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Modeling the Roles of *miRNA164* in Determining the Age-dependent Cell Death in Plant Leaves

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Senescence is a genetically controlled developmental process that leads to cell death and limits the life span at the cellular, tissue, organ, and organism levels [1]. Leaf senescence and the associated cell death, are evolutionary selected developmental processes to maximize plants' fitness by facilitating nutrient recovery and recycling [2]. For example, annual plants such as soybean, corn, or rice relocate nutrients from senescing leaves to reproducing seeds through leaf senescence, which ensures optimal production of offspring and better survival of plants in given temporal and spatial niches.

It is well known that the timing of leaf senescence is controlled by developmental age under the influence of other internal factors such as phytohormones and reproductive development and external factors including abiotic and biotic stresses [3, 4]. Therefore, it is obvious that multiple pathways responding to various endogenous and environmental factors are interconnected to form a highly complex regulatory network whose dynamic operation induces age-dependent leaf senescence by fine-tuning the initiation timing and progression rate.

For the past decade, major breakthroughs in the understanding of leaf senescence and the associated cell death have been achieved through isolation and

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characterization of various senescence-associated genes (*SAG*s) and senescence mutants [5-11]. These studies have revealed complex molecular regulatory networks underlying leaf senescence. A number of molecular and genomic approaches have been employed to isolate genes that are differentially expressed during senescence from several plant species, such as *Arabidopsis*, barley, *Brassica*, maize, and rice [5-7]. Especially, analyses of genome-wide gene expression changes during leaf senescence in *Arabidopsis* have led to the identification of more than 800 potential *SAG*s that are distinctively regulated during leaf senescence [12]. Although these *SAG*s might be useful to establish a rough sketch of a complex molecular regulatory network of leaf senescence, fundamental molecular mechanisms involved in the coordinated regulation of these *SAG*s during leaf senescence still need to be investigated. As an effort to unveil the regulatory mechanisms of leaf senescence, we have performed a genetic approach involving isolation and characterization of senescence mutants in *Arabidopsis thaliana*. In addition to the advantages of *Arabidopsis* as a plant model system, its leaves have other crucial advantages in leaf senescence research. *Arabidopsis* leaves undergo readily distinguishable developmental stages and also show well-defined and reproducible phenotypes of senescence and the associated cell death (Figure 1). Through the study on the *oresara1* (*ore1*, oresara means “long-living” in Korean), we recently reported that a trifurcate feed-forward pathway, involving EIN2 (Ethylene Insensitive 2), ORE1, and *miR164*, regulates age-dependent cell death and senescence in *Arabidopsis* leaves, which serves as a significant milestone for unraveling the elaborate regulatory mechanisms of leaf senescence and the associated cell death [13]. In summary, ORE1, a NAC (NAM, ATAF, and CUC) transcription factor, functions positively in aging-induced cell death. *ORE1*’s expression is up-regulated in an age-dependent manner by EIN2 but is negatively regulated by *miR164*. The *miR164* whose expression gradually decreases with leaf aging by EIN2 (Figure 1) ultimately leads to the up-regulation of *ORE1* expression. However, EIN2 also contributes to aging-induced cell death in another pathway that does not include ORE1. We proposed that plants utilize the trifurcate feed-forward pathway, a highly robust regulatory network, to ensure senescence and the accompanying cell death when leaves are aged as an evolutionary selected developmental process to maximize plants’ fitness.

We are further investigating the role of *miR164* in senescence and the associated cell death by employing a mathematical modeling of the trifurcate feed-forward pathway.

The trifurcate pathway is characterized by embedded coherent feed-forward loops including coherent type 1 & coherent type 4 feed-forward loops (Figure 2), according to the categorization of feed-forward loops existing in gene regulatory network by Mangan *et al*. [14]. Coherent type 1 and 4 structures involve different forms of activation from a molecule X to Z through Y. These feed-forward structures are widely distributed in gene regulatory networks, but an embedded structure of feed-forward loops and its functions in biological processes have been rarely reported.

To understand a function of the trifurcate pathway, we developed a mathematical model for the trifurcate pathway, as described in [14]. In this model,

we assumed that EIN2 and *miR164* simultaneously regulated *ORE1*, and EIN2 and ORE1 did *SAG12* in the same way. From this assumption, we used 'and-logic' to express *ORE1* and *SAG12* via the feed-forward loops, which allows us to explain delayed response of *SAG12* after a persistent activation of EIN2. We set parameter values to fit the model to experimental data from the wild type plant. The simulation results from the mathematical model shed several insights into functions of the trifurcate pathway and its underlying design principles. Figure 3A shows that the expression of *SAG12* gradually increases on a systematic, persistent action of EIN2 in the trifurcate feed-forward pathway, while it does not change on a nonsystematic, transient action of EIN2. Moreover, the simulation results suggest that the delayed response of *SAG12* may be due to the suppression of *ORE1* by *miR164*. When we assume the absence of *miR164* computationally, we can observe that *SAG12* increases rapidly on EIN activation (Figure 3B). This observation implies that *miR164* may play an important role in endowing the delayed response of *SAG12*, thus resulting in highly robust regulation of age-dependent cell death by the trifurcate pathway.

To rigorously investigate a role of *miR164* in age-dependent cell death, we plan to examine the response of the trifurcate pathway in the absence of double negative interactions involving *miR164* and confirm whether the role of *miR164* may be maintained under certain variations of the trifurcate pathway model (e.g. rates of inhibitions of *miR164* by EIN2 and also *ORE1* by *miR164*).

Senescence and the associated cell death in plants are affected not only by aging, but also by external stresses. For example, salt stress accelerates senescence and cell death in plants in *ORE1* and *EIN2*-dependent manner. Figure 4A shows that senescence and cell death is delayed in *ore* mutant lines under salt stress condition, compared to the wild type plant in the same stress. In addition, *ORE1* is induced by salt and affects salt-induced senescence (Figure 4B). Interestingly, the regulation of miR164, ORE1, and SAG12 expression by salt showed a clear dependence the age of leaves. We will investigate a detailed mechanism underlying the integration of salt stress into the trifurcate pathway, especially in relation to *miR164* using mutant lines genetically perturbing the trifurcate pathway (Figure 4). In addition to the role of *miR164* intrinsic to the trifurcate pathway, we will be able to explain a role of external factors such as salt stress extrinsic to the trifurcate pathway of age-dependent cell death.

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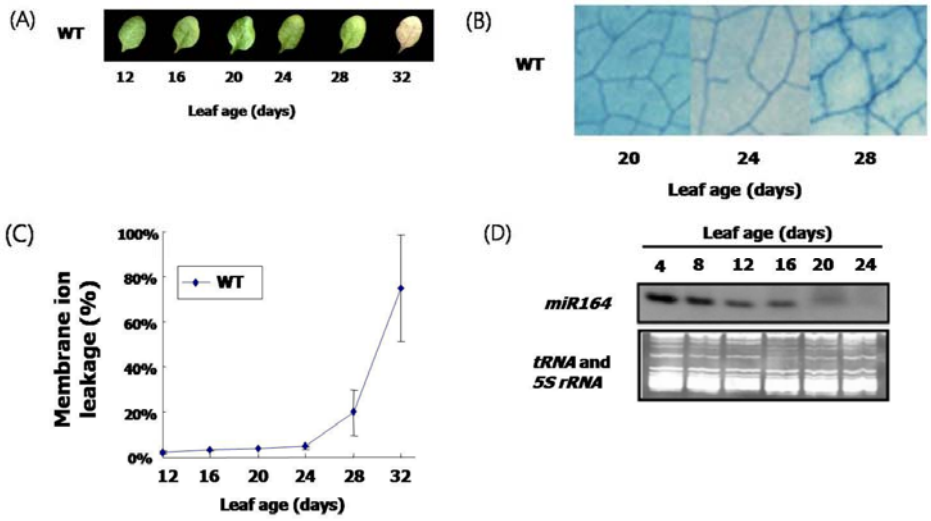

Figure 1: Age-dependent cell death analysis of *Arabidopsis* leaves. (A) The age-dependent senescence phenotype of wild type leaves. (B) Trypan blue-staining of wild type leaves during age-dependent senescence. (C) Membrane ion leakage in wild type leaves during age-dependent senescence. (D) Expression pattern of *miR164* with leaf aging

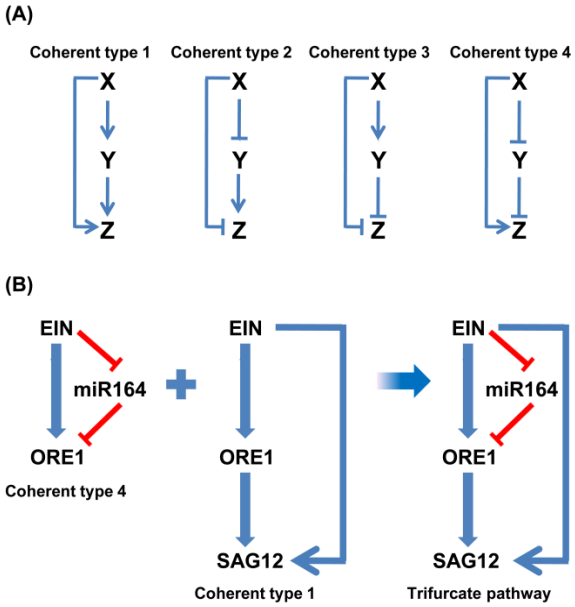

Figure 2: Coherent feed-forward structures and the trifurcate pathway (A) Structures of coherent feed-forward regulations [14]. (B) The trifurcate pathway contains two embedded coherent feed-forward loops (coherent type 1 & 4).

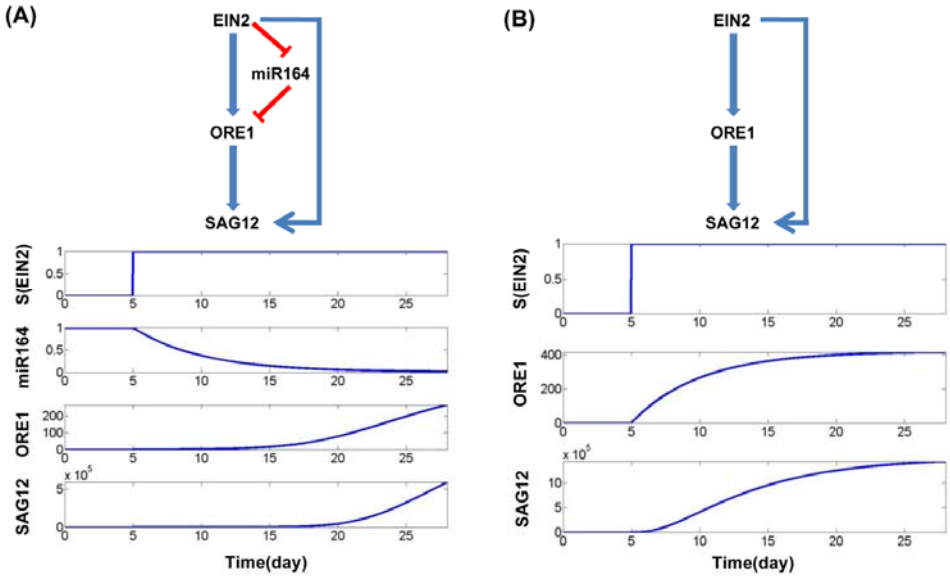

Figure 3: The role of *miR164* in age-dependent cell death. (A) The trifurcate pathway (above) and corresponding simulation result (below) showing a delayed response after a persistent activity of EIN2 with *miR164*-double negative activation. (B) A bifurcate pathway without *miR164* (above) and corresponding simulation result (below) showing a rapid response on the same EIN2 activation as (A).

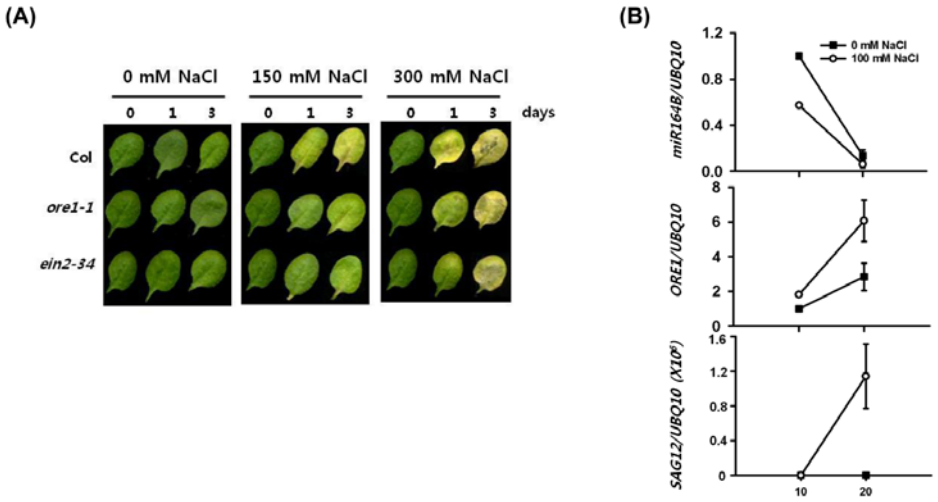

Figure 4: Salt stress affects leaf senescence. (A) Chlorophyll loss in the wild type (Col), *ore1-1*, and *ein2-34* under salt stress condition at the indicated days. (B) mRNA expression levels of *miR164B, ORE1, and SAG12* in the wild type plant with/without salt stress.