2011 International Conference on Food Engineering and Biotechnology
IPCBEE vol.9 (2011) © (2011)IACSIT Press, Singapore

Role of ABA in ethylene-independent Iris flower senescence

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Abstract. This project aims at investigating the role of ABA in ethylene-independent flower senescence. Our hypothesis is that ABA (abscisic acid) as a developmental factor activates PP2C (protein phosphatase 2C), an ABA signalling regulator. Activated PP2C probably dephosphorylates an interacting component essential for ABA signalling and turns on the pathway for "death protein" synthesis, which activates hydrolytic enzymes like proteases, leading to PCD and floral death. By using flag petal rims of cut *Iris* flowers, we have gathered physiological and molecular evidences suggesting a direct role of ABA in up-regulating the early steps of ethylene-independant petal senescence, and "Blue Magic" *Iris* could serve as a good model plant for studying age-dependent, ethylene-independant senescence of flowers, some of which are food crops.

Key words: *Iris*, ethylene insensitivity, flower senescence, PCD, ABA, PP2C.

1. Introduction

Flowers of "Blue Magic" *Iris* (*Iris* x *hollandica* Tub, cv. Blue Magic) senesce rapidly just a few days after opening in an age-dependent manner without an environmental cue. The senescence of *Iris* flowers is genetically programmed and controlled by endogenous factors that are propagated in contiguous cell populations. Unlike the group of ethylene-dependant flowers whose floral senescence is regulated at least in part by ethylene, *Iris* belongs to another group whose flowers are insensitive to ethylene [1]. The endogenous factors regulating the ethylene-independent plants are yet to be characterized.

The floral death of this ethylene-independent group is possibly regulated by another senescence-promoting hormone, abscisic acid (ABA). Some studies suggest that ABA may play an important role in the regulation of flower senescence. In ethylene-dependant flowers like carnation, exogenous ABA triggered endogenous ABA production and flower senescence; however, the effects of ABA might be mediated through an increase in ethylene production resulted from ABA application or through an activation of ethylene action [2]. Thidiazuron treatment doubled the ABA content but did not affect flower life, confirming the secondary role of ABA during flower senescence of petunia, another ethylene-dependant flower [3]. In contrast to its suspected secondary role in ethylene-dependant flower senescence, ABA might have a direct effect on the senescence of ethylene-independent flowers. In cocoa [4] and daylily [5], exogenous ABA, not ethylene, accelerated flower senescence. Endogenous ABA increased dramatically before any visible signs of senescence, and continued to increase during petal senescence in both these taxa. Treatment of fluridone, an inhibitor of ABA biosynthesis, decreased ABA levels and extended the longevity of cocoa flowers. However, in daffodil, a flower that could respond to exogenous ethylene but whose natural senescence is ethylene independent, exogenous ABA accelerated flower senescence but such an effect is considered to be mediated through stimulated ethylene production as in ethylene-dependant flowers; ABA was not the primary regulator of daffodil flower senescence since the increase in senescence-associated genes commenced before the rise in ABA content [6]. To our knowledge, so far there has been no conclusive

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report about the direct role of ABA in the regulation of ethylene-independent flower senescence, and the elements of the ABA early signal transduction pathway are yet unknown.

The objective of our study is to investigate the role of ABA in ethylene-independent flower senescence, and the early element of the ABA early signal transduction pathway. We used *Iris* flowers as a model system for testing our hypothesis as summarized in the above abstract. In order to test the involvement of ABA and PP2C in the early steps of *Iris* flower senescence prior to any visible symptoms, we first examined the effects of ABA, ABA antagonizer and inhibitor, and protein phosphatase inhibitors on senescence and on protease activities. Efforts have been taking to isolate senescence-associated PP2C gene(s) and study its role in petal senescence.

2. Materials and Methods

2.1. Plant material and treatments

Cut *Iris* (*Iris* x *hollandica* Tub, cv. Blue Magic) flowers were harvested at bud stage when the petals just emerged above the green sheath leaves (day 0). Homogeneous flowers with similar size at similar bud stage were recut to a length of 10 cm, keeping only flower buds and stems. They were individually placed in tubes with water or various treatment solutions on day 0 (0 h) and kept in a climate-controlled room at 20^{o}C, 60% relative humidity and a photosynthetic photon flux of 15 $\mu mol.m^{-2}.s^{-1}$ (12 $h.d^{-1}$). Symptoms and stages of senescence were observed twice daily. The rims of flag petals from 20 flowers per sampling time point per treatment were cut and immediately kept in liquid nitrogen, and stored at -80^{o}C (for sampling time points see Tab. 1) for determining protease activity, the isolation of senescence-associated PP2C gene and expression analysis. All the experiments were performed with at least two replicates.

BA (Sigma) at 0.1 mM, ABA (Sigma) at 0.5 mM, EDTA at 5 mM and NaF at 2 mM (in water) were applied on day 0 (0 h), day 1 (am-16 h and pm-24 h), day 2 (am-46 h and pm-54 h) and day 3 (am-70 h and pm-78 h). Prior to this experiment, 0.1-10 mM ABA was tested. At 0.5 mM and above ABA had obvious effect on petal senescence. At the higher concentrations of ABA the petals senesced more rapidly. At 5 and 10 mM petal edges appeared purple droplets during the late stages of senescence. EDTA at 1-10 mM and NaF at 0.1-10 mM were also previously tested. EDTA at 1 mM prevented petal inrolling but caused more purple discoloration between veins which is different from natural way of purple fading; at 10 mM it caused stem bending. Effective concentrations of NaF are 0.5 mM and above; however, at 5 mM and above petal opening was inhibited and centre whitening appeared probably due to the poisoning effect of NaF. Although EDTA and NaF are both not specific PP2C inhibitors, they were used in this study because no specific PP2C inhibitor has been reported or are available. To test if the effect of EDTA is due to the chelation of some other cation(s) instead of Mg^{2+}, $Ca(NO_3)_2$, $ZnCl_2$, $Mn(NO_3)_2$ and $CuSO_4$ at 0.5-2 mM applied on day 0 were individually tested. In addition, ABA-biosynthesis inhibitor norflorazon (Novartis) at 0.05, 0.1, 0.5, 1, 5, and10 mM applied on day 0 was examined.

Cut *Iris* flowers were stored dry with relevant controls (stored in water) at 20^{o}C in the climate-controlled room for 24 h. Stages of senescence were analyzed as stated above.

2.2. Protease activity assays

The petal edges of the above-stated treatments were harvested at intervals stated in Table 1, ground in liquid nitrogen, and freeze-dried. Resorufin-labelled casein was used as substrates for *in vitro* estimation of total protease activity as described previously [7].

3. Results

3.1. Natural petal senescence

Iris flag petals which were harvested at bud stage (day 0) continued to enlarge, and opened on day 1. Flag petals showed the first visible symptoms of senescence approaching late day 3 / early day 4. The distal edges of the flag petals first appeared water-logged (an indication of the loss of membrane semi permeability), then discolored (stage 1a) and started to inroll (stage 1b). Inrolling gradually proceeded until

the whole flag region was inrolled and discoloured on the following two days (stage 2-6). It took 5-6 days from flower opening to complete wilting. During the whole process, no sign of water stress was observed.

During natural senescence, PCD in *Iris* petals progressed from cells at the edge towards those at the centre, and was observed first in mesophyll cells followed by sub-epidermal and epidermal cells. On early day 3, about one day prior to inrolling, virtually all mesophyll cells of petal edge, which are located between the upper and lower epidermis, died and physically collapsed whereas epidermal cells died on day 4 and afterwards. Edge inrolling started when some epidermal cells subsequently lost turgor. Therefore, different regions of a petal undergo synchronized differential senescence.

3.2. Exogenous ABA induced and advanced the senescence

ABA induced and advanced senescence by approximately 15 hours if applied on day 0 (Table 1), and by approximately 10 hours if applied on day 1 and day 2. ABA applied on day 3 had no apparent effect on promoting senescence. Higher level of ABA induced more rapid senescence. All symptoms of senescence in ABA-treated flowers appeared the same as in naturally senescing flowers, except that ABA inhibited the expansion of flag petals during the opening stage.

Table 1. Stage of senescence of Iris flag petals corresponding to time from start of experiment on day 0.

Time		Sampling time points						
Hours	0	20	40	60	66	80	86	100
Day	0	1	2	3am	3pm	4am	4pm	5am
Treatments		*Stage of senescence*						
H_2O	0	0	0	0	0	0-1a	1a-1b	2(-3)*
NaOH 0.25 mM	0	0	0	0	0	0-1a	1a-1b	2(-3)
BA 0.1mM	0	0	0	0	0	0	0(-1a)	1a
ABA 0.5 mM	0	0	0	0	1a	(1b-)2	3	(4-)5
EDTA	0	0	0	0	0	0	0	0
NaF 2mM	0	0	0	0	0	0	0	0
Dry storage	0	0	0	0	0	1a	1b	3

The stages in parentheses are in minority.

3.3. Exogenous ABA upregulated activities of various hydrolytic enzymes

ABA-treated flag petals showed earlier and stronger increase in total protease activity (Fig. 1) than the natural ones, but the pattern of changes are the same. In addition to its role in prompting protein degradation & protease activity, ABA also upregulated the following parameters associated with natural senescence: 1) leakage of both ions and anthocyanins, 2) phospholipid degradation, and 3) RNA degradation & RNase activity (data not shown).

3.4. Dry storage accelerated the senescence

Dry storage advanced senescence by approximately half a day (Table 1), and prematurely accelerated protease activity (Fig. 2).

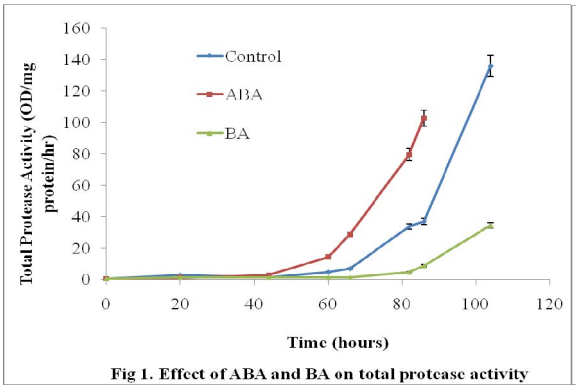

Fig 1. Effect of ABA and BA on total protease activity

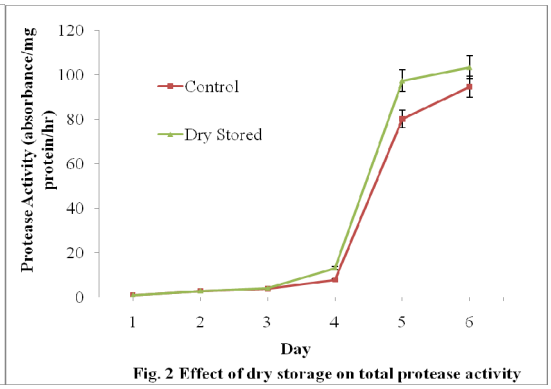

Fig. 2 Effect of dry storage on total protease activity

3.5. ABA antagonizer significantly delayed the senescence & downregulated protease activities

As an ABA antagonizer, BA delayed natural senescence to stage 1 by approximately one day and to later stages by about 1. 5 days, if applied on day 0. BA applied on day 1 delayed the onset of senescence by a few hours and to later stages by about 1 day. BA applied on day 2 did not apparently delay senescence to stage 1, but to later stages by about 0.5 day. BA applied on day 3 had no effect on senescence. BA also significantly inhibited the activities of proteases (Fig. 1). However, ABA biosynthesis inhibitor norflurazon at the tested concentrations did not delay senescence, but rather promoted senescence at 0.5 mM and above.

3.6. PP2C appeared to play a role in the senescence

Among the protein phosphatase inhibitors tested (NaF, EDTA, okadaic acid - an inhibitor of protein phosphatases-1 and **-2A** and Na_3VO_4 - a tyrosine phosphatase inhibitor) only EDTA and NaF had an effect on petal senescence. EDTA at 5 mM and above completely prevented normal petal senescence, if applied on day 0 and day 1. Petals remained at stage 0 for 6 days without any discoloration and inrolling, and wilted on day 7 when stems became yellow and bended. EDTA applied on day 2 did not apparently delay the onset of natural senescence, but significantly delayed the course of natural senescence, more and more toward the later stages. The senescence to stage 2 was delayed by approximately 1 day. The petals stayed at stage 2 without further discoloration and inrolling, until wilted on day 7. EDTA applied on day 3 virtually had no effect on senescence.

The cations Ca^{2+}, Cu^{2+} and Mn^{2+} at the tested concentrations did not have visible effect in *Iris* petal senescence, whereas Zn^{2+} at 1 mM delayed the onset and course of senescence by approximately half a day. Therefore the effect of EDTA is not due to the chelation of some other cation(s) instead of Mg^{2+} (PP2C activity is Mg^{2+} dependant).

Similar to EDTA, NaF at 0.5 mM and above completely prevented normal petal senescence if applied on day 0 and day 1, but no longer had such an effect if applied on and after day 2. NaF applied on day 2 did not delay early stages of normal senescence (petal edge discoloration and inrolling), but delayed senescence to stage 4 by approximately 1 day (applied on day 2 am) or half a day (applied on day 2 pm). NaF applied on and before day 2 prevented petal discoloration. NaF applied on day 3 did not have any visible effect.

A full length cDNA encoding an *Iris* PP2C was isolated and characterized. The differential expression of the PP2C showed an early increase prior to any visible sign of senescence (data not shown).

3.7. ABA upregulated the expression of senescence-associated genes

ABA strongly induced and promoted PP2C expression. BA did not appear to have a significant effect on the expression but only weakened the expression slightly. Protein phosphatase inhibitor completely inhibited the expression. ABA also upregulated the expression of RNase and UBQ-E2 genes (data not shown).

4. Discussion

4.1. ABA in relation to natural senescence processes in Iris petals

Natural PCD pathway and senescence (edge inrolling and discoloration) in *Iris* petals can be blocked by protein synthesis inhibitors cycloheximide [8] and narciclasine [9], hence depends on the synthesis of "death protein(s)". The "death protein(s)" was probably synthesized on or before day 2, since both cycloheximide

and narciclasine, as well as the effects of EDTA and NaF in our study, were effective in preventing Iris petal inrolling and discoloration only when their continuous application was started before day 2. If applied on and after day 2, these chemicals can only slightly delay natural senescence in a way similar to BA and $ZnCl_2$. It appears that PCD in *Iris*, as in apoptotic cells, has two phases. The "condemned phase", which can be stopped by biochemical means, is before the synthesis of "death protein(s)" on or before day 2. The subsequent "execution phase" is after the "death protein" is synthesized and virtually can no longer be blocked. Unlike apoptosis in animal cells, PCD in *Iris* is probably non-apoptotic since no DNA laddering, cell shrinkage and cell fragmentation were observed. *Iris* PCD seems to have a very different pathway from apoptosis, as it is not affected by the apoptosis suppressors including inhibitors of granzymes and caspases (data not shown).

Accompanying the morphological changes, a series of physiological and biochemical processes were also occurring prior to and during *Iris* petal senescence. On day 2 (two days prior to visible senescence), autophaging was observed in many cells, and in vivo phospholipase D activity is stimulated while phospholipid hydrolysis and membrane disassembly started to increase (unpublished results). As can be seen in Fig. 1 and Fig. 2, total protease activity started to increase very slightly on day 2 and day 3, and then dramatically on day 4 and onwards; such activities were up-regulated by ABA and down regulated by BA.

The results of our study collectively suggest that ABA might be the factor initiating the synthesis of the "death protein(s)" and the processes leading to PCD and senescence of *Iris* petals.

4.2. ABA plays a direct role in regulating Iris petal senescence

Exogenous ABA, not ethylene, accelerated morphological, biochemical (e.g. up-regulation of hydrolytic enzyme activities), and molecular changes (e.g. expression of ABA-induced genes) that occurred during natural senescence of *Iris* petals. The ABA responses in our experimental system are unlikely through an ethylene pathway, since the rate of ethylene production decreased during petal senescence [8] and ethylene appears to play no important role in the senescence of *Iris* [10]. Neither are the responses likely due to stress, as the senescence symptoms of the detached petals in our study occurred in the same manner as the petals left on intact flowers [8].

ABA antigonizer BA delayed visible senescence and inhibited protease activity. However, ABA biosynthesis inhibitor norflurazon which blocks synthesis of ABA precursor carotenoids [11] did not inhibit the senescence of *Iris* petals. Neither did norflurazon inhibit daylily senescence [5]. It is probably because during norflurazon treatment high amount of carotenoids might be already present which provide ample substrate for ABA synthesis, or ample ABA might have already been synthesized during bud development.

Another means of accumulating evidence for a role of ABA is to follow the endogenous levels of ABA during natural and ABA-induced senescence. However, hormonal effects need not be dependent on endogenous levels as cell receptivity is also an important determinant. The results of our dry storage experiment lends support for a role of ABA in promoting Iris petal senescence, since water deficiency leads to increased endogenous ABA level [12].

Several ABA-induced genes have been detected during *Iris* petal senescence; one of them is PP2C, a component of ABA signal transduction pathway. The presence of PP2C and the increase in its mRNA levels before the executing phase during normal and ABA-induced senescence provides further support for a regulatory role of ABA in senescence of *Iris* petals.

In conclusion, our results suggest that ABA plays an important, direct role in up-regulating ethylene-independent flower senescence, and that PP2C is likely a positive effecter of the ABA signalling. Our study further suggests that "Blue Magic" *Iris* could serve as a good model plant for studying age-dependent, ethylene-independent flower senescence due to the flowers' fast (yet not too fast like in daylily), synchronous PCD processes. In order to further elucidate the roles of ABA in the senescence, it will be important to study the functions and regulations of ABA-induced transcription. The development of mutants and specific inhibitors of ABA will provide powerful means for future efforts in understanding the regulation of the senescence of ethylene-independent flowers.

5. Acknowledgements

We thank Drs. W. G. van Doorn, C. van der Schoot and T. De Vrije for their contribution to this work.

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