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Suppressed Leaf Senescence in Chrysanthemum Transformed with a Mutated Ethylene Receptor Gene *mDG-ERS1(etr1-4)*

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Previously, Narumi et al. (2005) generated chrysanthemum plants transformed with a mutated ethylene receptor gene (*mDG-ERS1(etr1-4)*), and showed that the *in vitro* plantlets of the transformants grown aseptically in a small plastic container had a reduced sensitivity to ethylene resulting in reduced leaf yellowing after exposure to exogenous ethylene. In the present study we evaluated ethylene sensitivity of the transformants using soil-grown mature plants. When the shoots detached from soil-grown plants were treated with exogenous ethylene under continuous light, leaf yellowing (senescence) was delayed in the transformants as compared with the non-transformed plants. Furthermore, when the detached shoots were kept in darkness without ethylene treatment, the transformants showed reduced senescence as compared with those of the non-transformed plants. These results demonstrated that the mutated ethylene receptor gene *mDG-ERS1(etr1-4)* could confer reduced sensitivity to ethylene in the leaves of mature chrysanthemum plants. This gene may be useful to generate transgenic *Compositae* vegetables with leaves green for a longer time and thus having a longer shelf life.

Keywords: *Chrysanthemum morifolium* (*Dendranthema grandiflorum*), ethylene insensitive, leaf deterioration, leaf senescence, leaf yellowing, transgenic chrysanthemum

Ethylene is a primary plant hormone involved in the senescence of leaves and flowers of many plants (Abeles et al., 1992). It is produced at the onset of leaf and flower senescence. The increased ethylene production accelerates yellowing of leaves and withering and dropping of flower petals. Inhibition of the synthesis or action of ethylene delays the onset of senescence symptoms and extends the longevity of leaves and flowers.

Cut chrysanthemum is one of the most popular cut flowers and is of economic importance in the floriculture industry. Leaves of cut chrysanthemum frequently become yellow spontaneously, sometimes prior to the onset of flower senescence (Doi et al., 2003, 2004, Ferrante et al., 2003). Moreover, exposure to ethylene during storage, transportation or display of the cut flowers accelerates leaf yellowing although the sensitivity to ethylene varies with the cultivar (Reyes-Arribas et al., 2001, Doi et al., 2003). Leaf yellowing is a characteristic of leaf senescence; it makes cut flowers unsightly, lowers their quality, and shortens vase life. Therefore, transgenic chrysanthemum with reduced ethylene sensitivity is expected to have very green leaves and a greatly extended vase life.

Previously, Narumi et al. (2005) generated chrysanthemum plants transformed with a mutated ethylene receptor gene, derived from a chrysanthemum ethylene receptor (*DG-ERS1*) cDNA, and showed that *in vitro* plantlets, cultured aseptically in a plastic container, had reduced sensitivity to ethylene resulting in delayed leaf yellowing after exposure to exogenous ethylene. However, it remains uncertain whether the suppressed sensitivity to ethylene is expressed in the leaves of mature chrysanthemum plants. Recently the transformants were analyzed for natural senescence using shoots obtained from soil-grown plants and the results were reported in preliminary reports (Satoh et al., 2006, 2007). In the present paper, we characterize in detail the senescence of the shoots of soil-grown mature chrysanthemum transformed with *mDG-ERS1(etr1-4)* under ethylene treatment and in darkness without ethylene treatment.

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MATERIALS AND METHODS

Plant Materials

The chrysanthemum [*Dendranthema grandiflorum* (Ramat.) Kitamura] cultivar 'Sei-Marine' (SM) and seven strains of chrysanthemum (Nos 10, 14, 17, 19, 33, 39 and 41) transformed with *mDG-ERS1(etr1-4)* described previously (Narumi et al., 2005) were used. These transformants were previously selected from *in vitro* plantlets by observing reduced sensitivity to exogenous ethylene as compared with SM. In that experiment, the plantlets of Nos 14, 17 and 19 exposed to 10 μL L^{-1} ethylene showed yellowing in one or two of the lowermost leaves and those of Nos 10, 33, 39 and 41 did not show leaf yellowing, although the SM severe yellowing in the leaves on the basal half of the stem (6 leaves out of 12 leaves).

The plantlets of SM and each transformant strain (T0 plants), cultured *in vitro* were transplanted onto soil (Metro-Mix 350, Scotts-Sierra Horticultural Products, Marysville, OH) in a plastic container. They were grown for 1 month in a growth cabinet under a 14-h light period (100 μmol m^{-2} s^{-1} of white fluorescent light) at 25°C and a 10-h dark period at 18°C (long-day condition). Then, the plants were treated with a low temperature at 4°C for 1 month under continuous white fluorescent light (15 μmol m^{-2} s^{-1}). After the low

temperature treatment, they were grown in a glasshouse under a 14-h day (sunlight supplemented with light from mercury and sodium halide lamps, > 150 μmol m^{-2} s^{-1}) at 25°C and 10-h night at 18°C. Thereafter, the plants were grown under a short-day condition (10-h day at 25°C and 14-h night at 18°C) for 1 month, and then transferred back to the long-day condition described above and grown for about 2 months until flowering. During the vegetative growth period, shoots of 20-cm long were detached from plants of about 40 cm in height, and used for the experiment in which senescence in darkness without ethylene treatment was examined (Figure 3). After flowering, the remaining chrysanthemum plants were grown as perennial plants in plastic containers in a glasshouse at 25°C under a 16-h light period (sunlight supplemented with white fluorescent light at >100 μmol m^{-2} s^{-1}) and an 8-h night period. The plants were fertilized as necessary. The transgenic chrysanthemum plants were cultivated in containment glass houses.

Chrysanthemum plants were exclusively cultivated by vegetative propagation. The transgenic lines of chrysanthemum were propagated vegetatively in our study, too. Therefore, the transgenic lines used in this study were T0 plants. The experiments shown in Figures 3 and 4 were carried out in 2005-2006 at Tohoku University, and those shown in Figures 1 and 2 in 2007-2008 at Kyoto Prefectural Institute of Agricultural Biotechnology.

Ethylene-induced Senescence of Shoots

The response of the shoots of chrysanthemum plants to exogenous ethylene was determined, shoots of 20-cm long with 8-12 leaves were detached from the maintained perennial plants. Four or five shoots from each line were placed in a 50-mL plastic tube with their cut end in 40 mL water, and enclosed in a 57-liter glass container containing about 10 μL L^{-1} ethylene, and a 100 mL solution of 1 M NaOH as an absorbent of CO_2 evolved from the chrysanthemum plants. The concentration of ethylene in the container was monitored every 2 or 3 days and maintained at 8-12 μL L^{-1}. The plants were left for 9 days under a continuous light (10 μmol m^{-2} s^{-1} white fluorescent light) at 25°C. The experiments were repeated 4 times, each with 4 or 5 detached shoots. At the end of incubation, the leaf yellowing (senescence) was observed with the naked eye and photographed. The magnitude of senescence was scored as 0, 1, 2, 3 and 4 when leaf yellowing covered 0, 1-10, 11-40, 41-70 and 71-100% from the base of the shoot, respectively. Leaf yellowing score was shown as the average of 4 or 5 shoots, and data were shown as the mean ± SE of 4 experiments.

Senescence of Shoots in Darkness

Shoots (20-cm long) detached from the vegetative plants about 40 cm in height were enclosed in a plastic bag with minimum air space to prevent drying of the shoots. They were left in the dark at 25°C for one week to cause senescence (deterioration) of the leaves. At the end of incubation, the deterioration (yellowing, browning and rotting) of leaves was observed and photographed. The leaf deterioration was scored as described above for the leaf yellowing caused by ethylene treatment. The experiments were repeated 5 times, each with 5 detached shoots. Leaf deterioration score was shown as the average of 5 shoots, and data were shown as the mean ± SE of 5 experiments.

Statistical Analysis

Statistical analyses were carried out by Dunnett's multiple test using an on-line statistical analysis program MEPHAS (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/).

RESULTS AND DISCUSSION

Exogenous Ethylene-induced Leaf Senescence (Yellowing)

When *in vitro* plantlets, grown aseptically in a plastic culture vessel, were transplanted to soil in a plastic container, all plants of SM and transgenic lines grew vigorously and flowered almost normally.

Four or 5 shoots, 20-cm high with 8-12 leaves, were cut from soil-grown plants maintained as perennial plants in plastic containers, and treated with ethylene at 10 μL L^{-1} for 9 days. The shoots of some transgenic lines showed reduced leaf yellowing as compared with those of SM. A typical profile of leaf yellowing is shown in Figure 1, in which a shoot of SM showed severe yellowing (almost all the leaves showed yellowing), but a shoot of No. 33 line did not. Figure 2 shows the degree of leaf yellowing caused by exposure to exogenous ethylene in SM and seven transgenic lines. The leaf yellowing was significantly suppressed in shoots of transgenic lines, Nos 10, 17, 19 and 33, as compared with SM, whereas the reduction in leaf yellowing was

Figure 1. Leaf yellowing profiles of the non-transformed 'Sei-Marine' control and a transgenic line No. 33 after treatment with ethylene. Shoots of 20-cm long were detached from soil-grown plants, enclosed in a glass container with 10 μL L^{-1} ethylene, and left for 9 days at 25°C under continuous white fluorescent light.

not significant in shoots of the transgenic lines Nos 14, 39 and 41. These results suggested that the sensitivity to ethylene, shown by leaf yellowing, was reduced in the transgenic lines Nos 10, 17, 19 and 33.

Leaf Deterioration in Darkness without Ethylene Treatment

We examined the leaf deterioration (yellowing, browning and rotting) of cut shoots kept in darkness without ethylene treatment in SM and the transgenic lines. These experiments were preliminary shown in our previous paper (Satoh et al. 2006, 2007). Briefly, shoots (20-cm long with 8-12 leaves) cut from the soil-grown plants (about 40 cm in height) were enclosed in a plastic bag with minimum air space and left in the dark for one week at 25°C. This treatment caused leaf deterioration characterized by yellowing, browning and rotting in the shoots of SM. In some transgenic lines, however, leaf deterioration was alleviated showing less yellowing, deterioration and rotting of the leaves than in SM. In one experiment , the shoot of SM showed severe leaf deterioration, yellowing, browning and rotting, but the shoots of transgenic lines Nos 17 and 33 showed little or no leaf deterioration (Figure 2 in Satoh et al., 2006). In another experiment, the shoot of SM showed rotting in the lower leaves, but the shoots of transgenic line No. 17 remained sound (Figure 4 in Satoh et al., 2007). Reduced rotting probably resulted from a significant reduction in susceptibility to bacteria or fungi, because some pathogens often infect senescent tissues.

Figure 3 summarizes leaf deterioration after one week by enclosing shoots in a plastic bag. The leaf deterioration was significantly suppressed in shoots of transgenic lines, Nos 10, 17, 33 and 39, as compared with SM. However, leaf deterioration was not reduced significantly in shoots of the transgenic lines Nos 14, 19 and 41.

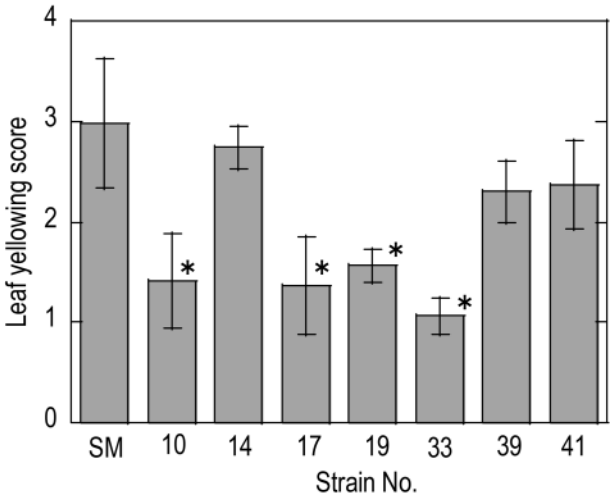

Figure 2. Leaf yellowing after treatment with ethylene in the 'Sei-Marine (SM)' and transgenic lines. Shoots of 20-cm long were detached from soil-grown plants of the SM and transgenic lines, enclosed in a glass container with 10 μL L^{-1} ethylene, and left for 9 days at 25°C under continuous white fluorescent light. Leaf yellowing score was determined as 0, 1 , 2, 3 and 4 when leaf yellowing covered 0, 1-10, 11-40, 41-70 and 71-100% from the bottom of the shoot, respectively. Leaf yellowing score was shown as the average of 4 or 5 shoots. Data were shown by the mean ± SE of 4 experiments. *shows significant difference from the value in SM at $p=0.05$ by Dunnett's multiple test.

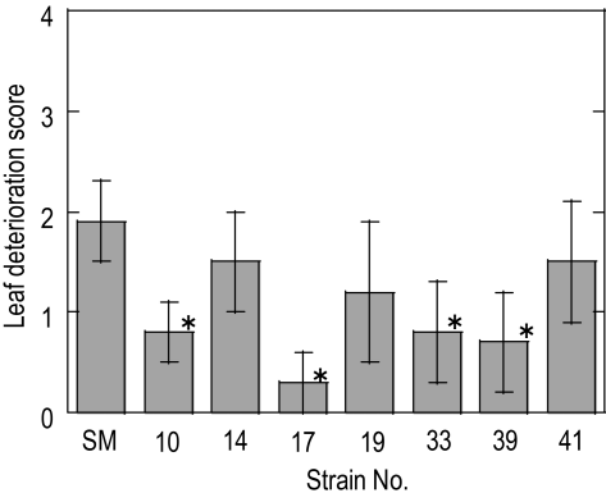

Figure 3. Leaf deterioration after senescence in darkness in 'Sei-Marine (SM)' and transgenic lines. Shoots of 20-cm long were detached from soil-grown plants of SM and transgenic lines, enclosed in a plastic bag with minimum air space and left for one week at 25°C in the dark. Leaf deterioration (yellowing, browning and rotting) score was determined as 0, 1 , 2, 3 and 4 when leaf yellowing covered 0, 1-10, 11-40, 41-70 and 71-100% from the bottom of the shoot, respectively. Leaf deterioration score was shown as the average of 5 shoots. Data were shown by the mean ± SE of 5 experiments.
*shows significant difference from the value in SM at $p=0.05$ by Dunnett's multiple test.

Leaf Abscission

When plants of transgenic lines were cultivated until flowering in a crowded condition in a glasshouse, the appearance of plants was different from that of SM plants as shown in Figure 4. In SM, leaves on the basal part of the stem had abscised during growth. In the transgenic line No. 17, leaves on the lower part of stem remained attached to the stem showing no sign of yellowing (senescence). This phenome-

Figure 4. Different plant appearance between 'Sei-Marine' and a transgenic line No. 17. Plants were cultivated until flowering in a greenhouse.

non was probably caused by the reduced capability of senescence in the leaves of the transgenic line No. 17. The same phenomenon was found in other transgenic lines (data not shown).

The present study revealed that the transgenic lines Nos 10, 17 and 33 showed reduced leaf yellowing in response to ethylene treatment and reduced leaf deterioration in darkness without ethylene treatment. On the other hand, line No. 19 showed reduced sensitivity to ethylene-induced senescence but not to dark senescence, while the reverse was found with line No. 39. Lines Nos 14 and 41 did not show any reduction in either ethylene-induced and or dark-induced senescence.

The ethylene sensitivity of the shoots of mature soil-grown plants was somewhat different from the previous experiment with *in vitro* plantlets, which showed reduced sensitivity to exogenous ethylene in all the transgenic lines (Narumi et al., 2005). The discrepancy might be caused by the difference between mature soil-grown plants and *in vitro* plantlets, but we did not further examine the cause of the difference.

Narumi et al. (unpublished results) detected the transcript of the mutated ethylene receptor gene *mDG-ERS1(etr1-4)* in leaves of all the transgenic lines, but did not find any relationship between the amount of *mDG-ERS1(etr1-4)* transcript and the magnitude of reduction in sensitivity to ethylene. The detection of the expression of the integrated *mDG-ERS1(etr1-4)* gene suggested that the mutated ethylene receptor gene conferred reduced ethylene sensitivity through a mechanism similar to that responsible for ethylene insensitivity of the *Arabidopsis etr1-1* mutant (Chang et al., 1993).

The present results clearly showed that the integration of the transgene *mDG-ERS1(etr1-4)* suppressed the senescence of leaves in the transgenic chrysanthemum plants, irrespective of ethylene-induced or dark-induced senescence. Chrysanthemum plants belong to the family *Compositae*. *Compositae* plants include leafy vegetables such as lettuce, garland chrysanthemum, chicory and butterbur. These vegetables are vulnerable to senescence, resulting in yellowing, browning and rotting of leaves. Usually, to prevent senescence, harvested vegetables are handled and stored under cool temperature or controlled atmosphere conditions. However, the *Compositae* vegetables cannot be stored for a long time in a cool temperature because of cold injury, which is possibly accelerated by the action of ethylene. Thus, the senescence of these vegetables could be preventable by the generation of transgenic plants with reduced leaf senescence. The present study suggests that *mDG-ERS1(etr1-4)* could be used to generate transgenic *Compositae* leafy vegetables with retarded leaf senescence resulting in healthy green leaves and a greatly extended shelf life.

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