Roles of catalases in response to gamma irradiation in Arabidopsis

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Abbreviations used

APX	ascorbate peroxidase
CAT	catalase
DAB	diaminobenzidine tetrahydrochloride hydrate
DSB	double strand breaks
LET	linear energy transfer
MES	2-(*N*-morpholino)ethanesulfonic acid
MDA	malondialdehyde
MMP	mismatch repair proteins
POD	Peroxidase
SSB	single strand breaks
SCGE	Single cell gel electrophoresis
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substance

CHAPTER 1

General Introduction

1.1 Plant physiology

Plant physiology is the study of the functions and processes occurring in plants. Its ultimate objective is to explain all life processes of plants by a minimal number of comprehensive principles founded in chemistry, physics and mathematics. It is divided into three major parts: a) the physiology of nutrition and metabolism b) the physiology of growth, development and reproduction and c) environmental physiology. Closely related fields include plant morphology, plant ecology, phytochemistry, cell biology and molecular biology. Fundmental processes of plant such as photosynthesis, respiration, plant nutrition, plant hormone functions, circadian rhythms, environmental stress physiology, seed germination, dormancy and stomata function and transpiration also are related fields.

1.2 Ionizing Radiation

Ionizing radiations are types of particle radiation (such as neutron, alpha particles, beta particles and cosmic ray) or electromagnetic (such as ultraviolet, X-rays and gamma rays) with sufficient energy to ionize atoms or molecules by detaching electrons from their valence orbitals. The degree and nature of such ionization depends on the energy of the individual particles or on frequency of electromagnetic wave. It is well known that exposure to ionizing radiation at sufficiently high doses results in various types of adverse biological effects. The biological effect of radiation involves direct and indirect actions. Both actions produce molecular changes that mostly need enzymatic

repair. Indirect effect involves the production of reactive free radicals which produce oxidative mutilation on the key molecules. The environmental sources of oxidative attack include, in particularly, specific exposures of the organism to ionizing radiations like X-, γ- or cosmic rays and α-particles from radon decay as well as UVA and UVB solar light. Ionizing radiations prevalent in space, involve a broad range of radiation types and energies from cosmic and unpredictable solar sources, representing a very diverse range of ionization qualities and biological effectiveness. Linear energy transfer (LET) is a measure of the energy transferred to tissue or cells as an ionizing particle travels through it. The LET of the potential radiations can cover several orders of magnitude from <1.0 keV μm_1 to > several 100 keV μm_1 (Blakely and Chang 2007) Low LET radiation causes damage through reactive oxygen species (ROS) production mainly by the radiolysis of water present in living system.

1.3 The effects of gamma radiation on plants

Gamma rays are a high-energy form of electromagnetic radiation.Gamma rays are photons of electromagnetic radiation emitted from unstable nuclei like those formed during nuclear fission. Their wavelengths are typically less than 10 ^ -12 meters, and their frequencies usually exceed 10 ^ 20 Hertz; consequently, they have sufficient energy to eject electrons from atoms and cause damage to tissues in living organisms. At high doses, gamma rays can harm plant life.

If doses are high enough, irradiation with gamma rays may be sufficient to kill most or even all of the plant species in a community. An Oak Ridge National Laboratory paper in 1995, for example, cited previous work studying past radiation releases in the Soviet Union like the Chernobyl disaster. Doses of radiation exceeding 500 rads (a unit measuring radiation and equivalent to 10 milli Gray per day completely killed off plants, even those that had higher tolerance levels. Doses of 10,000 rads per year caused complete destruction of exposed ecosystems and their plant inhabitants. Some species were more sensitive than others; pine trees, for example, fell victim to doses as low as 5 to 10 rads per day according to the report

Lower doses of radiation do not kill plants but can induce a range of abnormalities. Withered crowns, underdeveloped or misshapen leaves and unusual growth patterns such as gigantism excessive height and over-rapid growth characterize plants exposed to intermediate doses of gamma rays. When doses are sufficient to kill many of the existing plants, subsequent recovery may be slow. The gamma rays induce DNA damage and the higher the dose, the more damage to the plant's DNA they cause.

1.4 Molecular biology effects of gamma radiation due to free radical generation

As a matter of fact, gamma radiation penetrating living tissue and can damage all

important cellular components both through direct ionization and through generating ROS due to water radiolysis and induce oxidative damage. Radiation-induced oxidative stress was evaluated by three independent approaches; DNA damage, lipid peroxidation and protein oxidation.

1.5 Reactive Oxygen Speces (ROS)

Eukaryotic cells continuously produce ROS (such as H_2O_2 or $O_2^{\cdot-}$) as by-products of electron transfer reactions. Several major metabolic processes of plants including photosynthesis, respiration, and β-oxidation of fatty acids are responsible for the production of ROS during normal metabolism. It has generally been accepted that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radicals (-OH) and singlet oxygen, are produced by water radiolysis (De Vita et al., 1993; Dubner et al., 1995; Kovacs and Keresztes, 2002; Luckey, 1980, Miller 1987; Quintiliani 1986). Among these ROS, H_2O_2 is a normal metabolite in cells under the optimal plant growth conditions, are not particularly cytotoxic, but when its concentrations are increased by environmental stresses and ionizing radiation, it can lead to cell lethality (Halliwell, 1974). Considering that water radiolysis, the predominant effect of ionizing radiation in organisms, induces ROS formation as mentioned above, it is possible to assume that plants, microorganisms, and animals should have cellular protection systems against ionizing radiation (Zaka et al., 2002) Antioxidant enzymatic and non-enzymatic mechanisms effectively remove ROS from different cellular compartments, preventing cellular damage. Catalase (CAT),

Peroxidase (POD), and superoxide dismutase (SOD), represent the endogenous enzymatic defense of the plant cell, which become active during cell injury (shindo et al., 1994). Actually, it has been reported that the activities of scavenging enzymes, such as POD, CAT, SOD, and ascorbate peroxidase (APX), are generally increased in various plant species by the treatment of ionizing radiation (Kim et al., 2005; Kwon et al., 2001; Lee et al., 1999; Wada et al., 1998; Zaka et al., 2002).

1.6 DNA damage

Cells and their genomic constituent of the living organisms are continually exposed to oxidative attacks. Acute exposure to ionizing radiation can create oxidative stress in a cell and chronic exposure to this stress can result in permanent changes in the genome (Cooke et al., 2003). The main target of ionizing radiation has long since been indicated to be DNA which shows wide range of lesions. The oxidatively DNA damage commonly are apurinic/apyrimidinic (abasic) DNA sites, oxidized purines and pyrimidines, single strand (SSB) and double strand (DSB) DNA breaks and non-DSB (Kryston et al., 2011). Other initial chemical events induced in DNA by ionizing radiation include cross-links, oxidative base modification (Hutchinson, 1985) and clustered base damage (Goodhead, 1994), sugar moiety modifications, and deaminated and adducted bases (Cooke et al., 2003, Sedelnikova et al., 2010, Sutherland et al., 2000, Ward, 1994). The numbers of DNA lesions per cell that are detected immediately after a radiation dose of 1 Gy have been estimated to be approximately greater than 1000 base

damage, 1000 SSBs, 40 DSBs, 20 DNA–DNA links, 150 DNA–protein cross-links and 160–320 non-DSB clustered DNA damage and defective DNA mismatch repair proteins (MMP) (Martin et al., 2010). Recently, it is suggested that radiation dose and the type of DNA damage induced may dictate the involvement of the MMP system in the cellular response to ionizing radiation. In particular, the literature supports a role for the MMP system in DNA damage recognition, cell cycle arrest, DNA repair and apoptosis (Martin et al., 2010). In addition, The DNA oxidation products are a direct risk to genome stability, and of particular importance are oxidative clustered DNA lesions, defined as two or more oxidative lesions present within 10 bp of each other (Sedelnikova et al., 2010).

1.7 Comet Assay

A number of techniques for the detection of DNA damage have been used to identify substances with genotoxic activity. Of these, the most frequently used methods involved either the detection of DNA repair synthesis in individual cells, or the detection of DNA single strand breaks or alkali-labile sites in pooled cell populations using the alkaline elution assay (Tice et al., 2000). The first method provided information at the level of individual cells, however, the method is technically difficult to perform and requires the use of radioactivity and is not very sensitive. On the other hand, the second assay ignored the critical importance of intercellular differences in DNA damage and required relatively large number of cells (Tice et al., 2000). In

recent years, a new molecular-based assay, the Comet or single cell gel electrophoresis (SCGE) has been introduced to plant and mycological sciences for detecting the induced DNA damage (Collins and Harrington, 2002; Gichner et al., 2003; Lin et al., 2007) to overcome this limitation. Although this technique has been primarily applied to human and animal cells (Sing et al., 1988; Mitchelmore and Chipman, 1998) such as sperm and blood cells, the incorporation of this technique with plant tissues has enabled us to fast determination of level of DNA damages in plants. Use of this technique also extends the utility of plants in basic and applied studies in environmental mutagenesis. In theory, comet assay can be applied to every type of eukaryotic plant cell. The basic principle of this assay is to determine the DNA breaks by measuring the DNA damage which is quantified by the proportion of DNA, which migrates out of the nuclei towards the anode when individual cells or isolated nuclei **(**Dikilitas et al., 1919) embedded in a thin agarose layer (Menke et al., 2001). The formation of comet or "comet-like" shape (with a head, the nuclear region and a tail which contains DNA fragments) of nuclei followed by electrophoresis enables quantification of DNA in comet tails after staining with an appropriate fluorochrome such as propidium iodide or ethidium bromide (Bhanoori and Venkateswerlu, 1998; Olive and Banath, 2006). Diameter of nuclei of the studied species and the degree of DNA denaturation under assay conditions would indicate the condition of DNA, which is responsible for many metabolic activities. Comet assay was first described by Swedish researches Östling and Johansson (1984), then it was modified by Singh et al.,

(1988) as ‘alkaline comet assay’ and after that numerous modifications have been made to date (Fairbairn et al., 1995; Lin et al., 2007; Gichner et al., 2008). Comet assay has 2 commonly used versions; neutral (neutral unwinding/neutral electrophoresis, N/N) and alkaline (alkaline unwinding/alkaline electrophoresis, A/A). In recent studies, alkaline-neutral (alkaline unwinding/neutral electrophoresis, A/N) assay was also employed by Lin et al., (2007). The N/N assay (pH of lysing and electropheretic solutions are approximately 9) is useful to assess DNA double strand breaks (Östling and Johanson, 1984). This method was then developed by Olive et al., (1990) to detect single strand breaks.

Alkaline version of the comet assay, A/A, (pHs of lysing and electrophoretic solutions are 10 and 13, respectively) can quantitatively measure DNA damage, including single strand breaks, double strand breaks, alkali labile sites (primarily aprunic and apyrimidinic sites) incomplete excision repair sites and DNA cross links (Singh et al., 1988; Gichner and Plewa, 1998; Lin et al., 2007). The Singh and Olive methods are identical in principle and similar in practice, but Singh method appears to be one or two orders of magnitude more sensitive. The A/N method (pH of unwinding and electrophoresis solutions are 10 and 8.5, respectively) also useful to measure both double- and single strand breakages of DNA (Lin et al., 2007). In many works, various combinations of neutral and alkali pH solutions prior to and during electrophoresis or addition of antioxidant to the lysing/electrophoretic buffer, and precipitation of DNA with ethanol and the use of sensitive dyes (e.g. YOYO-1, DAPI) have enhanced the

sensitivity of assay techniques to screen for low level DNA damages in variety of cells (Singh, 1996; Angelis et al., 1999). The comet assay in the nuclei of various higher plants has been applied before, such as in *vicia faba* (Koppen and Verschaeve, 1996; Koppen and Angelis, 1998; Koppen et al., 1999), *Allium cepa* (Navarrete et al., 1997; Pincheira et al., 2003), *Nicotiana tabacum* (Koppen et al., 1999; Stavreva et al., 1998; Gichner et al., 2000; ptacek et al., 2001; Stavreva and Gichner, 2002; Restivo et al., 2002), *Calamagrostis epigejos* (Ptacek et al., 2002), *Impatiens balsamina* (Poli et al., 2003). *Arabidopsis thaliana* (Menke et al., 2001) and barly (Jovtchev et al., 2001).

1.8 Lipid peroxidation

Lipid peroxidation is the process where ROS remove electrons from the lipids in the cell membranes thereby damaging the cells. This process occurs in three stages: initiation, propagation, and termination. During initiation phase, hydroxyl, alkoxyl, peroxyl radicals abstract the first hydrogen atom. Phospholipids containing polyunsaturated fatty acids are susceptible to peroxidation as they contain multiple double bonds and the methylene group that lies within is prone to abstraction of hydrogen atom. The initial reaction with polyunsaturated fatty acids produces a lipid radical. The lipid radical produced abstracts hydrogen from neighboring fatty acids to produce lipid hydroperoxide (LOOH) and a second lipid radical. The LOOH undergoes reductive cleavage by reducing metals and produces alkoxyl radical. Both alkoxyl and peroxyl radicals create a chain reaction by abstracting additional hydrogen atoms.

Thiobarbituric acid reactive substances (TBARS), the cytotoxic product of lipid peroxidation, is normally considered as the major TBA-reacting compounds that indicate the magnitude of the oxidative stress (Qadir et al., 2004; Qureshi et al., 2007). During the process of lipid peroxidation, the malondialdehyde (MDA) is formed by the decomposition of polyunsaturated fatty acids which reacts with thiobarbituric acid. The basic effect of radiation on cellular membranes is believed to induce lipid peroxidation by the production of free radicals (Leyko and Bartosz, 1986). Lipid peroxidation products in leaves of Arabidopsis thaliana L. present highest at full flowering and decreased with higher g-exposure at this growth stage. At the other two growth stages, lipid peroxidation products were unaffected by gamma treatment (Vandenhove et al., 2009). The Malondialdehyde (MDA) content was observed only under the highest irradiation dose, in soybean (Glycine max Merill.) seeds. The MDA quantity increase of 21.6%, compared with the non-irradiated control (Štajner et al., 2009).

1.9 Arabidopsis mutant

Arabidopsis thaliana is a model plant having a large collection of mutants with defect in different defence and stress related signaling pathways and use of these mutants makes it possible to determine which pathways are controlling an observed response (Glazebrook, 1997). Pharmacological, cell biological, genetical and electrophysiolocal studies have elucidated the multiple regulatory protein components, enzymes amd second messengers and biosynthesis (Schroeder et al., 2001; Munemasa

et al., 2007). In my experiments, *cat3-1, cat2,* and *cat1cat3* mutantts were used to clarify the functions of catalases in response to irradiated plants

1.10 CAT mutants

Catalase (CAT) (H_2O_2: H_2O_2 oxidoreductase; EC 1.11.1.6) is a tetrameric iron porphyrin protein that catalyzes the dismutation of H_2O_2 to water and oxygen. Catalase was the antioxidant enzyme to be discovered and charecterized. The typical catalase reaction is the dismutation of two molecules of H_2O_2 to water and oxygen. Information from genome sequencing has confirmed the presence of three CAT genes in *Arabidopsis,* two located on chromosome1 (*CAT1* and *CAT3*) and one located on chromosme 4 (*CAT2*) (Frugoli et al., 1996). All three-translation products consist of 492 amino acids, with high similarity between sequences (Frugoli et al., 1996; Mhamdi et al., 2010). In plant, classical subcellular fractionation studies as well as in situ activities staining have established that peroxisomes contain high CAT activity and import of CAT into these ornanelles has been experimentally demonstrated (Mullen et al., 1997). Proteomic analysis of highly purified mitochondria from *Arabidopsis* cells identified CAT2 and CAT3 peptide sequences (Heazlewood et al., 2004). *Arabidopsis* genome initiative numbers for catalase genes are *CAT1* (At1G20630), *CAT2* (At4G35090) and *CAT3* (At1G20620). All three transcripts can be detected in mature Arabidopsis rosettes though *CAT3* and *CAT2* transcripts are much more abundant than those of *CAT1* (Frugoli et al., 1996, McClung, 1997).

1.11 Purposes of the study

The aim of this study is to obtain novel information about the functions of catalases in response to gamma irradiation in Arabidopsis

Specific aims were as follows:

1. To investigate the functions of CAT1 and CAT3 in response to gamma irradiation-induced DNA damage, H_2O_2 accumulation, and lipid peroxidation in Arabidopsis

2. To investigate the function of CAT2 in response to gamma irradiation-induced H_2O_2 accumulation and lipid peroxidation in Arabidopsis

CHAPTER 2

Catalases, CAT1 and CAT3, are not key enzymes to alleviate gamma irradiation-induced DNA damage, H_2O_2 accumulation, or lipid peroxidation in *Arabidopsis thaliana*

2.1. Abstract

Gamma irradiation increased catalase activities at 0.1 kGy and decreased at 10 kGy in Arabidopsis wild-type and catalase deficient mutants, *cat3-1* and *cat1 cat3*. Irradiation induced DNA damage, H_2O_2 accumulation and lipid peroxidation in both mutants as well as wild-type. Hence, catalases may not be a key enzyme to protect gamma irradiation-induced damage.

2.2. Introduction

Gamma irradiation has been used in the biological studies which reveals that low-doses stimulats and high-doses inhibits (Ribeiro and Machado, 2007). Gamma irradiation affects growth and development due to cytological, genetical, biochemical, physiological and morphogenetic changes in cells and tissues of plants (Gunckel et al., 1961). The exposure to gamma irradiations can have stimulatory effects on specific morphological parameters and can increase the yield of plants in terms of growth (e.g., taller plants), reproductive success (e.g., formed seeds) and ability to withstand water shortage (Dishlers and Rashals, 1977; Zaka et al., 2002; Maity et al., 2005; Yu et al.,

2007; Melki and Dahmani, 2009). A more detailed description of morphological abnormalities were documented by Gunckel (1957) and Sparrow (1966). It was generally observed that low doses of γ-rays stimulated cell division, growth, and development in various organisms, including animals and plants. The morphological, structural, and functional changes depend on the strength and duration of the gamma irradiation dose applied. The symptoms frequently observed in plants irradiated with a low or high dose were enhancement or inhibition of germination, seedling growth, and other biological responses (Kim et al., 2000; Wi et al., 2005). Although no conclusive explanations for the stimulation effects of low dose gamma irradiation have been available until now, papers support a hypothesis that the low dose irradiation will induce growth stimulation by changing the hormonal signalling network in plant cells or by increasing the antioxidative capacity of cells to easily overcome daily stress factors such as fluctuation of light intensity and temperature in growth conditions (Kim et al., 2004; Wi et al., 2007). In contrast, the growth inhibition induced by high-dose irradiation has been attributed to the cell cycle arrest at the G2/M phase during somatic cell division and (or) varying damage to the entire genome (Preuss and Britt, 2003). The relationship between growth of irradiated plants and the dose of γ-irradiation has been manifested by investigating the morphological changes and seedling growth of irradiated plants.

Also, it has been shown to enhance the production of reactive oxygen species (ROS) in a variety of cells resulting oxidative stress (Repine et al., 1981; von Sonntag,

1987; Xienia et al., 2000). Reactive oxygen species play an important role in the action of ionizing radiation (Ewing and Jones, 1987; Alaoui et al., 1992). ROS are the byproducts of many degenerative reactions in crop plants, which will affect the regular metabolism by damaging the cellular components (Foyer and Noctor, 2002). Extensive study on oxidative stress has demonstrated that exposure of plants to adverse environmental conditions induces the overproduction of reactive oxygenspecies (ROS), such as superoxide radical (O_2), H_2O_2 and hydroxyl radical (HO) in plant cells (Wise and Naylor, 1987). In addition, ROS are highly reactive to membrane, lipids, protein and DNA. They are believed to be the major contributing factors to stress injuries and to cause rapid cellular damage (O'Kane et al., 1996; El-Beltagi et al.,2008; Salama et al., 2009; Mohamed et al., 2009; El-Beltagi et al., 2010; El-Beltagi and Mohamed, 2010 and Aly and El-Beltagi, 2010), particularly when plants are exposed to stress conditions such as chilling stress, salt stress, Fe deficiency, cadmium stress, Lead toxicity and ionizing radiation. H_2O_2 is an important ROS under radiation stress (Vanhouht et al., 2011). Gamma irradiation increases hydrogen peroxide accumulation and lipid peroxidation (El-Beltagi et al., 2011). Ionizing radiation can damage DNA by directly ionizing DNA itself and by indirect processes in which DNA reacts with numerous radiolytic reactive products includling OH, H, O_2 and H_2O_2, that are generated in aqueous fluid surroundings DNA (O'Neill and Fielden, 1993). Plant cells can tolerate ROS by endogenous protective mechanisms involving non-enzymic as well as enzymatic system (Asada, 1994). As a consequence, plants evolved cellular adaptive

responses like up-regulation of oxidative stress protectors and accumulation of protective solutes (Horling et al., 2003). Antioxidant defense enzymes such as superoxide distumase (SOD), Catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) are the system designed to minimize the concentrations of superoxide and hydrogen peroxide, SOD catalases the dismutation of superoxide into oxygen and hydrogen peroxide. H_2O_2 is eliminated by catalase and peroxidases, which include both enzymic and non-enzymic H_2O_2 degradation (peltzer et al., 2002). Catalase dismutates H_2O_2 into water, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Blkhina et al., 2003). Catalase is one of antioxidant enzymes to scavenge H_2O_2 and irradiation changes catalase activities (Martinez-Solano et al., 2005). Hence, it is thought that increasing activity of catalases is favorable to alleviation of damage caused by irradiation (De Freitas et al., 2012). The Arabidopsis genome contains three *CAT* genes, *CAT1*, *CAT2* and *CAT3*, which are differentially expressed and can form up to six different isozymes (Frugoli et al., 1996). Catalase are highly specific to H_2O_2 over other chemical species of ROS, such as superoxide and hydroxyl radical. CAT1 is an important player for removal of H_2O_2 generated under various environmental stresses. CAT3 was major H_2O_2 scanvengers to contribute to ROS homeostasis in light or darkness, respectively (Du et al., 2008). However, it remains to be clarified roles of catalases in irradiated plants. We examined the effects of

gamma irradiation on Arabidopsis catalase-deficient mutants, *cat3-1* and *cat1 cat3* to elucidate roles of catalases in responses to gamma irradiation.

2.3. Materials and Methods

2.3.1. Plant materials and growth conditions

Arabidopsis thaliana wild type (WT, Wassilewskija ecotype) and *cat3-1, cat1 cat3* mutant lines were used. *cat3-1* mutant possesses a T-DNA insertion in *CAT3* locus of the Wassilewskija (WS) accession and isolated by a genomic PCR screening of the pooled DNA from random T-DNA inserted populations provided by Ohio State University. The *cat1 cat3* double mutant was identified in a poputation of WS that had been subjected to fast neutron bombardment. To define the limits of the deletion, we developed PCR primers to amplify genes flanking the linked *CAT1* (At1G20630) and *CAT3* (At1G20620) loci. In addition to *CAT1* and *CAT3*, the two genes immediately downstream of CAT1, At1G20650 (encoding a RWP-RK family protein) and At1G20660 (encoding a protein serine threonine kinase) were missing, but the third gene, At1G20670 (encoding a putative bromo-domain containing protein), was retained. Similarly, the next gene upstream of CAT3, At1G20619 (Encoding CYCLIN B2;3), was also retained. We then designed primers to amplify across the deletion and determied the DNA sequence of the amplified fragment. The deletion eliminates 20,625 base pair (bp), from position 7141093, 2052 bp upstream of CAT3, to position 7161718, 1182 bp upstream (5') of At1G220660 and 2574 bp downstream of At1G20670 (nucleotide numbers are based on the Columbia-0 reference genome).

Plants were grown in soil in a plant growth chamber (LPH-350SP; Nihonika Co., Osaka, Japan) at 22°C under a 16-h-light/8-h-dark photoperiod at a photosynthetic photon flux density of 80 μmol m^{-2} s^{-1} and watered twice a week with Hyponex solution (0.1%).

2.3.2. Measurement of catalase activity in whole leaves

For measurement of CAT activity leaves were homogenized in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.5 mM EDTA, and phenylmethylsulfonyl fluoride with a mortar and a pestle. The homogenate was centrifuged at 15,000x*g* for 10 min at 4ºC. The supernatant was used for CAT activity measurement.

Catalase activity was assayed according to the method of Aebi (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 20 mM H_2O_2 and 50 μL crude enzyme sample. The reaction was started by the addition of H_2O_2. The activity was calculated from the decline in absorbance at 240 nm for 60 s. The extinction coefficient was 39.4 $M^{-1}cm^{-1}$. Protein contents were measured as described by Bradford (1976) using bovine serum albumin as the standard.

2.3.3. Measurement of DNA damage in mesophyll cells by neutral comet assay

DNA integrity of primary leaves was analysed with the Single Cell Gel Electrophoresis (SCGE) assay (comet assay) (3 replicates). Cells and/or nuclei embedded in agarose are lysed to remove nuclear membranes and proteins and then submitted to electrophoresis for a short time. DNA structural changes or DNA damage (strand breaks, incomplete excision repair sites or crosslinks) cause a change in DNA migration capacity in the electric field. Small DNA molecules and free DNA loops can migrate away from the residual nucleus. When DNA is stained with a fluorescent dye and viewed using an epifluorescence microscope, the nucleus resembles a comet with a 'head' and a 'tail'. Usually the more DNA integrity is disturbed, the bigger tails are. The comet analysis protocol was done according Koppen et al. with some slight modifications. To extract the DNA, the frozen plant leaves tissue (0.1 - 0.2 g) was chopped with a razor blade in 300 mL ice-cold PBS (Phosphate Buffer Saline) buffer. The mixture was filtered over an 80 mm nylon sieve in an ice-cold eppendorf. 10-20 ml of this crude nucleus suspension was then mixed with 250 mL LMAgarose gel and layered on a microscope slide . All the steps are completed on ice and under protection from UV light. The slides were put in a neutral lysis solution for at least 1 h to lyse nuclei and to permit deproteination of DNA. The slides were washed for 10 min in icecold TBE solution, immersed in an electrophoresis chamber filled with TBE and processed for 20 min at 1 V cm^{-1} and 30 mA. Then they were washed in cold (4^0 C) distilled water for 10 min. After staining with 10 mg ml^{-1} SYBR green for 10 min, the slide is rinsed with water

and analysed with a fluorescence microscope (excitation filter of 515-560 nm and barrier filter of 590 nm).

2.3.4. Measurement of H_2O_2 accumulation in whole leaves

The rosette leaves of Arabidopsis plants were analyzed using 3,3-diaminobenzidine tetrahydrochloride hydrate (DAB) (Tokyo Chemical Industries, Tokyo, Japan) as described previously with some modification (Hossain et al., 2013). Excised irradiated rosette leaves were floated on medium containing 5 mM KCL, 50 μM CaCl2, and 10 mM MES-Tris (pH 6.15) with 0.05% Tween20 and incubated for 2 h under light (80 μmol m^{-2} s^{-2}). Then, the leaves were transferred in 1 mg mL^{-1} DAB solution and gently infiltrated in a vacuum for 4 h. After incubation, the leaves were cleared in boiling ethanol (99%) for 10 min. Localization of H_2O_2 is visualized as a reddish-brown coloration. Then, the leaves were mounted on cover glass and pictures were taken. The intensity of coloration was quantified using Adobe photoshop CS2 software (Adobe Systems Inc., San Jose, CA, USA).

2.3.5 Measurment of lipid peroxidation in whole leaves

The MDA content of plant leaves was used as a measure of lipid peroxidation. Plant tissue was homogenized with 2 ml 0.1% TCA buffer per 100 mg plant material using a mortar and pestle. After centrifugation at 15000 *g* for 10 min, 0.5 ml of the supernatant was added to 2 ml 0.5% TBA. This mixture was heated at 95^0 C for 30 min

and quickly cooled in an ice bath. After centrifugation at 15000*g* for 10 min, the absorbance of the supernatant was measured spectrophotometrically at 532 nm corrected for unspecific absorbance at 600 nm according to Dhindsa et al., (1981).

2.3.6 Isolation of total RNA from whole leaves

Total RNA from whole leaf was carried out with Trizole Reagent (Invitrogen) according to the producers manual.

2.3.7 Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows: Single strand complementary RNA was synthesized with MMLV reverse transcriptase according to the manufacturer's manual from total RNA isolated whole rosette leaves of 4-6-weak-old *Arabidopsis* plants. Polymerase chain reaction was carried out using gene-specific primer pairs as listed in Table 1 with a 30-cycles reaction steps: 94°C for 30 s, 53°C for 30 s and 72°C for 60 s. BIOTAQ DNA polymerase (Bioline, Bio-21040) was used.

Table 1. Primer sequences used for RT-PCR of catalase genes.

Primers	Sequence
Common forward primer	5′-GGTATCCCACAAGATTACAGGCACATGGA-3′
CAT1 reverse primer	5′-ACAGGAACTAGTACCCTTCTTTAAGCGTT-3′
CAT2 reverse primer	5′-AGGCCAATCAAGAATTCTTTCACTCGT-3’
CAT3 reverse primer	5′-GATAGATCGATGAGATTGATTGTACCTCA-3′.

2.3.8 Statistical Analysis

Significance of differences between mean values was assessed by Student's t-test or one-way ANOVA with Dunnett's test. Differences at $p < 0.05$ were considered significant.

2.4. Results

2.4.1 Catalase activities in Arabidopsis rosette leaves irradiated by gamma rays

Inhibition of CAT activity was reported under irradiation stress (Ye et al., 2000 Stajner et al., 2009, Vandenhove et al., 2009). CAT in *Nicotiana tabacum* increased in response to gamma irradiation treatment (Wada et al., 1998). Here, I examined catalase activities in Arabidopsis rosette leaves irradiated by gamma rays. Fig. 2.1 (A) shows that under control condition, its means at 0 kGy, the *Cat3-1* mutation and *Cat1 Cat3* mutation significantly reduces the CAT activities in whole leaves compare with wild type in agriment with the results of Jannat *et al*., (2011). These results indicate that disruption of CAT in the whole leaf of mutants. Fig.2.1 (B) shows that, irradiation at o.1 kGy significantly increases the catalase activity in the wild type plant, *cat3-1* and *cat1 cat3* mutants compare with control. At doses of 1 kGy, there were no changes in catalase activities in the wild type, *cat3-1* and *cat1 cat3* mutants compared with the control (Fig. 2.1 C).

Irradiation at 10 kGy significantly decreases the catalase activities in the wild type plants, *cat3-1* and *cat1 cat3* mutants and there were no significant differences in

catalase activities among the wild type and both mutant at 10 kGy (Fig. 2.1 D), which is similar to previous results (Martinez-Solano et al., 2005). According to Lee et al., (1991) (Kim et al., 2005; Lee et al., 1999) the oxidative defence and stress in plants induced by acute exposure to relatively high radiation doses produced low CAT enzyme activity. These results suggest that lower doses of gamma radiation increases the catalase activities and higher doses of gamma radiation decreases the catalase activities in wild type plant and *cat3-1* and *cat1 cat3* mutants. And there were no significant differences in catalase activity among the wild type and both mutants at 10 kGy.

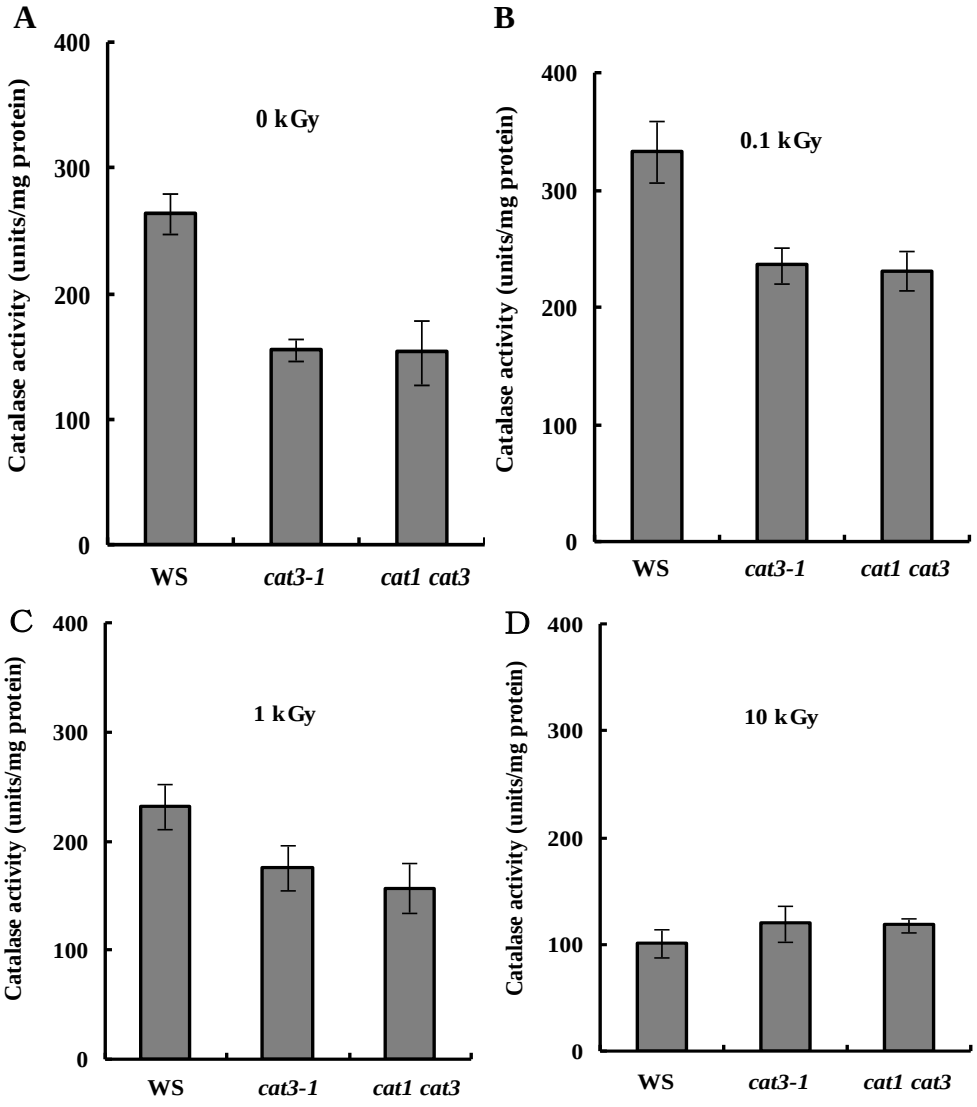


Fig. 2.1 Catalase activities in Arabidopsis rosette leaves irradiated by gamma rays.

Rosette leaves of wild type, *cat3-1*, and *cat1 cat3* mutant were irradiated with gamma rays at 0 kGy (A), 0.1 kGy (B), 1 kGy (C), and 10 kGy (D). Averages for three independent experiments are shown. Error bars represent standard deviations. Values

indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.2 Catalase activities in Arabidopsis whole leaves incubated without irradiation.

Consequently, leaves were irradiated for 1 h, 10 h, and 100 h for irradiation of 0.1 kGy, 1 kGy, and 10 kGy, respectively. I examined the effect of incubation period of 1 h, 10 h, and 100 h on catalase activity in the wild-type and both munants. This figure shows that incubation for up to 100 h without irradiation did not affect catalase activities in the wild type, *cat3-1*, and *cat1 cat3* mutants compare with control.

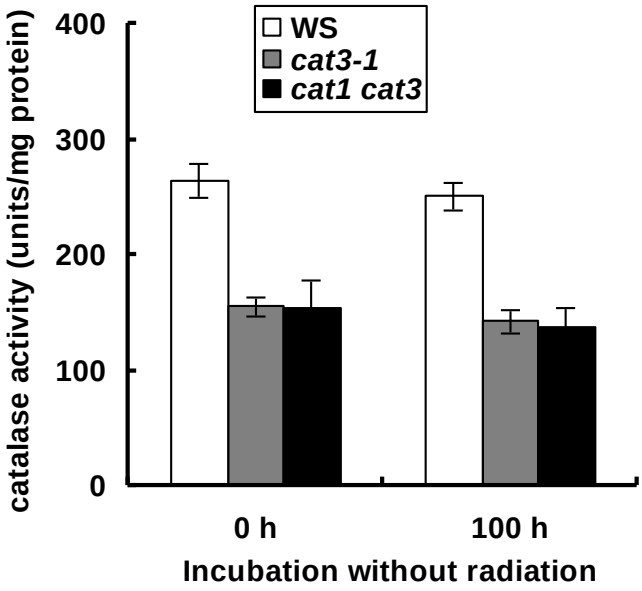


Fig. 2.2 Catalase activities in Arabidopsis rosette leaves incubated without radiation.

Rosette leaves of wild type, *cat3-1*, and *cat1 cat3* mutant were incubated without gamma irradiation at 0 h and 100 h. Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.3 Gamma radiation-induced DNA damage in mesophyll cells of Arabidopsis

It is well established that ionizing radiation induces DNA double strand break, and that this lesion is critical for the induction of cell death (Harms et al., 1996). The dose-response for DNA double strand break could vary markedly between cell lines, and an important cause of differences in radiosensitivity (Harms et al., 1996; Radford, I. R. and Murphy T. A. 1994). I examined gamma radiation-induced DNA damage in the *cat3-1* and *cat1 cat3* mutants and wild type plants. Under control condition, there are no differences in DNA damage in the wild type and both mutants. Irradiation at o.1 kGy, there are no differences in DNA damage in the wild type and both mutants compare with control (Fig. 2.3 B). At doses of 1 kGy, there are no differences in DNA damage in the wild type and both mutants compare with control ((Fig. 2.3 C).

Irradiation at 10 kGy significantly increases DNA damage in the wild type plants, *cat3-1* and *cat1 cat3* mutants compare with control and there were no significant differences in DNA damage among the wild type and both mutants at 10 kGy (Fig.

2.3D). These results indicate that gamma irradiation-induced DNA damage significantly elevated at doses of 10 kGy but not at 0.1 kGy and 1 kGy in the wild type, *cat3-1*, and *cat1 cat3* mutants.

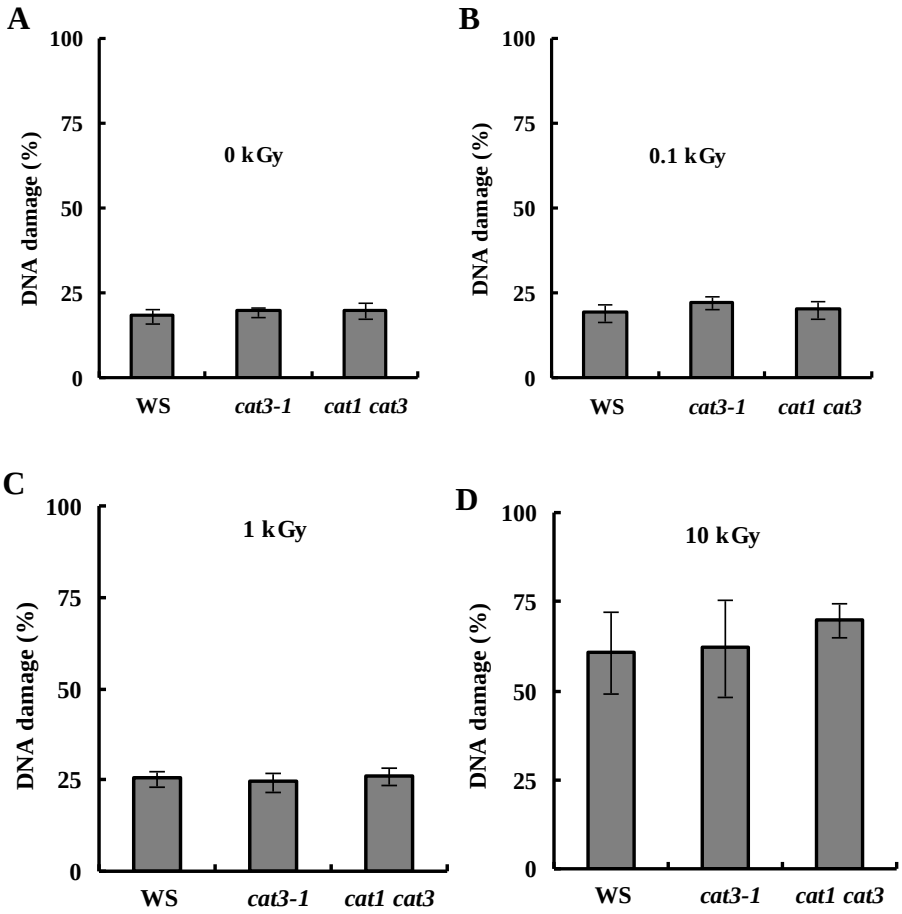


Fig. 2.3 DNA damage in Arabidopsis mesophyll Cells irradiated by gamma rays.

Rosette leaves of wild type, *cat3-1* and *cat1 cat3* mutant plants were irradiated with gamma rays at 0 kGy (A) 0.1 kGy (B), 1 kGy (C) , and 10 kGy (D). DNA damage (%) was evaluated by the ratio of the tail area to the sum of tail area and head

area of the comet image. Averages for three independent experiments (more than 100 comets per bar) are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.4 DNA damage in Arabidopsis mesophyll cells incubated without irradiation.

Consequently, leaves were irradiated for 1 h, 10 h, and 100 h for irradiation of 0.1 kGy, 1 kGy, and 10 kGy, respectively. I examined the effect of incubation period of 1 h, 10 h, and 100 h on DNA damage in the wild type and both munants. Incubation for up to 100 h without irradiation did not affect DNA Damage in the wild type, *cat3-1*, and *cat1 cat3* mutants compare with control.

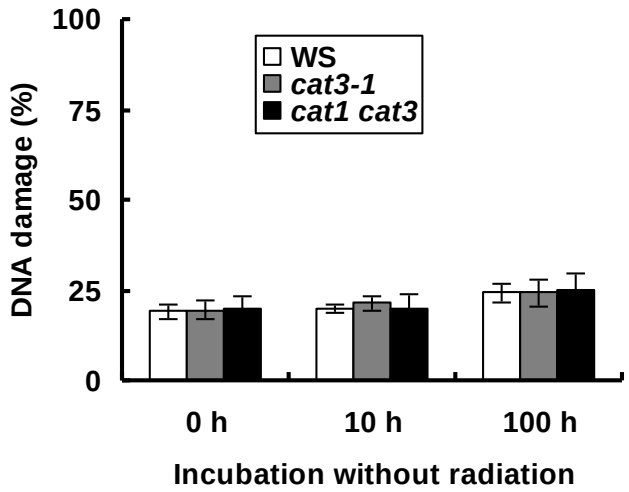
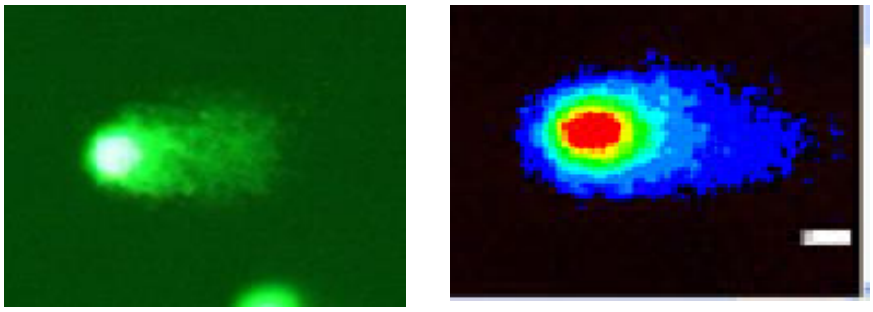


Fig.2.4 DNA Damage in Arabidopsis Mesophyll Cells Incubated Without Radiation.

Rosette leaves of wild type, *cat3-1* and *cat1 cat3* mutant plants were incubated without gamma irradiation at 0 h, 10 h, and 100 h. DNA damage (%) was evaluated by the ratio of the tail area to the sum of tail area and head area of the comet image. Averages for three independent experiments (more than 100 comets per bar) are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.



Typical analytical comet

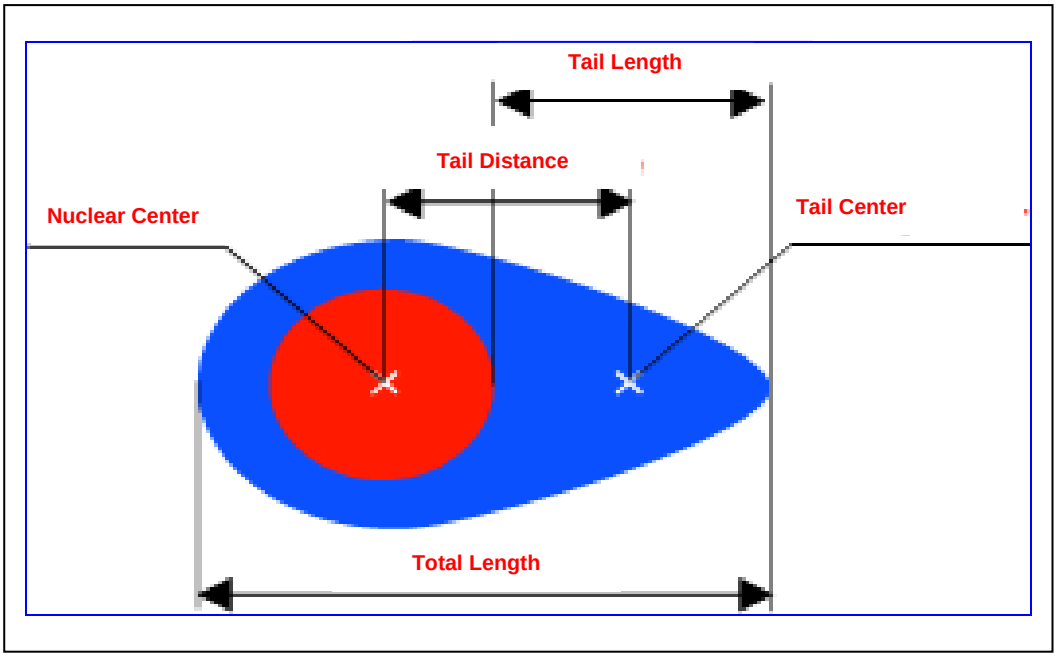


Diagram of an analytical comet

Fig. 2.5 A simple model for the measurment of DNA damage

Calculation of the DNA damage

$$\% \text{ DNA damage} = \frac{\text{Tail length of the comet}}{\text{Total length of the comet}} \times 100$$

2.4.5 Gamma radiation-induced H_2O_2 accumulation in whole leaves of Arabidopsis

The concentration of H_2O_2 increased in various pumpkin tissues sush as leaves, petioles and hypocotyls after gamma-irradiation (Wi et al., 2007). An increase in the endogenous H_2O_2 level has been reported to be associated with the promotion of leaf senescence (Mondal and Choudhuri, 1981). Fig.2.6 (A) and (B) indicate that 0 kGy and 0.1 kGy did not significantly increase H_2O_2 accumulation in the wild type, *cat3-1*, and *cat1 cat3* mutants. Irradiation at 1 kGy also did not significantly increase H_2O_2 accumulation in the wild type, *cat3-1*, and *cat1 cat3* mutants (Fig.2.6 C). Irradiation at 10 kGy significantly increases H_2O_2 accumulation in the wild type plant, *cat3-1* and *cat1 cat3* mutant and there are no significant differences in H_2O_2 accumulation among the wild type and both mutants (Fig.2.6D). These results indicate that H_2O_2 accumulation increased by irradiation at 10 kGy but not at 0.1 kGy or 1 kGy in the wild type, *cat3-1*, and *cat1 cat3* mutants. There were no significant differences in H_2O_2 accumulation, among the wild type, *cat3-1*, and *cat1 cat3* mutants at doses of 10 kGy gamma irradiation.

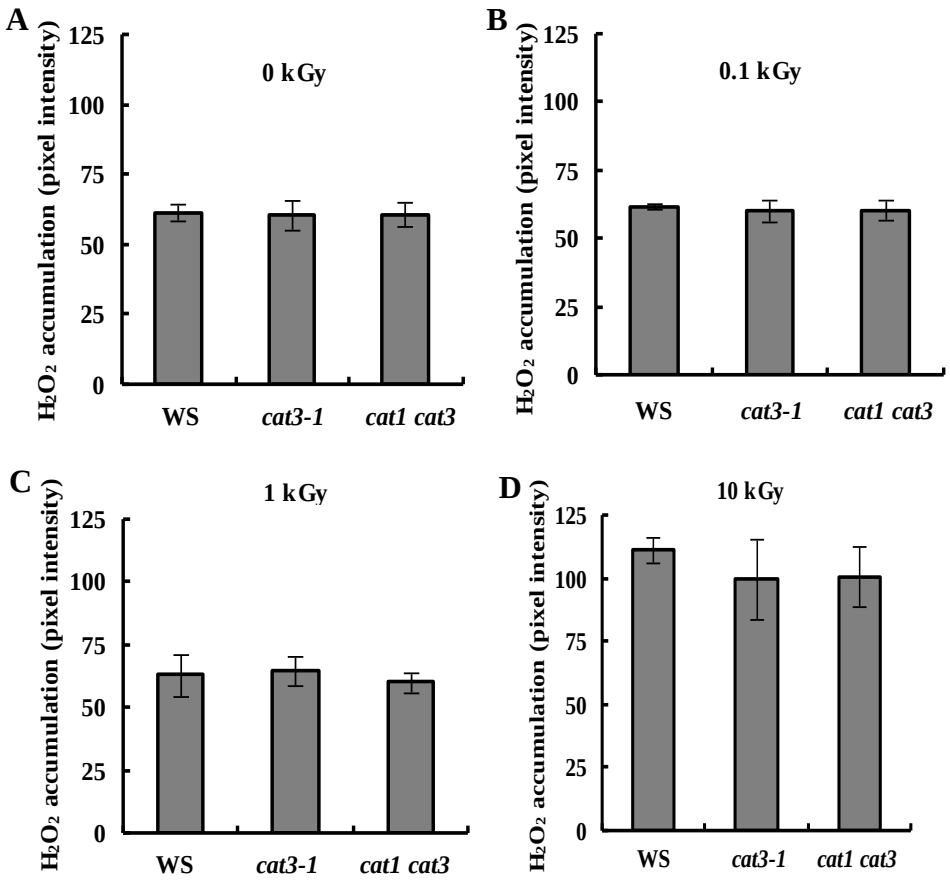


Fig. 2.6 Hydrogen peroxide accumulation (A, B, C, and D) in Arabidopsis rosette leaves irradiated by gamma rays.

Rosette leaves of wild type, *cat3-1* and *cat1 cat3* mutant plants were irradiated with gamma rays at 0 kGy (A), and 0.1 kGy (B), 1 kGy (C), and 10 kGy (D). Hydrogen peroxide accumulation was detected by 3,3'-diaminobenzidine . Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.6 H_2O_2 accumulation in Arabidopsis whole leaves incubated without irradiation

Consequently, leaves were irradiated for 1 h, 10 h, and 100 h for irradiation of 0.1 kGy, 1 kGy, and 10 kGy, respectively. I investigated the effects of incubation period of 1 h, 10 h, and 100 h on H_2O_2 accumulation in the wild type and both munants. Incubation for up to 100 h without irradiation did not affect H_2O_2 accumulation in the wild type, *cat3-1*, and *cat1 cat3* mutants.

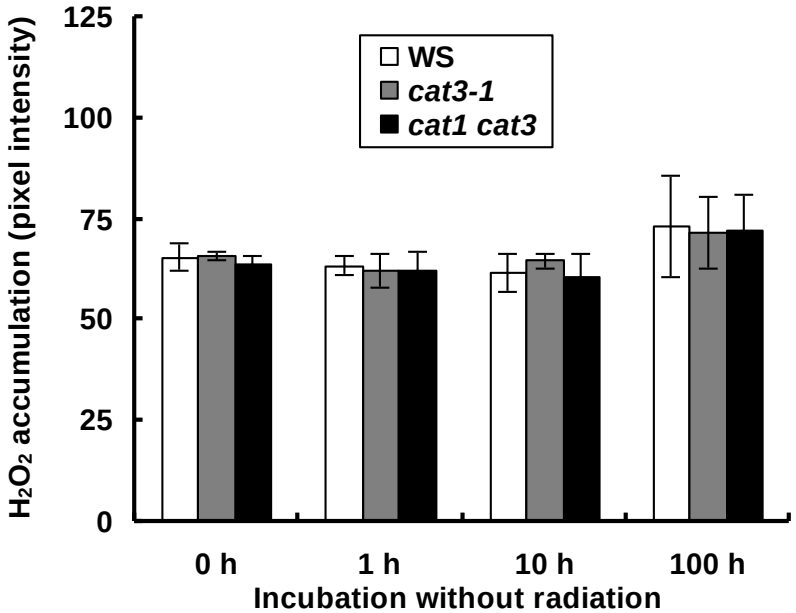


Fig. 2.7 H_2O_2 accumulation in Arabidopsis rosette leaves incubated without radiation.

Rosette leaves of wild type, *cat3-1* and *cat1 cat3* mutant plants were incubated without gamma irradiation at 0 h, 1 h, 10 h, and 100 h. H_2O_2 accumulation was

detected by 3,3'-diaminobenzidine. Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.7 Lipid peroxidation induced by gamma irradiation in whole leaves of Arabidopsis

The lipid peroxidation contents (MDA) of the irradiated samples were significantly higher than that of control (El-Beltagi H. S. 2011). We examined the lipid peroxidation induced by gamma irradiation in whole leaves of Arabidopsis Fig. 2.8 (A) indicate that under control condition there were no changes in lipid peroxidation in the wild type, *cat3-1*, and *cat1 cat3* mutants. Fig. 2.8 (B) indicates that irradiation at 0.1 kGy, there were no changes in lipid peroxidation in the wild type, and both mutants compare with control. Fig.2.8 (C) indicates that, Irradiation at 1 kgy, there were no changes in lipid peroxidation in the wild type, and both mutants.

Irradiation at 10kGy significantly increases lipid peroxidation in the wild type, *cat3-1* and *cat1 cat3* mutant compare with control and there were no significant difference in lipid peroxidation among the wild type and both mutant at 10 kGy (Fig. 2.8 D). These results indicate that lipid peroxidation increased by irradiation at 10 kGy but not at 0.1 kGy or 1 kGy in the wild type, *cat3-1*, and *cat1 cat3 mutants*. There were no significant differences in lipid peroxidation levels among the wild type, *cat3-1*, and *cat1 cat3* mutants at doses of 10 kGy gamma irradiation. The observed changes in the

MDA content were consistent with the previous results observed by Fu and Huang (2001), El-Beltagi et al. (2008), Salama et al., (2009) and Aly and El-Beltagi (2010); the authers stated that an enhanced level of lipid peroxidation of grasses, flax and *Vicia faba*, under environmental stress (drought, Fe deficiency, salt stress and radiation) indicated oxidative damage to plants; it means lipid peroxidation may be a consequence of generation of reactive oxygen species (OH, O_2, and H_2O_2).

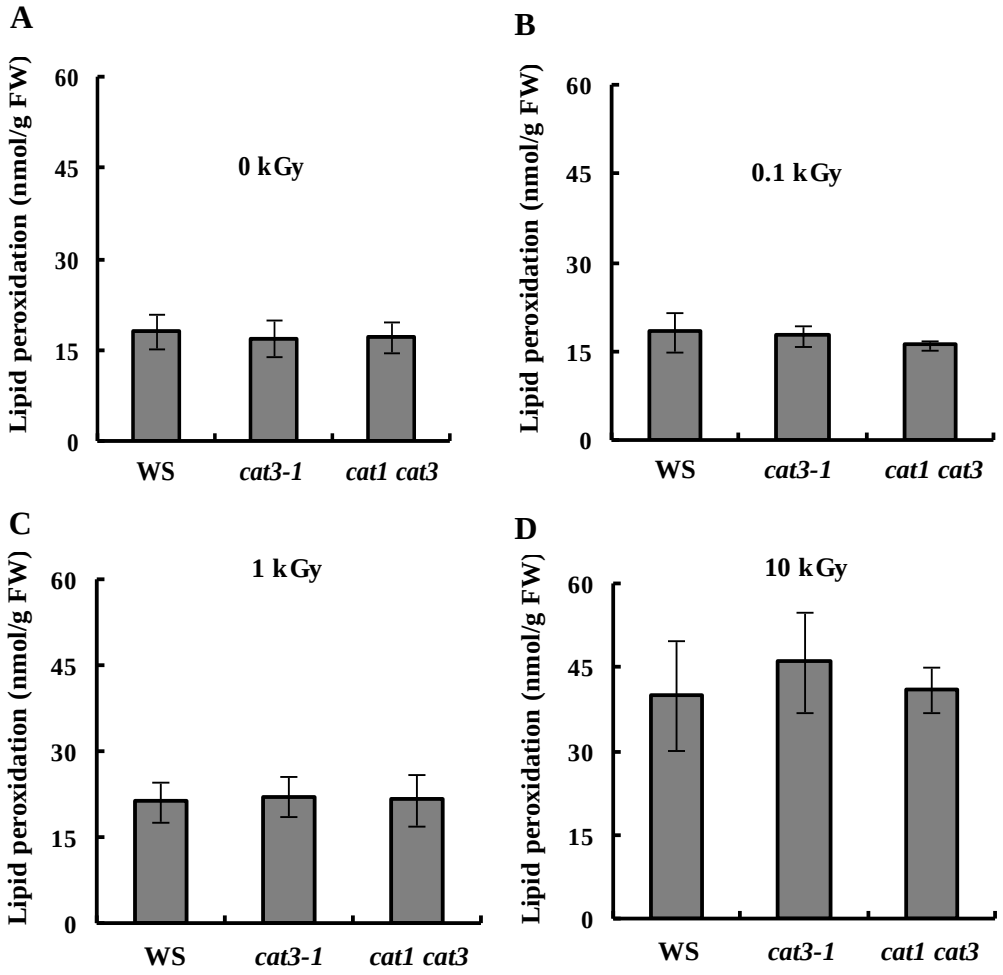


Fig. 2.8 Lipid peroxidation (A, B, C, and D) in Arabidopsis rosette leaves irradiated by gamma rays.

Rosette leaves of wild type, *cat3-1* and *cat1 cat3* mutant plants were irradiated with gamma rays at 0 kGy (A), 0.1 kGy (B), 1 kGy (C) and 10 kGy (D). Lipid peroxidation was evaluated using thiobarbituric acid. Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.8 Lipid peroxidation in Arabidopsis whole leaves incubated without radiation

Consequently, leaves were irradiated for 1 h, 10 h, and 100 h for irradiation of 0.1 kGy, 1 kGy, and 10 kGy, respectively. I investigated the effects of incubation period of 1 h, 10 h, and 100 h on Lipid peroxidation in the wild type and both munants. Incubation for up to 100 h without irradiation did not affect lipid peroxidation in the wild type, *cat3-1*, and *cat1 cat3* mutants.

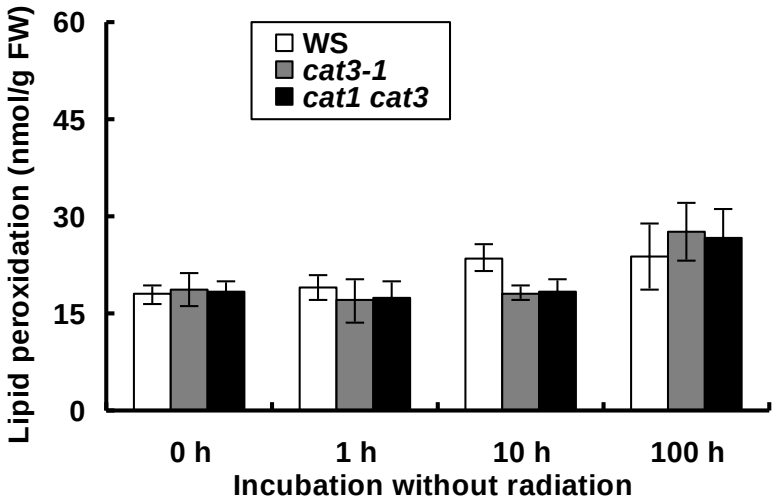


Fig. 2.9 Lipid peroxidation in Arabidopsis rosette leaves incubated without radiation.

Rosette leaves of wild type, *cat3-1* and *cat1 cat3* mutant plants were incubated without gamma irradiation at 0 h, 1 h, 10 h, and 100 h. Lipid peroxidation was evaluated using thiobarbituric acid. Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.9 The mRNA levels of the catalase isoforms in response to gamma irradiation

I examine the mRNA levels of the catalase isoforms in response to gamma irradiation. Reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from whole leaves as the template showed that *CAT1*, *CAT2*, and *CAT3* were expressed in whole leaves at doses of 0.1 kGy, and 1 kGy gamma irradiation. Diurnal

regulation of *CAT1* and *CAT2* mRNA abundance was apparent in all conditions and day/night *CAT1* and *CAT2* expression patterns were modified by mild and severe drought (Luna et al., 2004). The abundance of *CAT1* transcripts was regulated by circadian controls that persisted in continuous darkness, while *CAT2* was modulated by light. Drought decreased abundance, and modified the pattern, of *CAT1* and *CAT2* mRNAs (Luna et al., 2004).

It is shown that the expression of *CAT1* and *CAT2* is modulated by light in wheat as it is in maize and Arabidopsis (McClung, 1997). Wheat *CAT1* expression shows characteristics of circadian control, as indicated by the persistence of the rhythm in darkness, and its expression pattern is equivalent to the clock-regulated maize and Arabidopsis *CAT2* genes (Zhong et al., 1994). Instead, *CAT2* expression does not appear to be clock-regulated in wheat, similar to results reported for maize *CAT2* and Arabidopsis *CAT1* (McClung et al., 1997). *CAT2* expression is down regulated during leaf senescence, whereas *CAT3* expression is induced by age and senescence (Zimmermann et al., 2006; Xing et al. 2007). Interestingly, the mRNA abundance of CAT is also accumulated under drought, abscisic acid (ABA) and salt treatments (Xing et al. 2007). *CAT1* and *CAT2* transcript abundance is highest in the light and broadly correlated with H_2O_2 formation in photorespiration.

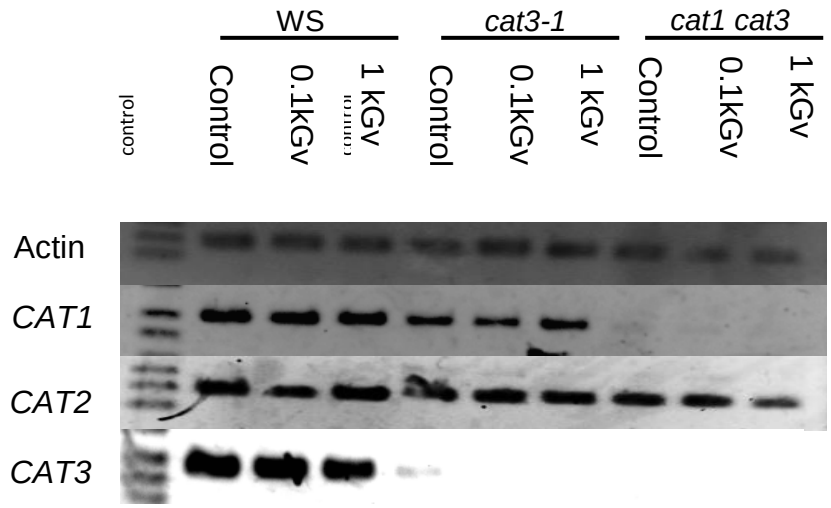


Fig. 2.10 The mRNA levels of the catalase isoforms in response to gamma irradiation.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *CATALASE* mRNAs; *CAT1*, *CAT2* and *CAT3* in whole leaves of *Arabidopsis*.

Rosette leaves of wild type, *cat3-1* and *cat1 cat3* mutant plants were irradiated with gamma rays at 0 kGy, 0.1 kGy, and 1 kGy.

2.5 Discusstion

Catalase activities of non-irradiated wild-type were higher than those of non-irradiated *cat3-1* and *cat1 cat3* mutants (Fig. 2.1 A), in accordance with previous results (jannat et al., 2011). Moreover, catalase activities of the non-irradiated *cat1 cat3* mutants was as high as those of the non-irradiated *cat3-1* mutants (Fig. 2.1), suggesting that CAT1 is not expressed in rosette leaves, in agreement with previous results (Queval et al., 2007). Catalase activities were increased by irradiation at 0.1 kGy in the wild type

and both mutants and were decreased by irradiation at 10 kGy (Fig. 2.1), which is similar to previous results (Martinez-Solano et al., 2005). When leaves were irradiated at 0.1 kGy and 1 kGy, catalase activities of the wild type were higher than those of the *cat3-1* and *cat1 cat3* mutants, but when at 10 kGy, there were no significant differences in catalase activities among the wild type and both mutants (Fig. 2.1).

Double-strand DNA break is one of the most detrimental damage caused by ionizing radiation in plant cells (West et al., 2000). Gamma rays can damage DNA by directly ionizing DNA itself and by indirect processes in which DNA reacts with creating free radicals (O'Neill et al., 1993). H_2O_2 can induce double-strand breaks (Driessens et al., 2009). Compared with DNA damage level of non-irradiation groups, irradiation at up to 1 kGy did not significantly increase DNA damage level in the wild type and *cat3-1* and *cat1 cat3* mutants (Fig. 2.3). In tobacco protoplasts, gamma irradiation-induced double-strand break was linearly increased from 0.2 kGy to 0.8 kGy, (Yokota et al., 2005) suggesting that protoplasts are more sensitive to gamma irradiation than intact cells, which may be accounted for by the absence of cell wall. Irradiation at 10 kGy significantly elevated DNA damage level in the wild type and both mutants (Fig. 2.3). There were no significant differences in DNA damage level among the wild type and both mutants.

Hydrogen peroxide accumulation and lipid peroxidation levels were not significantly increased by irradiation at 0.1 kGy or 1 kGy (Fig. 2.6 A,B and 2.8 A,B) but were significantly increased by irradiation at 10 kGy (Fig. 2.6 D and 2.8 D). There

were no significant differences in H_2O_2 accumulation or lipid peroxidation level among the wild-type and both mutants (Fig. 2.6 and 2.8). Hence, scavenging of H_2O_2 may not be attributed to catalases.

Neither DNA damage levels (Fig. 2.3) nor H_2O_2 accumulation (Fig. 2.6) was changed by gamma irradiation at up to 1 kGy despite differences of catalase activities (Fig.2.1), whereas DNA damage levels (Fig. 2.3) and H_2O_2 accumulation (Fig.2.6) were increased by gamma irradiation at 10 kGy along with reduction of catalase activities in wild type and catalase deficient mutants (Fig. 2.1).

Taken together, CAT1 and CAT3 may not be key enzymes to protect cellular damage induced by gamma irradiation in Arabidopsis, while another catalase isoform, CAT2, may play a crucial role in response to gamma irradiation because CAT2 rather than CAT1 or CAT3 more considerably contributes to the oxidative stress tolerance (Queval et al., 2007; Hu et al., 2010).

CHAPTER 3

Catalase, CAT2, is not involved in mitigation of gamma irradiation-induced H_2O_2 accumulation or lipid peroxidation in *Arabidopsis thaliana*

3.1 Abstract

In Arabidopsis wild type (Col-0 ecotype) and *cat2* mutant plants, gamma radiation induced H_2O_2 accumulation and lipid peroxidation at 10 kGy but not at 0.1 to 1 kGy. Hence, CAT2 may not be a key enzyme to protect gamma irradiation-induced damage.

3.2 Introduction

Gamma irradiation affects growth and development due to cytological, genetical, biochemical, physiological and morphogenetic changes in cells and tissues of plants (Gunckel JE and Sparrow AH, 1961). Gamma irradiation induces hydrogen peroxide accumulation and lipid peroxidation (Yokota et al., 2005). Catalase is one of antioxidant enzymes to scavenge H_2O_2 and irradiation changes catalase activities (El-Beltagi et al., 2011). Hence, it is thought that increasing activity of catalases is favorable to alleviation of damage caused by irradiation (Martinez-Solano et al., 2005). In *Arabidopsis*, three CAT genes (*CAT1*, *CAT2*, and *CAT3*) and six enzymatically distinguishable isoforms have been identified (Frugoli et al., 1996). The enzyme activity and expression levels of CAT2 were highest among CATs in the leaves (Mhamdi et al., 2010). In cat2 knockouts,

leaf catalase activity is only about 10% of Col-0 wild-type plants (Mhamdi et al., 2010) .Double *cat2 cat1* and *cat2 cat3* mutants have similar decreases in leaf catalase activity to those in *cat2* and CAT2 are the major isoforms in Arabidopsis rosette tissue (Mhamdi et al., 2010). While the relative contribution of the different genes to overall leaf catalase activity changes with the developmental stage of the plant (Zimmermann et al., 2006), the approximately additive nature of cat2 (90% decrease in leaf activity) and cat3 (20% decrease in leaf activity) mutations suggests that the formation of hetero-oligomeric proteins from more than one catalase gene product is a minor phenomenon in vivo. However, it remains to be clarified roles of catalases in irradiated plants. We examined effects of gamma irradiation on Arabidopsis catalase-deficient mutants, *cat2* to elucidate roles of catalases in responses to gamma irradiation.

3.3 Materials and Methods

3.3.1 Plant materials and growth conditions

Arabidopsis thaliana wild type (WT) (ecotype Columbia-0) and cat2 (salk_076998) plants, were grown in a growth chamber following previous method discussed in chapter2.

3.3.2 Measurement of H_2O_2 accumulation in whole leaves

H_2O_2 accumulation was measured as described previously in chapter 2 (Hossain et al., 2013).

3.3.3 Measurement of lipid peroxidation

Lipid peroxidation were measured as described previously in chapter 2 (Dhinsha et al., 1981).

3.3.4 Statistical Analysis

Significance of differences between mean values was assessed by Student's t-test or one-way ANOVA with Dunnett's test. Differences at $p < 0.05$ were considered significant.

3.4 Results

3.4.1 Gamma radiation-induced H_2O_2 accumulation in whole leaves of wild type and *cat2* mutants

An increase in the endogenous H_2O_2 level has been reported to be associated with the promotion of leaf senescence (Mondal and Choudhuri, 1981). I examined the gamma radiation-induced H_2O_2 accumulation in whole leaves of Arabidopsis. Irradiation at 0 kGy and 0.1 kGy did not significantly increase H_2O_2 accumulation in the wild type and *cat2* mutants. Irradiation at 1 kGy also did not significantly increase H_2O_2 accumulation in the wild type, and *cat2* mutants. Irradiation at 10 kGy significantly increases H_2O_2 accumulation in the wild type and *cat2* mutants and there are no significant differences in H_2O_2 accumulation among the wild type and mutants. These results indicate that H_2O_2 accumulation increased by irradiation at 10 kGy but not at

0.1 kGy or 1 kGy in the wild type and *cat2* mutants. These results agreement with the previous results, it has been reported that, the concentration of H_2O_2 increased in various pumpkin tissues sush as leaves, petioles and hypocotyls after gamma-irradiation (Wi et al., 2007). There were no significant differences in H_2O_2 accumulation, among the wild type and *cat2* mutants at doses of 10 kGy gamma radiation. Hence, scavenging of H_2O_2 may not be attributed to catalases.

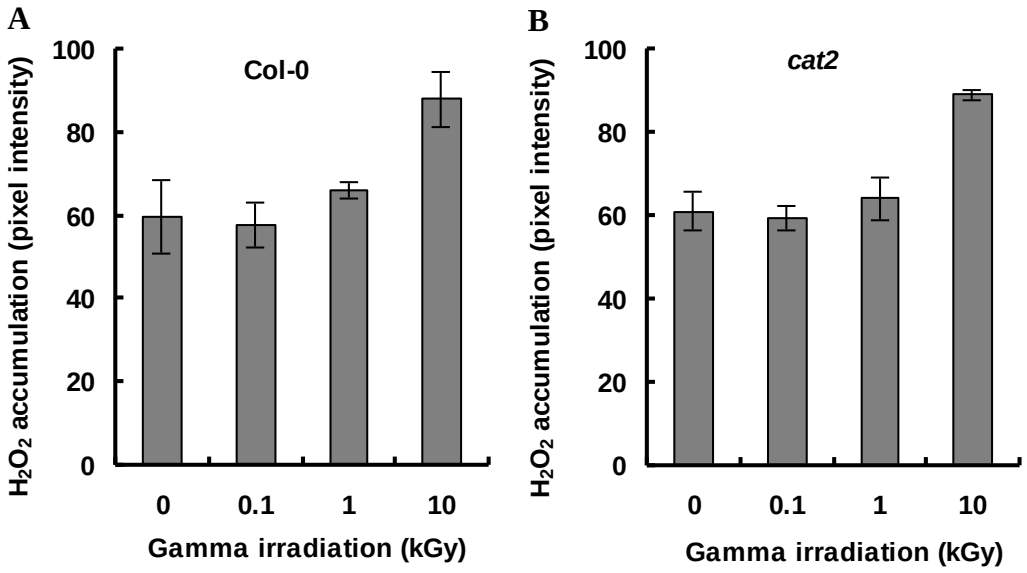


Fig. 3.1 Accumulation of H_2O_2 (A and B) in Arabidopsis rosette leaves irradiated by gamma rays.

A-B) Rosette leaves of wild type and *cat2 m*utant plants were irradiated with gamma rays at 0 kGy, 0.1 kGy, 1 kGy, and 10 kGy. H_2O_2 accumulation was detected by 3,3'-diaminobenzidine Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

3.4.2 H_2O_2 accumulation in whole leaves of wild type and *cat2* mutants incubated without irradiation

Consequently, leaves were irradiated for 1 h, 10 h, and 100 h for irradiation of 0.1 kGy, 1 kGy, and 10 kGy, respectively. I investigated the effects of incubation period of 1 h, 10 h, and 100 h on H_2O_2 accumulation in the wild type and *cat2* munants. Incubation for up to 100 h without irradiation did not affect H_2O_2 accumulation in the wild type and *cat2* mutants.

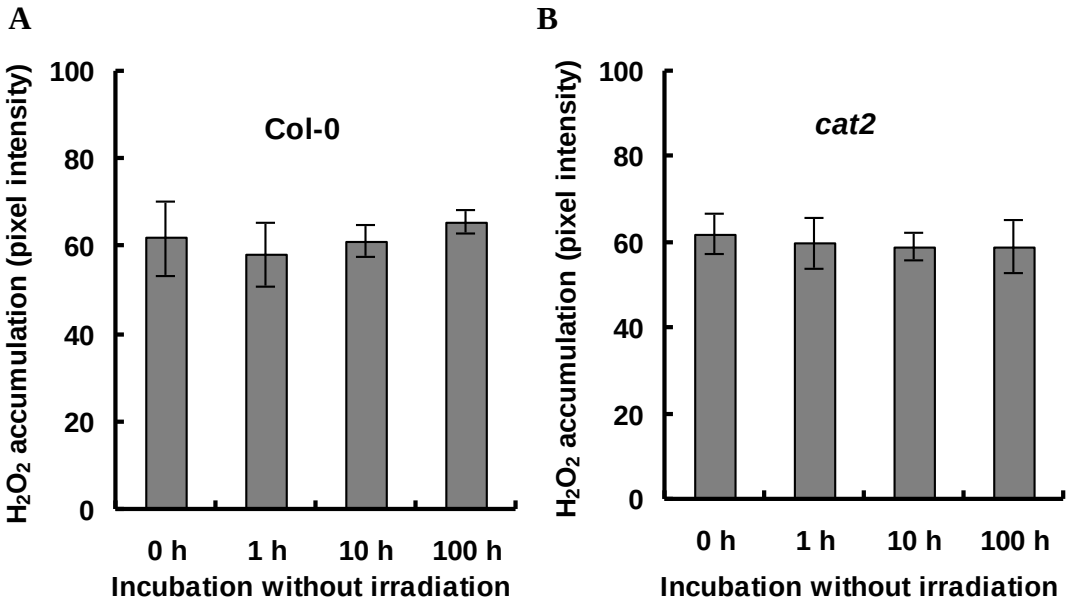


Fig. 3.2 H_2O_2 accumulation in Arabidopsis rosette leaves incubated without radiation.

Rosette leaves of wild type and *cat2* mutant plants were incubated without gamma irradiation at 0 h, 1 h, 10 h, and 100 h. H_2O_2 accumulation was detected by 3,3'-diaminobenzidine. Averages for three independent experiments are shown. Error

bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

3.4.3 Lipid peroxidation induced by gamma irradiation in wild type and *cat2* mutants

The lipid peroxidation contents (MDA) of the irradiated samples were significantly higher than that of control (El-Beltagi H. S. 2011). I examined the lipid peroxidation induced by gamma irradiation in whole leaves of Arabidopsis. Under control condition there were no changes in lipid peroxidation in the wild type, and *cat2* mutants. Irradiation at 0.1 kGy, there were no changes in lipid peroxidation in the wild type and mutants compare with control. Irradiation at 1 kGy, there were no changes in lipid peroxidation in the wild type, and *cat2* mutants.

Irradiation at 10kGy significantly increases lipid peroxidation in the wild type plant, and *cat2* mutant compare with control and there were no significant difference in lipid peroxidation among the wild type and *cat2* mutant at 10 kGy. These results indicate that lipid peroxidation increased by irradiation at 10 kGy but not at 0.1 kGy or 1 kGy in the wild type and *cat2* mutants. There were no significant differences in lipid peroxidation levels among the wild type, and *cat2* mutants at doses of 10 kGy gamma irradiation. The observed changes in the MDA content were consistent with the previous results observed by Fu and Huang (2001), El-Beltagi et al. (2008), Salama et al. (2009) and Aly and El-Beltagi (2010); the authers stated that an enhanced level of

lipid peroxidation of grasses, flax and *Vicia faba*, under environmental stress (drought, Fe deficiency, salt stress and radiation) indicated oxidative damage to plants; it means lipid peroxidation may be a consequence of generation of reactive oxygen species (OH, O_2, and H_2O_2).

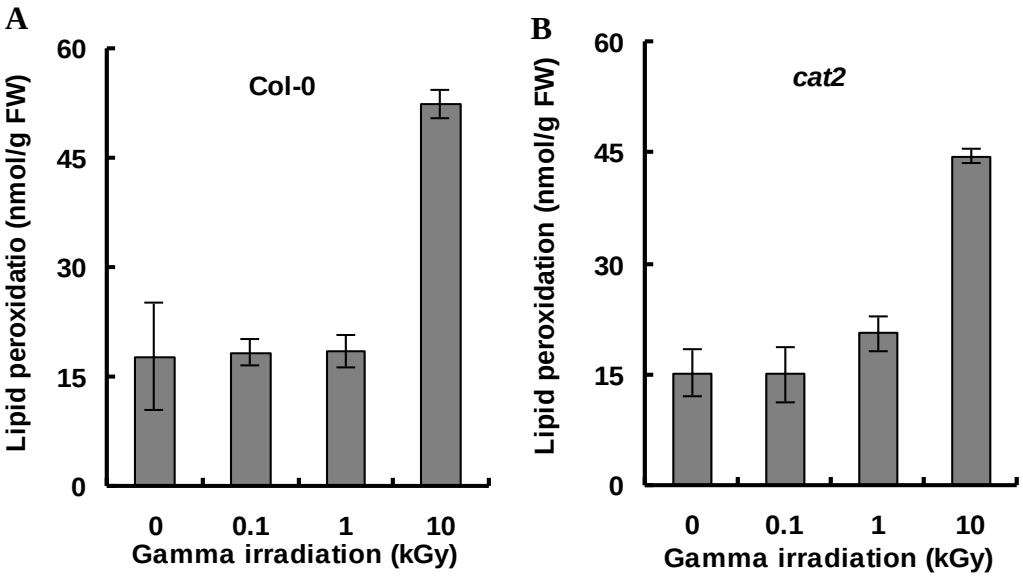


Fig. 3.3 Lipid Peroxidation (A and B) in Arabidopsis rosette leaves irradiated by gamma rays.

A-B), rosette leaves of wild type and *cat2* mutant plants were irradiated with gamma rays at 0 kGy, 0.1 kGy, 1 kGy, and 10 kGy; Lipid peroxidation was evaluated using thiobarbituric acid. Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

3.4.4 Lipid peroxidation in whole leaves of wild type and *cat2* mutants

incubated without radiation

Consequently, leaves were irradiated for 1 h, 10 h, and 100 h for irradiation of 0.1 kGy, 1 kGy, and 10 kGy, respectively. I investigated the effects of incubation period of 1 h, 10 h, and 100 h on lipid peroxidation in the wild type and both munants. Incubation for up to 100 h without irradiation did not affect lipid peroxidation in the wild type and *cat2* mutants.

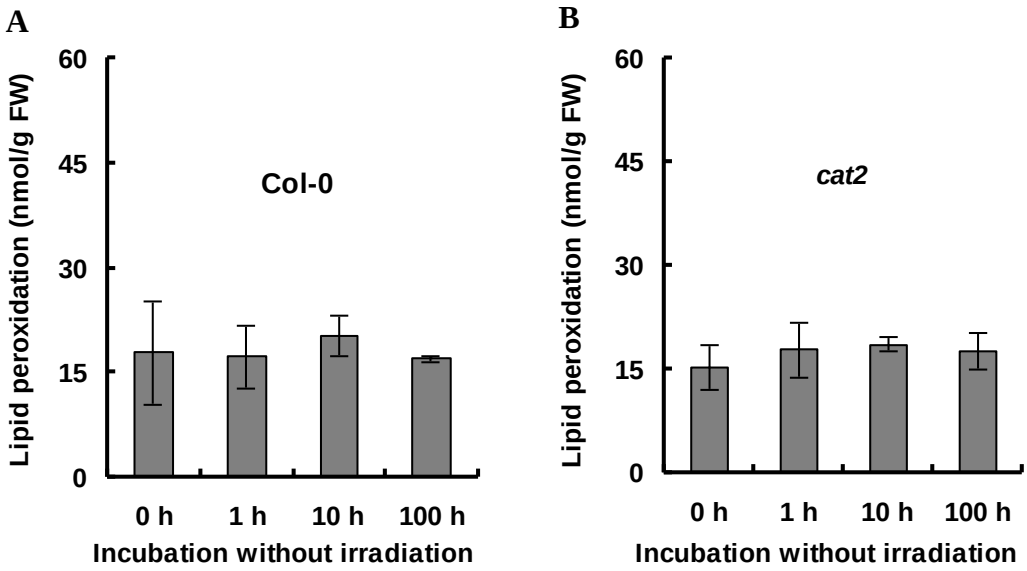


Fig. 3.4 Lipid peroxidation (A and B) in Arabidopsis rosette leaves irradiated by gamma rays. A-B), rosette leaves of wild type and *cat2 m*utant plants were incubated for 0 h, 1h, 10h, and 100h. Lipid peroxidation was evaluated using thiobarbituric acid. Averages for three independent experiments are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

3.5 Discussion

CAT2 is one of three catalase isoforms in *Arabidopsis thaliana* and is more dominant than CAT1 and CAT3 (Queval et al., 2007). Moreover, CAT2 rather than CAT1or CAT3 more considerably contributes to the oxidative stress tolerance (Queval et al., 2007; Hu et al., 2010). However, this study suggests that CAT2 is not a key enzyme to mitigate H_2O_2 accumulation and lipid peroxidation induced by gamma irradiation in Arabidopsis.

We recently have reported that CAT1 and CAT3 are not key enzymes to protect cellular damage induced by gamma irradiation in *A. thaliana* (Sultana et al 2013). Hence, catalases may not be key enzymes to protect cellular damage induced by gamma irradiation although it is thought that increasing activity of catalases is favorable to alleviation of damage caused by irradiation (Driessens et al., 2009).

General summary

Gamma radiation, composed of high energy photons, is an important type of ionizing radiation capable of penetrating and interacting with plants. Gamma irradiation affects growth and development due to cytological, genetical, biochemical, physiological and morphogenetic changes in cells and tissues of plants. Gamma irradiation induces DNA damage and increases hydrogen peroxide accumulation and lipid peroxidation. Catalase is one of antioxidant enzymes to scavenge H_2O_2 and irradiation changes catalase activities. Hence, it is thought that increasing activity of catalases is favorable to alleviation of damage caused by irradiation. However, it remains to be clarified functions of catalases in irradiated plants.

Catalase allow plant cells to remove H_2O_2 energy-efficiently because CAT decomposed H_2O_2 without consuming cellular reducing equivalents. The Arabidopsis genomecontains three CAT genes, *CAT1, CAT2, CAT3,* which are differentially expressed and can form up to six different isozymes.

I examined the effects of gamma irradiation on Arabidopsis catalase-deficient mutants, *cat3-1, cat2,* and *cat1 cat3* to elucidate functions of catalases in responses to gamma irradiation. In chapter 2, I investigeted the functions of CAT1 and CAT3 in response to gamma irradiation-induced DNA damage, H_2O_2 accumulation, and lipid peroxidation in Arabidopsis. Gamma irradiation at 0.1 kGy but not at 1 kGy increased catalase activities and irradiation at 10 kGy decreased catalase activities in the wild type, *cat3-1,* and *cat1 cat3* mutants. There were no significant differences in catalase

activity among the wild type, *cat3-1*, and *cat1 cat3* mutants at doses of 10 kGy gamma irradiation.

Gamma irradiation-induced DNA damage significantly elevated at doses of 10 kGy but not at 0.1 kGy and 1 kGy in the wild type, *cat3-1*, and *cat1 cat3* mutants. H_2O_2 accumulation increased by irradiation at 10 kGy but not at 0.1 kGy or 1 kGy in the wild type, *cat3-1*, and *cat1 cat3* mutants. Lipid peroxidation increased by irradiation at 10 kGy but not at 0.1 kGy or 1 kGy in the wild type, *cat3-1*, and *cat1 cat3* mutants. There were no significant differences in catalase activities, DNA damage, H_2O_2 accumulation, and lipid peroxidation levels among the wild type, *cat3-1*, and *cat1 cat3* mutants at doses of 10 kGy gamma irradiation.

I also investigated the effects of incubation period of 1 h, 10 h, and 100h on catalase activities, DNA damage, H_2O_2 accumulation, and lipid peroxidation in Arabidopsis. Incubation for up to 100 h without irradiation did not affect catalase activities, DNA damage, H_2O_2 accumulation, or lipid peroxidation in the wild type, *cat3-1*, and *cat1 cat3* mutants. I examined the mRNA levels of the catalase isoforms in response to gamma irradiation in Arabidopsis catalase-deficient mutants, *cat3-1* and *cat1 cat3*. Reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from whole leaves as the template showed that *CAT1*, *CAT2*, and *CAT3* were expressed in whole leaves of wild type as well as *cat3-1* and *cat1 cat3* mutants at doses of 0.1 kGy and 1 kGy gamma irradiation. These findings indicate that catalases, CAT1

and CAT3, did not alleviate gamma irradiation induced DNA damage, H_2O_2 accumulation, or lipid peroxidation in *Arabidopsis thaliana*.

In chapter 3, I examined the functions of CAT2 in response to gamma irradiation-induced H_2O_2 accumulation and lipid peroxidation in Arabidopsis. H_2O_2 accumulation significantly increased by irradiation at 10 kGy but not at 0.1 kGy or 1 kGy in the wild type and *cat2* mutants. Lipid peroxidation levels significantly increased by irradiation at 10 kGy but not at 0.1 kGy or 1 kGy in the wild type and *cat2* mutants. There were no significant differences in H_2O_2 accumulation and lipid peroxidation levels between the wild type and *cat2* mutants at doses of 10 kGy gamma irradiation. I also investigated the effects of incubation period of 1 h, 10 h, and 100h in H_2O_2 accumulation and lipid peroxidation in Arabidopsis. Incubation for up to 100 h without irradiation did not affect H_2O_2 accumulation, or lipid peroxidation in the wild type, and *cat2* mutants. These results indicate that catalase, *CAT2*, did not mitigate gamma irradiation-induced extracellular H_2O_2 accumulation and lipid peroxidation in whole leaves of Arabidopsis.Thus, my findings indicate that catalases are not key enzymes to protect cellular damage induced by gamma irradiation in Arabidopsis.

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