in this study shows significant regulation by glucose. Also *EIN2* might be an important player in crosstalk between ethylene and glucose signaling

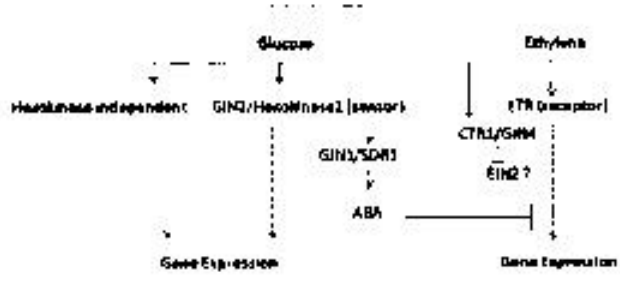

Fig. 3. Simple model of sugar repression of ethylene sensitivity according to Leon and Sheen (2003). Glucose apparently inhibits the ethylene transduction pathway through production of low ABA concentration.

particularly during flower development and senescence. However, the mechanism of how the glucose affects the expression of *EIN2* and accumulation downstream components is needs investigation. Complex patterns of these components' accumulation indicate an intricate interplay between ethylene signaling and production, developmental events and glucose signaling pathway in petunia.

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