Identification of Three Genetic Loci Required for Progression of Leaf Senescence in *Arabidopsis thaliana*

Sung Aeong OH, Joon Hyun PARK, Gyu In LEE, Kyung Hee PAEK[1], Soon Ki PARK, Hong Gil NAM*

Department of Life Science and School of Environmental Engineering, Pohang University of Science and Technology, Pohang, Kyungbuk, Korea; and [1]Department of Agronomy, Korea University, Anam-Dong, Seoul, Korea

Three key genetic loci required for proper progression of leaf senescence were identified in *Arabidopsis thaliana*. Mutations in these loci cause delay in all senescence symptoms examined, including both anabolic and catabolic activities, during natural senescence and upon artificial senescence induced by various senescence-inducing treatments. The result provides a decisive evidence that leaf senescence is a genetically programmed phenomenon controlled by several monogenic loci in *Arabidopsis*. The result further indicates that leaf senescence caused by various senescence signals occurs, at least in part, through common pathways in *Arabidopsis* and that the three genetic loci function at the common steps.

Leaf development ends with senescence consisting of deteriorative events that lead to cell death (1). During leaf senescence, the cells experience dramatic changes in metabolism and cellular structure. The most visible one is the color change in autumn leaves and in the leaves of monocarpic plants, which is due to preferential breakdown of chlorophyll and synthesis of the other pigments. This phenomenon occurs concomitantly with chloroplast degradation (1, 2) and with attenuated anabolic activities such as photosynthesis and protein synthesis (1). Instead, catabolism such as nucleic acid breakdown and proteolysis becomes active through induction of a number of hydrolytic enzymes (1, 3). Leaf senescence, although a deteriorative cellular process in its nature, is assumed to be an evolutionarily acquired, active genetic trait that makes an important contribution to fitness of plants, for example by remobilizing nutrient from vegetative tissues to reproductive organs (1). Elucidating the genetic mechanism of leaf senescence should be essential for the understanding of the senescence phenomenon itself and also for practical purposes such as the improvement of plant productivity, pre- or post-harvest storage, stress tolerance, etc. However, in spite of the biological and practical importance of leaf senescence, the genetic mechanism controlling the leaf senescence process remains poorly understood. We, therefore, undertook a systematic genetic screening for identification of the genes that control leaf senescence, using *Arabidopsis thaliana* as a model system. *Arabidopsis*, as a representative monocarpic plant, displays a relatively reproducible leaf senescence pattern (4, 5) along with its short life span and is readily amenable to genetic analysis.

Mutant plants with delayed leaf senescence were screened from seed pools mutagenized by ethylmethanesulfonate (EMS) (6) or by T-DNA insertion (7, 8, 9). Initial screening was carried out by visual evaluation of the degree of leaf yellowing caused by chlorophyll loss either during natural *in planta* senescence (*in planta* screening) or during incubation of detached leaves in darkness (dark screening). The latter scheme has been widely adopted in studies of leaf senescence (1, 3) and was employed here to accelerate leaf senescence in a more consistent manner. From the dark screening, we have isolated four mutants from approximately

25,000 M2 plants. These mutant lines were named *any1, 2, 3,* and *11,* respectively: *any* stands for *Arabidopsis* non-yellowing. From the *in planta* screening, we have identified one mutant from approximately 9,000 M2 plants. This mutant line was named *any9.*

Although chlorophyll loss and the concomitant yellowing of leaves are convenient and distinctive indications of leaf senescence, these phenotypes can be uncoupled from "unctional" leaf senescence (3).

For example, the naturally occurring "stay green" varieties of a few crop plants show delay in chlorophyll loss but not in loss of anabolic activities (10). To evaluate the degree of functional senescence in the mutant lines we have isolated, we examined the integrity of photosystem (PS) II as well as the chlorophyll contents. The integrity of PS II was tested by measuring the photochemical efficiency of PS II, a sensitive and effective parameter of functional leaf senescence (11, 12, 13). A preliminary assessment showed that loss of chlorophyll contents was delayed in all the mutant lines. Loss of the photochemical efficiency was delayed in the *any1, 2, 3,* and *9* mutant lines, but not in the *any11* line (data not shown). The result indicated that the delayed yellowing phenotype of the *any11* mutant was likely due to a lesion in chlorophyll metabolism rather than due to delay in functional senescence. The *any11* mutant line was not studied any further.

Examination of the genetic behavior of the 4 mutant lines, *any1, 2, 3,* and *9,* is summarized in Table 1. The delayed yellowing phenotype in all of the mutant lines was inherited as monogenic recessive Mendelian genetic traits. The genetic complementation test (Table 1) revealed that the mutant lines fell into 3 complementation groups. We designated the genes for the 3 monogenic complementation groups as *ANY1, ANY3* and *ANY9,* respectively (see below for genetic mapping). The phenotypes of *any1, any3,* and *any9* mutant plants (14) are shown in Figure 1.

The senescence behavior of the mutant plants was then examined in further detail during natural *in planta* senescence and artificial senescence induced by darkness. The data in Figure 2 clearly show that, in all of the three mutant plants, both of the chlorophyll contents (Figure 2A) and the photochemical efficiency (Figure 2B) are retained at much higher levels during natural and dark-induced senescence. The retention of cellular functions during senescence in the mutant leaves was further demonstrated by examining the relative amount of the large subunit of ribulose bisphosphate carboxylase oxygenase (Rubisco) complex as an indication of photosynthetic activity in leaf tissue. As shown in Figure 2D, relatively higher amounts of the protein are retained in the mutants than in wild type plants during both natural and artificial senescence. The results indicated that functional senescence was delayed in the 3 mutant lines.

While the senescence parameters examined above indicated that the mutants are delayed in their loss

Table 1. Genetic analysis of the mutations. The phenotypes of the progenies were scored visually for the delayed yellowing phenotype during both natural and dark-induced senescence in each plant. In all the progenies examined, the two phenotypes cosegregated. The χ^2 value is for an expected ratio of 3:1 (wild type:delayed senescence).

Cross	Type	Total	Phenotype [a] +	Phenotype [a] −	χ^2
any1/any1 × ANY1/ANY1	F1	30	30	0	
	F2	432	319	113	0.309 (p)0.5)
any2/any2 × ANY2/ANY2	F1	89	89	0	
	F2	230	177	53	0.470 (p)0.1)
any3/any3 × ANY3/ANY3	F1	22	22	0	
	F2	130	98	32	0.010 (p)0.9)
any9/any9 × ANY9/ANY9	F1	25	25	0	
	F2	196	142	54	0.680 (p)0.1)
any2/any2 × any3/any3	F1	25	0	25	
any1/any1 × any3/any3	F1	32	32	0	
any1/any1 × any9/any9	F1	29	29	0	
any3/any3 × any9/any9	F1	25	25	0	
ein2/ein2 × EIN2/EIN2	F1	42	42	0	
	F2	78	57	21	0.154 (p)0.5)
any3/any3 × ein2/ein2	F1	32	0	32	
any2/any2 × ein2/ein2	F1	29	0	29	

[a] +, wild type: −, delayed senescence.

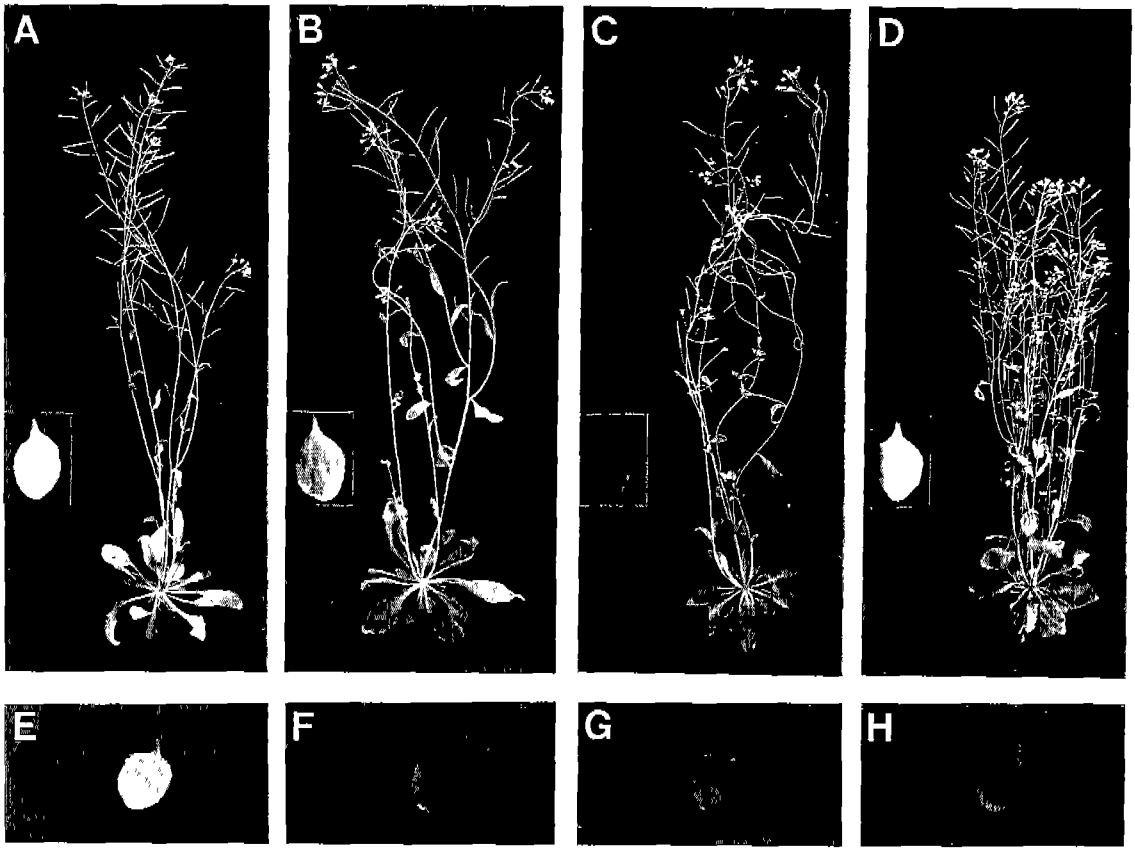

Figure 1. (A-D) Phenotypes of a whole plant and a representative fourth foliar leaf of wild type (A) and *any1* (B), *any3* (C), and *any9* (D) mutants at 50 days after germination (DAG). The plants were grown in 16 hour light/8 hour dark cycle at 23°C in a growth chamber. Neither the age of the leaves, as measured from the time of leaf emergence, nor the time of flowering was significantly different enough between the wild type and mutant plants to account for the apparent phenotypic difference in leaf senescence (15). (E-F) Phenotype of a fourth foliar leaf of wild type (E) and *any1* (F), *any3* (G), or *any9* (H) mutant upon induction of senescence by incubation in darkness. The leaves at a same age (21 DAG for wild type, *any3*, and *any9*; 22 DAG for *any1*) was detached and incubated on 3 mM 2-N-morpholinoethanesulfonic acid buffer (MES, pH 5.8) for 4 days in darkness.

of components for anabolic (photosynthetic) activities, leaf senescence is known also to involve the activation of catabolic (or hydrolytic) activities for degradation of cellular constituents. For example, senescence-associated RNase activity was found in a number of plant species and is thought to be involved in the remobilization of phosphate to nonsenescing organs (16, 17). We examined RNase activity, as a representative catabolic activity, in the mutant plants during leaf senescence. The results in Figure 2C show that the total cellular RNase activity, examined under neutral pH (16), increased to a lesser extent in the mutant plants than that in the wild type leaf during both natural and dark-induced senescence.

Leaf senescence also involves the disintegration of cellular ultrastructure in addition to metabolic changes. The disintegration of chloroplast ultrastructure in particular is one of the earliest symptom of leaf senescence (3). We have examined the chloroplast ultrastructure of the mutant and wild type leaves upon dark-induced senescence. As shown in Figure 3, the mutants retained substantially more of the granar structure than did the wild type. In

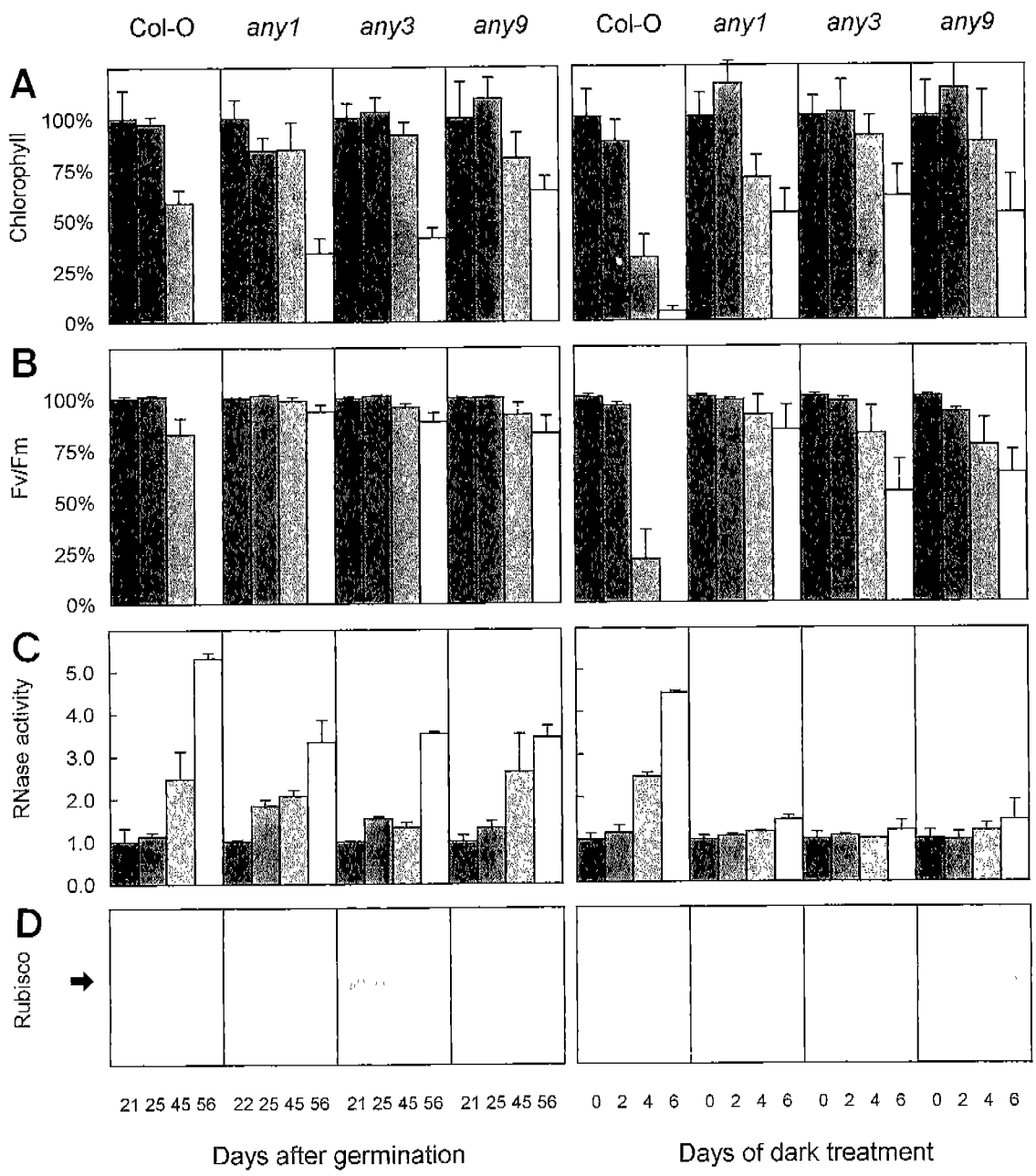

Figure 2. Examination of senescence parameters in the delayed senescence mutants. Chlorophyll content (A), photochemical efficiency (B), total cellular RNase activity (D) and the relative amount of the large subunit of Rubisco complex (C) were examined at several developmental stages (left) or at the given times after incubating detached leaves in darkness (right), using the third and fourth foliar leaves of wild type (Col-O), *any1*, *any3* and *any9* plants. The sampling time was chosen in relation to the timing of flowering and the advancement of senescence in wild type plants (12). The leaves of wild type, *any3*, and *any9* were assayed at the indicated times. The leaves of *any1* were assayed 1 day later than those of the others, to equalize the age of the leaves (15). The dark-induced senescence experiments were performed with the leaves at 21 DAG (wild type, *any3*, and *any9*) or at 22 DAG for *any1*. Shown is the relative value as percentage or fold of the initial point value for each experiment. The vertical bars denote standard deviations. Chlorophyll contents per fresh weight of leaf tissue were determined from individual leaves as described (4, 5, 12). The data were collected from 6 leaves for each experiment. The photochemical efficiency (Fv/Fm, 11) was determined from 12 leaves for each experiment. Total RNase activity assayed by measuring the release of acid-soluble material from yeast rRNA (16) under neutral pH was obtained from 6 individual leaves for each experiment. The relative amount of the large subunit of the Rubisco complex (arrow) in total soluble cellular protein was examined by SDS-polyacrylamide gel electrophoresis and then by staining the gel with Coomassie brilliant blue.

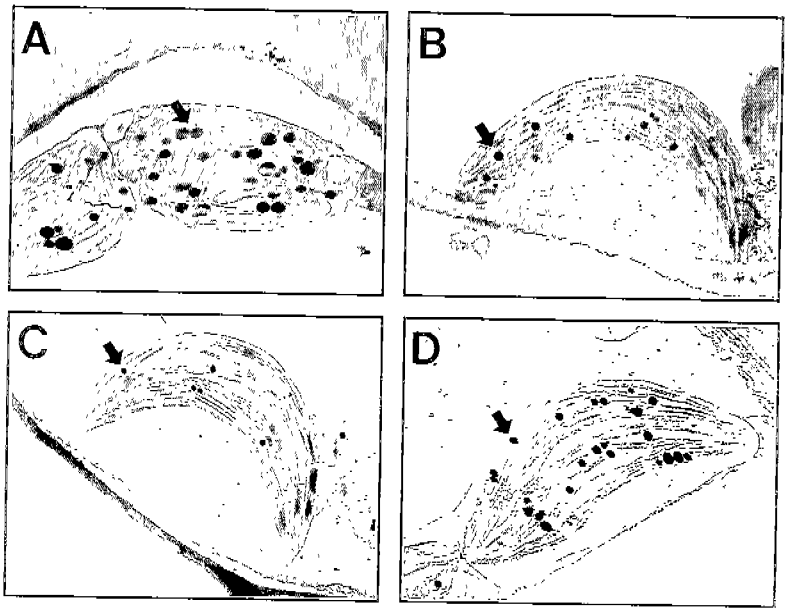

Figure 3. Transmission electron micrograph of chloroplast ultrastructure. The fourth foliar leaf at 21 DAG (A, wild type: C, *any3*: and D, *any9*) or 22 DAG (B: *any1*) was incubated in darkness for 6 days and prepared for transmission electron micrography. The electron dense particles indicated by arrows are plastoglobuli.

Table 2. Senescence responses of the mutants to treatment with senescence-hastening plant hormones. The photochemical efficiency (11) was measured with the detached fourth foliar leaves incubated in darkness in the presence or absence of each hormone (19). The data presented are expressed as percentages relative to each control experiment without hormone treatment. The values in parenthesis are standard deviations.

	ABA	Ethylene	MeJA
Col-0	50.3 (9.6)	52.4 (7.2)	53.6 (6.6)
any1	97.1 (2.9)	98.3 (2.0)	95.1 (6.5)
any3	93.3 (3.9)	97.7 (1.8)	95.2 (8.0)
any9	87.1 (7.0)	96.4 (1.7)	90.8 (2.4)

addition, the number of plastoglobuli, an indication of chloroplast membrane disintegration (2), is also lower in the mutant leaves than in wild type leaves.

The fact that the mutations identified here delay a broad spectrum of senescence symptoms including both anabolic and catabolic activities suggests that the genes defined by these mutations are most likely the key regulatory genetic components involved in the progression of functional leaf senescence. The result reported here, thus, presents a clear genetic evidence that functional leaf senescence in *Arabidopsis* is a genetically controlled event involving several monogenic genetic elements.

Leaf senescence is regulated by several endogenous factors, such as developmental age and plant hormones (1), as well as by exogenous factors. Especially three plant hormones, ethylene, abscisic acid (ABA) and methyl jasmonate (MeJA) are known to strongly hasten leaf senescence (1). We have examined a senescence response of the mutants to these hormones. The results in Table 2 show that the senescence response of the mutant leaves to all three hormones is highly reduced, when compared to that of wild type leaves.

The fact that the mutants display delayed senescence in response to several senescence-inducing signals (developmental age, dark, ABA, ethylene and MeJA) suggests that leaf senescence caused by these factors may proceed, at least in part, through common pathways in *Arabidopsis*. The results further suggest that the genetic loci we have identified here function in the common pathways. It should be noted that senescence is only delayed in these mutations, but it ultimately does occur. This could be explained if the mutations are weak alleles of the genes or alternatively if senescence proceeds through several parallel pathways. Accepting the

notion that senescence is important for fitness of plants, remobilizing nutrients from senescing tissue to young or reproductive organs, the strategy of having genetically redundant pathways would be advantageous for plants to promote the fidelity of the senescence process (20).

A plant hormone can affect a wide variety of plant processes, including senescence (21, 22, 23). The reduced senescence response of the mutants to the three plant hormones prompted us to examine if the mutations we identified are alleles of known plant hormone mutations. We found that the *any1* and *any9* mutations have normal response to the three hormones in seedling development (24, data not shown), separating them from the known mutations in these hormonal pathways. Furthermore, the chromosomal mapping data (25) showed that the two genes are located at positions away from that of any of the known mutations in the three hormonal pathways. These results suggest that we have identified two new genetic loci involved in leaf senescence. Seedlings with the *any3* mutation showed normal response to ABA or MeJA, but insensitivity to ethylene treatment (24). The genetic complementation test (Table 1) showed that *any3* is, in fact, allelic to the *ein2-1* mutation (26), an ethylene-insensitive mutation. While it has been stated that some of the mutations in the ethylene signal pathway affect leaf senescence (26, 27, 28), only the *etr1* mutation was analyzed in detail to show that the mutation causes delay in several senescence-related symptoms (28). Our results show that *EIN2* gene also has a dual function in both leaf senescence and seedling development, adding *EIN2* as another ethylene pathway gene involved in leaf senescence.

REFERENCES

1. L. D. Noodén et al., *Senescence and Aging in Plants*, L. D. Nooden and A. C. Leopold, Eds. (Academic Press, San Diego, 1988); H. Thomas and J. L. Stoddart, Plant Physiol. 31, 83 (1980); P. Matile, in Crop *Photosynthesis: Spatial and Temporal Determinants*, N. Baker and H. Thomas, Eds. (Elsevier, Amsterdam, 1992), pp. 413-440; K. V. Thiman, Ed., in *Senescence in Plants* (CRC Press, Boca Raton, 1980), pp. 85-115.
2. U. C. Biswal and B. Biswal, in *International Review of Cytology*, G. H. Bourne, K. W. Jeon, M. Friedlaner, Eds. (Academic Press, San Diego, 1988), vol. 113, pp. 271-321.
3. C. M. Smart, *New Phytol.* 126, 419 (1994).
4. L. L. Hensel, V. Grbic, D. A. Baumgarten, A. B. Bleecker, *Plant Cell* 5, 553 (1993).
5. K. N. Lohman, S. Gan, M. C. John, R. M. Amasino, *Physiol. Plant.* 92, 322 (1994).
6. Approximately 40,000 seeds (M1) of each of Col-O and La-O ecotype were mutagenized by treatment with 0.33% EMS solution for 8 hours. M2 seeds were obtained by self-fertilization of the M1 plants.
7. The T-DNA insertional lines used in these experiments were obtained in part from the Ohio seed stock center or were generated in part in our laboratory using the *in planta* transformation procedure (8).
8. S. S. Chang et al., *Plant* J. 5, 551 (1994).
9. K. A. Feldmann, M. D. Marks, *Mol. Gen. Genet.* 208, 1 (1987).
10. H. Thomas, C. M. Smart, *Ann. Appl. Biol.* 123, 193 (1993).
11. The photochemical efficiency of PS II of the mutant plants was deduced from the characteristics of chlorophyll fluorescence as described (12). The ratio of Fv (maximum variable fluorescence)/Fm (maximum yield of fluorescence), the potential quantum yield of photochemical reactions of PS II, was employed as the value of the photochemical efficiency of PS II (13).
12. S. A. Oh, et al., *Plant Mol. Biol.* 30, 739 (1996).
13. V. Raggi, *Physiol. Plant.* 93, 540 (1995); I. John et al., *Plant J.* 7, 483 (1995).
14. *any1* was isolated from EMS-mutagenized La-O ecotype seeds. The others were from EMS-mutagenized Col-O ecotype seeds. The mutant plants were back-crossed twice to Col-O. The descendants of a single progeny derived from each backcross were used for the experiment in Figure 1 and all others, except the genetic analyses.
15. The fourth and sixth foliar leaves of *any1* emerged 1 day and 3 days later than those of wild type, respectively. The leaves of *any3* emerged at an almost same time as those of

wild type. The third and fourth foliar leaves of *any9* emerged at the time as those of the wild type, while the sixth foliar leaf of *any9* emerged 2 days earlier than the corresponding wild type leaf. The time of flowering, measured as the time of the first visual appearance of the floral inflorescence, was 3 days later and 2 days earlier in *any1* and *any9* mutants, respectively, than in wild type, respectively. The flowering time of *any3* mutant was same as that of wild type.

16. A. Blank, T. A. McKeon, *Plant Physiol.* 97, 1409 (1991).
17. C. B. Taylor et al., *Proc. Natl. Acad. Sci. U.S.A.* 90, 5118 (1993).
18. A. R. Spurr, *J. Ultrastruct. Res.* 26, 31 (1969).
19. Detached leaves of plants at 21 DAG (wild type, *any3*, and *any9*) or 22 DAG (*any1*) were incubated by floating on 3 mM MES buffer (pH 5.8) with or without hormones. The leaves were incubated for 2 days in the ABA (10 μM)-containing medium and for 3 days in ACC (100 μM) or MeJA (50 μM)-containing medium in darkness. ACC produces ethylene upon hydrolysis.
20. J. H. Thomas, *Trends Genet.* 9, 395 (1993).
21. C. E. Benedetti, D. Xie, J. G. Turner, *Plant Physiol.* 109, 567 (1995).
22. M. Koornneef, G. Reuling, C. M. Karssen, *Physiol. Plant.* 61, 377 (1984); J. Giraudat et al., *Plant Cell* 4, 1251 (1992).
23. J. R. Ecker, *Science* 268, 667 (1995); F. B. Abeles, P. W. Morgan, M. E. Saltveit Jr., *Ethylene in Plant Biology* (Academic Press, New York, ed. 2, 1992).
24. The seedling responses examined are seedling growth inhibition by MeJA (21), germination inhibition by ABA (22) and the "triple response" by ethylene (23). Surface-sterilized seeds were plated on a medium (21) with or without 1-aminocyclopropane-1-carboxylic acid (ACC, 10 μM, Sigma), ABA (10 μM, Sigma), MeJA (5 or 50 μM, Bedoukian Research). Seeds plated on the ACC-containing medium were incubated in darkness and seeds plated on the ABA- or MeJA- containing medium were kept under continuous light. The *any1* or *any3* seedlings responded to the three hormones in a manner similar to wild type seedlings. The *any3* seedlings also responded normally to ABA and MeJA but had longer hypocotyl and root and reduced development of apical hook in the presence of ethylene, when compared to wild type seedlings.
25. The chromosomal mapping was done by the CAPS mapping strategy [A. Konieczny, F. M. Ausubel, *Plant* J. 4, 403 (1993)]. *ANY1* is located 16.5 ± 3.6 cM away from *DFR* loci on chromosome 5. *ANY9* is located 21.7 ± 4.5 cM away from *GPA* loci on chromosome 2.
26. P. Guzman, J. R. Ecker, *Plant Cell* 2, 513 (1990).
27. L. Zacarias, M. S. Reid, *Physiol. Plant.* 80, 549 (1990).
28. V. Grbic, A. B. Bleecker, *Plant* J. 8, 595 (1995).
29. We thank Gisela Hoschek for proofreading this manuscript. This work was supported by an internal grant of POSTECH, by a grant from the Ministry of Education to the School of Environmental Engineering, POSTECH, and in part by a grant from PMBBRC. H. G. Nam dedicates this paper to T. J. Park, former president of POSCO and the late Dr. H. G. Kim, former president of POSTECH, who together founded POSTECH with a great vision toward progress of science and technology.