Auxin induced programmed cell death in mustard seedlings

K. TULASI RAMA KRISHNAN AND V. SIVAKUMARI

Asian Journal of Environmental Science (June to November, 2009) Vol. 4 No. 1 : 95-99

See end of the article for authors' affiliations

Correspondence to :
V. SIVAKUMARI
Department of of Environmental and Herbal Sciences, Tamil University, THANJAUR (T.N.) India

SUMMARY

Programmed cell death is a process of developmental aspect in plants. The objective of the present study was to understand the hormonal basis of senescence, in particular 2, 4-D induced senescence along with the identification and characterization of the biomolecules and elucidation of their role in this terminal in the life process of all plants. This study explained the auxin induced senescence (AIS) pathway on the basis the different hormones, cellular components, signals, death signals and markers of senescence.

Key words : Phytohormones, Auxins and IAA oxidase

Accepted : ***May, 2009***

Phytohormones are known to regulate senescence in higher plants. Senescence among various plant groups, such as annuals, biennials, monocarpic, polycarpic and perennials are known to represent distinct patterns. In annuals and monocarpic plants, the plant undergoes necrosis (leaf yellowing/vein clearing) followed by senescence, resulting in the total death of plants. However, in the other group of plants comprising biennials, perennials and polycarpic plants, the selective elimination of plant organs, tissues and cells take place periodically, so that, rejuvenation takes place in these plants. The developmental processes of plant are collectively termed as programmed cell death (PCD).

Plant growth and development is regulated by hormones, of which auxins are one of the classical five types, together with ethylene, gibberellins, (+) – abscisic acid (ABA) and cytokinins (Kende and Zeevaart, 1997). Senescence is the final phase of plant vegetative and reproductive development, preceding the widespread death of cells and organs recapture of cellular material for use in other organs (Nooden, 1988; Bleecker and Patterson, 1997).

In the present study, 2, 4 – D mediated responses in the seedling of black-mustard has been studied. 2, 4 –D and IAA activities were studied at relatively high concentration. Experiments were carried out with young seedling of black mustard because of the following reasons:

– Members of Brassicaceae, to which black mustar belongs, are sensitive to differential concentration of IAA.

– IAA at lower concentration in this system acts as a cell elongation factors.

– IAA at relatively high concentration is growth inhibitory in this system.

This differential response to IAA is the basis of the present study. Hence, mustard seedling was chosen as the experimental system.

MATERIALS AND METHODS

Seeds of black mustard (*Brassica nigra* L.) were allowed o germinate at room temperature 35 ± 2°C on a thin layer of cotton. ABA, 2, 4 – D + AgNO3 was added on the 3rd, 5th and 7th days similarly on 0´ d, 1´ d, 3´ d and 4´ d. The root and shoot of the plants were measured in cm and the samples were collected from the above days.

Determination of root-shoot length:

One week old mustard seedling were uprooted and the roots and shoots were stretched with the help of a scale and root and shoot lengths were measured for every treatment in 3rd, 5th and 7th days similarly on 0´ d, 1´ d, 3´ d and 4´ d. Chlorophyll (Arnon, 1949), carotenoids (Mackinneys, 1964), lipid (Chapman, 1998), thin layer chromatography (Manoharan, 1981), phospholipids (Wagner *et al*., 1962), genomic DNA from plant tissue by the method of CTAB, agarose Gel Electrophoresis and catalase activity were analysed.

RESULTS AND DISCUSSION

Effect of ABA on the growth of mustard seedling:

The objective of the present study is to understand the hormonal interplay on the regulation of senescence. Auxin induced senescence via ethylene of ABA is the focus of the study. Accordingly ABA effect on mustard seedling was undertaken. ABA at 10 µm concentration was worked out. Among the parameters determined in the young seedling shoot weight, specific shoot growth and the content of chl a, chl b and total chlorophyll was significantly reduced in the ABA treated seedlings.

Effect of endogenous native Auxin, IAA on the growth of seedling:

This experiment was undertaken to study the effect of IAA on various basic cellular parameters, morphometric parameters, biochemical parameters of young seedlings. Results showed that IAA even at the supraoptimal concentration of 500 µm was stimulatory on seedling elongation. The "elongation factor" role of IAA could be seen on shoot growth, root growth as well as on the seedling height. There was also an increase in shoot weight of these seedlings. There was remarkable increase in the specific growth of shoot in contrast to that of the roots.

Acetone positive control:

Since 2, 4 – D and IAA solutions for various experiments solution were prepared in 1% acetone, its effect was studied in the young mustard seedlings during the experimental period. Among the parameters determined in the young seedlings, there was no significant difference in the growth of the seedlings.

Effect of supplementation of 2, 4 –D on he phospholipids composition of mustard seedling:

Membrane lipids are known to be the target for degradative enzymes during senescence. In order to characterize changes associated with the auxin – induced senescence in the membrane lipid composition, the following analysis was carried out. Control on the 0' and 2' day where included in the experiment. These 2 controls revealed any possible change due to aging under experimental condition (experimental aging) 2, 4-D supplemented seedlings were compared with water grown seedling (control).

The results indicated that the total content of PL showed a marginal increase. Among the PL species, PC showed about 1.25 fold increased due to 2, 4-D, also there was increase in PA and PS. Among the major membrane lipids, PC showed significant decrease. Among the PL qualitatively 6 molecular species were identified by the TLC system employed in the present study. Accordingly the following are the PL species in the control and 2, 4-D treated seedlings: PC, PI, PS, PE and PA (Table 1).

Effect of 2, 4 –D an seedlings the galactolipids composition of mustard:

A set of experiments were undertaken to find out the change in chloroplast specific galactolipid due to 2, 4 –D induced senescence. It was the aim of the experiment to mark the appearance of death signals and markers of chloroplast degradation during senescence. The results showed that 2'd 2, 4 –D treated seedlings did not show any significant change in the total amount of galactolipids. There was also no significant change in the molecular species of galactolipids, namely: MGDG, DGDG and SL. There was a marginal decrease in MGDG content due to 2, 4 – D in 1'd plant.

Effect of 2, 4-D on the MGDG: DGDG ratio of mustard seedlings:

An analysis was carried out to determine the MGDG: DGDG ratio in lipid samples prepared from the shoot tissue. MGDG and DGDG are the specific chloroplast lipids localized in the internal (grana and stroma membrane) and external (chloroplast envelope membrane), respectively. MGDG: DGDG ratio would provide insight into the intactness / degradation of the chloroplast membrane. Accordingly, 0'd and 2'd control and 2, 4-D supplemented samples were analysed for these parameters.

The results showed that there was no significant change in the MGDG: DGDG ratio of the 2'd 2, 4-D treated plants as compared to the control. This would reflect the intactness of the chloroplasts *insitu* and no degradation of the chloroplast membrane biomolecules due to 2, 4-D (Table 1).

Catalase activity and its relationship to senescence:

The catalase activity was determined in the shoot tissue of mustard seedlings which were subjected to auxin induced senescence. Catalase, an enzyme involved the

Table 1 : Effect of 2, 4-D on the MGDG: MGDG ratio of 2'd mustard seedlings

Sr. No.	Sample	MGDG / DGDG
1.	0'day control	2.22
2.	2'day control	2.26
3.	2' day 2, 4-D	2.03

ROS scavenging was positively correlated with senescence. Experiments were undertaken to study catalase activity to enzyme content and H_2O_2 concentration in 4'd seedlings.

Catalase activity in relation to senescence of seedlings:

One of the ROS scavenging enzymes, namely catalase was assayed in the ultimate stage in the present experimental conditions of auxin induced senescence (AIS) in mustard seedlings. 2, 4 – D supplemented to 4'd old seedlings for the subsequent 4'd with 500 µm 2, 4-D was the test system of the present study. Catalase activity was calculated on the basis of depletion of H_2O_2 in the assay mixture and its unit of expression was on the basis of specific activity of catalase. The results showed that there was about 3-fold stimulation in the catalase activity in the 2, 4-D treated seedlings.

Effect of 2, 4-D and 2, 4-D+ AgNO3 on the induction and reversal of nucleases activity and associated pattern of nDNA degradation during senescence:

A set of experiments were undertaken to characterize the nucleolytic changes in relation to 2, 4-D supplementation. Nucleases activity was visualized in the form of nDNA degradation by observing the DNA fragments in 0.75% agarose gels. The pattern of nDNA fragments in different days of 2, 4-D induced senescence was compared with their respective controls. AgNO3 was employed in order to reverse the 2, 4-D effect either completely or partially. 0'd, 2'd and 4'd control and 4'd 500 µm 2, 4-D treated seedlings were compared for the pattern for the pattern of nDNA fragmentation.

The nDNA in 4'd 2, 4-D treated seedlings showed the onset of nucleases activities and appearance of nDNA fragments in a wide range of sizes, as compared to 1'd control. When 0'd and 4'd controls were compared there was no aging related nDNA fragmentation under the experimental conditions of the present study, except in one of the samples. The RNA present in the samples got to the front of the gel. The 1'd control however did not have significant RNA contamination.

The nuclear DNA in the samples has a moleculars size of 23 kb as compared to the Hind III digested ëDNA. This 23kb nDNA appeared as a discrete band in all the samples, which represented the intact and under graded genomic DNA of mustard. The 4'd 2, 4-D treated plants also showed distinct pattern of nDNA degradation which fell in the range of 23 downwards to about 9kb as compared to the marker DNA.

2, 4-D induced senescence was reversed by $AgNO_3$ in 2'd and 4'd plants. 2, 4-D at 500 µm and $AgNO_3$ at 0.1mm were employed in the experiments. The results showed

– $AgNO_3$ reversal of senescence was not clearly seen in the pattern of nDNA fragmentation pattern in both samples.

– In one of the samples the 2'd control sample was a clear preparation without any significant amount of RNA contamination.

– Even in 4'd 2, 4-D + $AgNO_3$ treated seedling there was no reversed senescence as visualized by the nDNA fragmentation.

There was possibly a problem due to weak activity of silver ions in the inhibition of 2, 4-D induced senescence due to photo bleaching of silver nitrate under the study experimental condition. A suitable system to control $AgNO_3$ photo bleaching needs to be worked out.

Senescence is a type of programmed cell death (PCD) that occurs at the life cycle of a plant is known as terminal senescence. Terminal senescence is a PCD and all other senescence that occur prematurely constitute induced PCD. Examples of untimed senescence include necrosis, tissue lesion and damage due to environmental disturbances and pathogen attack. Control and regulation of premature senescence is relevant in crop protection and also crop productivity.

Pathway of auxin induced senescence and control points:

Auxin induced senescence takes place in response to native auxins such as IAA, IBA and NAA and also due to synthetic auxins such as 2, 4- dichlorophenoxy acid (2, 4-D) and 2, 4, 5- trichlorophenoxy acetic acid (2 4, 5-T). *In situ* plants are capable of degrading native auxins when they accumulate beyond a threshold concentration by IAA oxidase. Also there is a mechanism of conversion of endogenous native auxins into their conjugated forms, such as, IAA- amino acids, IAA – sugars and IAA –myo inositol. These conjugated forms are not the effective form of phytohormones within the cell.

Auxin induced senescence is a mostly due to synthetic auxins in contrast to native ones. In the present study, observations, showed that IAA supplemented externally even at 500 µm did not induce senescence. However, 2, 4 –D at equimolar concentration operated the senescence pathway in mustard seedlings. Relative insensitivity of the plants to native auxins in the operation of senescence pathway is due to change in the concentration of metabolic pool of the auxin in the cells. Metabolic pool of the auxin refers to the physiologically active form of the phytohormone, acetone positive control and no inhibitory

growth of acetone on mustard seedlings.

It is an experimental requirement to have positive control for chemicals which are employed as solvents, other than water to evaluate its interference with the experimental system. The results of acetone positive control showed no significant inhibition of the seedling growth. Thus the acetone is proved to be of no consequence in the results involvement of Ethylene and ABA in the 2, 4-D induced senescence.

Auxins in general and 2, 4-D in particular are known a hormonal interplay or hormonal interaction in the expression of senescence pathway. This is also termed as "hormone cross talk". Involvement of ethylene and ABA (abscisic acid) in the progression and termination of senescence in plants is known well. There are several mutants available in the model plant *Arabidopsis thaliana* in which mutants are insensitive to ethylene due to defect in the ethylene perception or in the subsequent signal transduction. *Arabidopsis thaliana* is a weed in the European countries which is a close relative of black mustard. It is relevant to mention that the whole genome sequence of *Arabidopsis thaliana* has been completed in the year 2000.

In the present study usage of 2, 4-D and several inhibitors of ethylene biosynthesis pathway, namely $AgNO_3$, $COCl_2$ and Na tungstate, revealed the involvement of ethylene as a intermediate signal in the senescence pathway. It is known that control on the ethylene biosynthesis is on the availability of S-Adenosyl Methionine (SAM) and the synthesis of 1-aminocyclopropane carboxylic acid (ACC). SAM is a methyl donor and ACC synthase is involved in the penultimate stage on the biogenesis of ethylene. Thus there is a defined locus in the control and regulation of ethylene mediated senescence pathway in plants. The results of the present study illustrated the role of ethylene in senescence on the basis of reversal of Auxin induced senescence by inhibit of senescence due to ethylene inhibitors.

Membrane matrix responsive to 2, 4-D induced senescence:

Biomembrane is a seat of high metabolic activity, especially in the generation of several signal molecules, operation of a signal transudation pathway and in the production of several death signals during senescence. Signal molecules generated from the biomembrane include phosphatidice acid (PA), phosphatidyl serine (PS), diacylglyceride (DAG), phosphatidyl inostol (PI) and inositol phosphates. These signal molecules are involved in a 2 components regulatory system on 2-component signal transduction pathway. 2-components of the signal molecules include hydrophobic membrane based signals and membrane lipid derived hydrophilic molecules. In the present study results showed the presence of membrane based signals which are sensitived to 2, 4-D supplementation.

Besides the signal molecules, biomembrane in the senescence system also generates a few death signal molecules. These include PC, PA, MGDG and DGDG. Catalase, a ROS scavenger enzyme, also serves as death signal senescence. These parameters in the present study revealed the onset of senescence in a organelle specific manner especially MGDG and DGDG in the chloroplast. Catalase served as a general inductive response due to senescence. It relevant to mention that PA was both a signal molecule both inductive and death revealing.

Terminal stage of programmed cell death and nucleolytic attack:

In higher plants, senescence at the terminal stages is marked by shrinkage of cell, disassembly of organelles, nucleosome disassembly and fragmentation of nDNA. Fragmentation of nDNA takes place towards the terminal stage of senescence in a sequential manner. Nuclear DNA fragment is brought by de novo synthesis of nucleases namely, Nuclease I (nuc I), Nuclease II (nuc II), Nuclease III (nuc III). Inductions of nucleases during senescence were shown in *Arabidopsis thaliana* and tobacco. It is known that once the nucleases are induced the senescence becomes irreversible thus nDNA fragmentation analysis by agarose gel electrophoresis showed that these parameters were sensitive to 2, 4-D in mustard seedlings.

Thus, the observation of the present study revealed hormonal induction of senescence, hormonal interactions, involvement of signal molecules and death signals in te 2, 4-D induced senescence pathway in mustard seedlings. The results also highlighted efficacy of $AgNO_3$ as an inhibitor of ethylene. Several of the parameters employed in the present study elucidated senescence.

Abbreviation:

2, 4-D: 2, 4-dichlorophenoxy acetic acid; ABA: Abscisic acid; ACC: Amino cyclo propane carboxylic acid; AgNO3: silver nitrate; HRS: Hyper sensitive response; IAA: Indole 3 acetic acid; MGDG: Mono Galactosyl Di Glycerite; PA: Phosphatidic acid; PCD: Programmed Cell Death; PE: Phosphatidyl ethanolamine; PLD: Phospholipase D; PS: Phosphatidyl Serine; PC: Phosphatidyl Colline; ROS: Reactive oxygen species; SAGs: Senescence associated Genes; SOD:

Superoxide dismutase.

Authors' affiliations
K. TULASI RAMA KRISHNAN, Department of Biotechnology, Marudhu Pandiyar College, THANJAVUR (T.N.) INDIA

REFERENCES

Arnon, D.I. (1949). Copper enzyme in isolated chloroplasts: Polyphenoloxidase in *Beta vulgaris. Plant Phyiol.,* **24**: 1-15.

Bleecker, A.B. and Patterson, S.E. (1997). Characterization of the ABA deficient tomato mutant *notabilis* and its relationship with maize. *Plant J*., **17**: 427-431.

Chapman, R. (1998). Phospholipase activity during plant growth and development and in response to environmental stress. *Trends in Plant Sci.,* **11**: 419-426.

Kende, H. and Zeevaart, A.D. (1997). Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Plant Physiol*., **92** : 322-328.

Mackinneys, R. (1964). Separation of plant phospholipids and glycolipids. In: James, A.T and Mossus, L.J (eds.), *New Biochemical Separatists*, Van Nostrand, Princeton. pp. 321-337.

Manoharan, K. (1981). Chromatographic characterization of polar lipids of *Pisum sativum* L. var bonneille. M. Phil. Thesis, Jawaharlal Nehru University, New Delhi.

Nooden, L.S.D. (1988). Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. *J. Cell Physiol*., **154**: 519-526.

Wagner, H., Lissan, A. Holzi, Z. and Horhammer, L. (1962). Estimation of inorganic phosphate in perchloric acid digests of phospholipids. *J. Lipid Res*., **3** : 177-180.