RESEARCH PAPER

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Neutron irradiation affects the expression of genes involved in the response to auxin, senescence and oxidative stress in Arabidopsis

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Abbreviations: ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; qPCR, quantitative polymerase chain reaction

We report, in *Arabidopsis thaliana* plants, the effect of neutron irradiation on the transcription of a set of genes belonging to different physiological groups: auxin action, senescence, oxidative stress and some aspects of photosynthesis. The results indicated that, in the wild-types, the effect on the *ARF1, ARF2* and *19* genes was of downregulation, whereas of the tested *AUX/IAA* only *AUX/IAA7* showed upregulation. Different results were obtained as regards the irradiation of the auxin transport mutants *aux1* and *eir1* because, in these cases, the *ARF* genes were upregulated, whereas *AUX/IAA7* was downregulated in *eir1*. On the other hand, the senescence activated genes *SAG12* and *SAG13*, and those connected to oxidative stress were upregulated in the wild-type, but downregulated in *aux1*. The gene *CAB1*, connected to photosynthesis, was also downregulated in the wild-type, but upregulated in *aux1*. Gene expression recovered in many cases almost to the initial condition in a time lapse of 24 hours, even though some effect persisted for a longer time. In particular, the state of juvenility of *arf2* was extended by irradiation, whereas, in all the other cases, senescence was accelerated. The research indicates that through mutant selection or genetic engineering a true possibility exists of producing organism more suitable for life in space.

Introduction

The literature regarding the effect of radiation on plants is not very large, even though its volume is increasing in the quest for extended space flights. However most of the available literature, regards the effect of ionising radiation,[1,2] and not that of neutrons,[3] even though this kind of particles pose a serious problem to the human flights, because so far no significant protection has been found from them.[4,5] On the other hand, intensive ionising radiation has been shown to induce variations in the general physiological processes in plants, including respiration, ethylene emission, accumulation of sucrose, and photosynthesis.[6-9] In particular, as regards the literature on Arabidopsis, some large scale analyses on the variations of expressed genes after irradiation with ionising agents, have been reported.[9-11] In these cases most of the altered genes were involved in cell growth and division, development, signal transduction and stress related responses. In addition, high doses of radiation have been shown to induce the activation of genes involved in the biosynthesis of enzymes that scavenge the toxic accumulation of reactive oxygen species (ROS),[12,13] which in turn cause enhanced lipid and protein oxidation,[8,14-16] and specific morphology changes in plants.[17] On the basis of the presently available data, it appears that the study of the effect of radiation on plants could lead to a better knowledge of the mechanisms that induce general stresses,[18] and to the adoption of countermeasures. In this work, we concentrated on studying the effect of radiation on genes activated by the phytohormone auxin, which controls a large part of the morphogenetic processes in plants,[19] and, at the same time, of genes involved in the activation of defence against stress and senescence. To overcome stress plants have developed highly-efficient defence systems, involving both enzymatic and non-enzymatic constituents. Whereas lipophylic antioxidants (tocopherols and carotenoids) are active in cell membranes, in the enzymatic ROS-scavenging pathways superoxide dismutase (SOD) converts O_2^- directly to hydrogen peroxide (H_2O_2), and catalase (CAT) is one of the main H_2O_2 scavenging enzymes resulting in its dismutation into water and O_2.[20-22] The role of ROS in stress biology is complex, involving oxidative damage, as well as signalling responses.[15] In general, the antioxidant status is reduced during senescence: the level of ROS is enhanced, many antioxidant enzymes show reduced activity[21] and the level of lipid peroxidation increases.[23]

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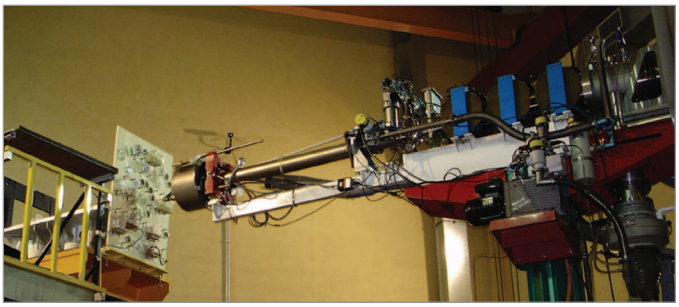

Figure 1. Experimental system used for neutron irradiation of Arabidopsis plants. Arabidopsis plants (15-day-old seedlings in Petri dishes and 35-day-old plants in pots) were mounted on a vertical panel and irradiated with neutrons at the Frascati Neutron Generator (FNG). The distances between plant samples and the generator was selected to deliver three different absorbed doses of neutrons: 30, 50 and 76 mGy.

In this paper we present the first data on the effect of neutron irradiation, administered at three different energy levels on Arabidopsis plants. The effect was measured by *real-time* PCR on the expression of genes involved in the movement and action of auxin, in senescence, under oxidative stress, and in photosynthesis, both in the wild-type and in two auxin mutants. In addition, we measured the production of thiobarbituric acid reactive substances (TBARS), the chlorophyll content and chlorophyll fluorescence, after exposing plants to neutron irradiation. The results showed notable variations in up or downregulation of the expressed genes, as well as showing the possibility of recovery of the plants within a few days of the irradiation.

Results

The effect of neutron irradiation on plant senescence was studied by treating both Arabidopsis seedlings (15-day-old) and mature plants (35-day-old), from two ecotypes (Col and Ws), and two auxin transport mutants (*aux1* and *eir1*), with different doses of radiation, i.e., 30, 50 and 75 mGy at the Frascati Neutrons Generator (FNG, Frascati, Rome, Italy) for 3 hours. The treatment was replicated five times under the same conditions (see Materials and Methods). Expression profiles of genes involved in specific metabolic pathways was determined by quantitative *real-time* qPCR analysis. The analyzed genes, about which we report only the effect of irradiation at 50 mGy, behaved similarly at the other dosages employed (30, 75 mGy), can be divided into four different groups: auxin connected (*ARF1, ARF2, ARF19, AUX/IAA3, AUX/IAA6, AUX/IAA7, AUX1, EIR1*), senescence connected (*SAG12* and *SAG13*), oxidative stress response connected (*CAT1* and *CAT3,* Fe*SOD1*), and photosynthesis connected (*CAB1, RUBS-1B*).

We chose to study particularly the effect of radiation on auxin connected genes, in consideration of the fundamental role played by this hormone in the morphogenesis and metabolism of plants.[24] Three of the most significant *ARF* genes were chosen, i.e., *ARF1, ARF2* and *ARF19*, since a recent study revealed their involvement in various aspect of plant development and physiology.[25-27] We also chose three of the most significant *AUX/IAA* genes, i.e., *AUX/IAA3, AUX/IAA6* and *AUX/IAA7*,[28] whose mutants, respectively, are *shy1*, *shy2*,[29] and *axr2*.[30] In our experiments (**Fig. 2**), after neutron irradiation, the gene expression of the auxin response factors *ARF1, ARF2* and *ARF19* was downregulated, in the wild-type, as well as the expression of the auxin-activated genes *AUX/IAA3*, *AUX/IAA6*, with the exception of *AUX/IAA7*, that was consistently upregulated. In contrast, a different response was seen in the auxinic mutant *aux1*, whose mutated gene was shown to be a facilitator of the auxin influx into cells.[31,32] In the *aux1* background, in fact, all the auxin response factors analyzed (*ARF1, ARF2* and *ARF19*) were upregulated after the treatment, whereas of the *AUX/IAAs* only *AUX/IAA7* was clearly upregulated.

The other Arabidopsis mutant on which the effect of neutron radiation was investigated was *eir1/pin2/agr1/wav6*. This is considered a mutant of the auxin efflux facilitator protein from the cells.[33] Similar to *aux1*, the mutant *eir1* also showed an upregulation of the auxin response factors *ARF1, ARF2* and *ARF19*, whereas *AUX/IAA7* showed strong downregulation after irradiation.

On the other hand, the genes *SAG12* and *SAG13* (**Fig. 3A–C**) considered to be involved in senescence processes, were significantly upregulated in their transcription in the wild-type, whereas in the mutant *aux1* they were downregulated (especially *SAG13*). In this case, however, in the mutant *eir1* upregulation was seen. The research was then extended to genes involved in processes connected with stress, i.e., *CAT1, CAT3* and Fe*SOD1*, which, as reported in **Figure 3D–F**, are conspicuously upregulated in the wild-type and in the mutant *eir1*. In contrast in the mutant *aux1* all the above considered genes were downregulated.

To study the effect of neutron radiation, in addition to the mRNA expression analysis, on the general process of senescence, some specific biochemical tests were performed, namely a test on the lipidic state of the membranes, i.e., a measure of the level of the thiobarbituric acid reactive substances (TBARS) in the wild-type (**Fig. 4A**), and a test on the efficiency of the photosynthetic system II performed on wild-type Col leaves (**Fig. 4B**). Wild-type plants displayed an increase of the TBARS content after the treatment at 50 mGy neutron dose with respect to the controls. On the other hand, chlorophyll fluorescence (Fv/Fm ratio) measurements on wild-type leaves showed a significant decrease of the Fv/Fm ratio (*ca* 15%) compared to the controls (**Fig. 4B**). These results suggest, that wild-type Arabidopsis plants are negatively affected by neutron radiation at the molecular and physiological level, and that neutrons are able to induce damage even at relatively low doses. In addition, the genes *CAB1* and *RUBS-1B* were downregulated showing, with the exception of *aux1*, negative effects of irradiation on the photosynthetic apparatus (**Fig. 4C**).

Early recovery from neutron irradiation. The possibility of recovery from the effects of neutron irradiation was studied in the plants as early as 12 and 24 hours after the treatment. The data showed a consistent recovery of the transcripts in all the considered genes (**Figs. 2–4**). This however was more evident in the wild-type in the case of *ARF1, ARF19, AUX/IAA7,*

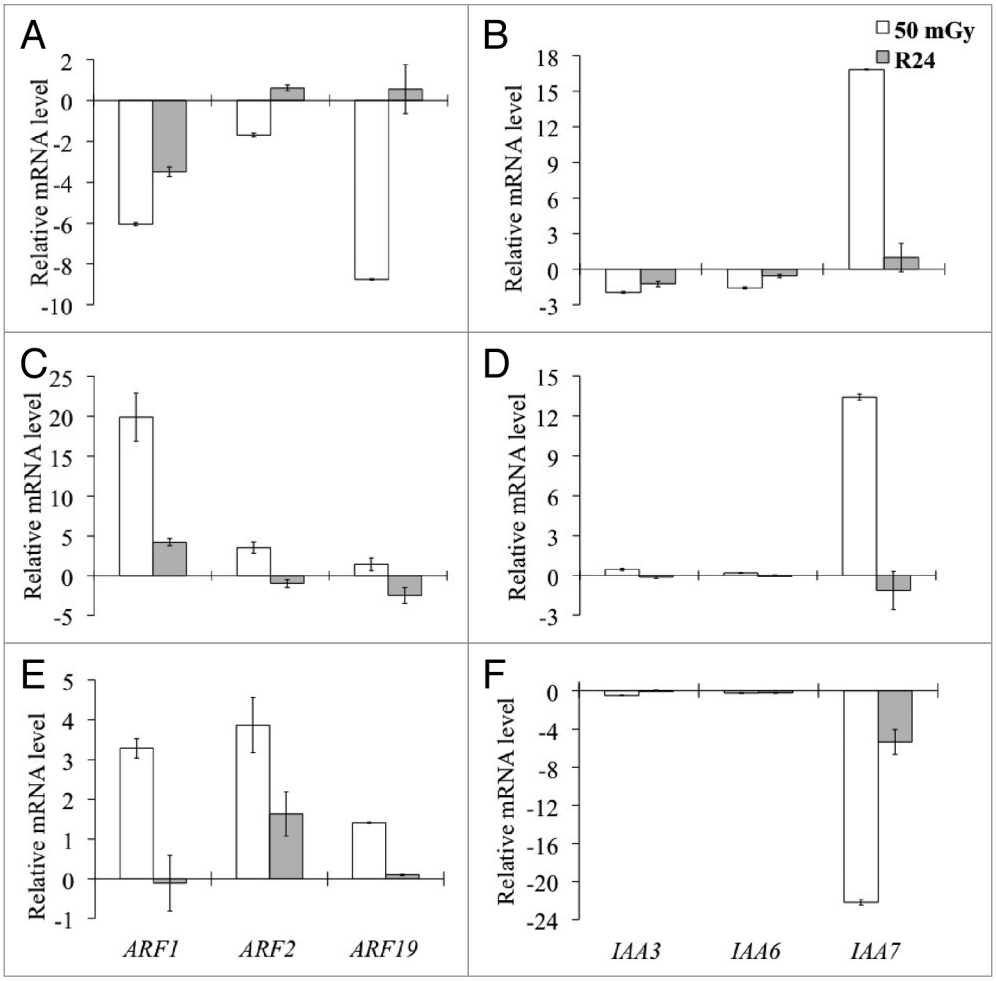

Figure 2. Neutrons affect the expression of different auxin and senescence related genes in Arabidopsis plants. To study the effect of neutron radiation, 35-d-old plants, were irradiated with different doses of neutrons (30, 50 and 76 mGy). The expression profile of auxin response factors (*ARF1, 3, 19*), and auxin activated genes (*AUX/IAA3, 6, 7*), are shown in wild-type Col plants (A and B), together with that of two Arabidopsis auxin transport mutants: *aux1* (C and D), and *eir1* (E and F). Note = this assay was performed by a quantitative real time qPCR analysis 1 hour after the end of neutron irradiation at three different doses: 30, 50 and 76 mGy, and after 24 h of recovery (R24, gray bars). Only results obtained at 50 mGy (gray bars) neutron dose are shown. Relative amounts were normalized with respect to *ACTIN8* expression level as log2 of the relative mRNA level, calibrated to the not-treated control plants (= 0). Log2 < 0 represents a downregulation, and log2 > 0 represents an upregulation of gene expression. Bars represent the means SD (n = 4–7).

SAG13, CAT3, CAB1. In the mutant *aux1* with *ARF1*, *AUX/IAA7, SAG13, CAT3,* Fe*SOD1, CAB1*, and in the mutant *eir1* regarding *ARF1, ARF2, ARF19, AUX/IAA7, CAT3,* Fe*SOD1* and *CAB1*. The effect was gradual, i.e., more visible at 24 hours than at 12. This seems to indicate that recovery mechanisms, even after exposures of as little as 3 hours were already at work.

Late effects of neutron irradiation. To investigate the late effects of neutron irradiation, both wild-type (Col and Ws), and the auxinic mutants *eir1*, *arf1-3* and *arf2-6* plants were irradiated at 50 mGy and left to grow firstly for 20 days, and then for 4 further weeks in the growth chamber together with controls. At the end of the 20 days, the plants were analyzed for the expression of senescence activated genes *SAG12* and *SAG13*, and lipid peroxidation efficiency by measuring the TBARS content (**Fig. 5**). After 20 days of recovery the irradiated plants showed no apparent specific phenotypic alteration, even though biochemical and molecular parameters suggested increased oxidative stress and senescence. In fact, 20 days after the treatment, *SAG12* and *SAG13* were significantly upregulated with respect to non-treated plants. This was especially marked in the wild-type, and less in the mutants *eir1* (in this case only *SAG13*), and *arf1-3*. In contrast, the mutant *arf2-6* after recovery showed a downregulation of both the senescence genes *SAG12* and *SAG13*. On the other hand, lipid peroxidation status, displayed a small increase in the wild-type and *arf1-3* mutant, whereas this value was larger in the mutant *eir1*, and especially in the mutant *arf2-6*.

Four weeks after the irradiation however, all the irradiated plants also started to show phenotype effects (**Fig. 6**), i.e., an accelerated senescence, shown by the earlier flowering and seed formation with respect to controls. The only exception was the mutant *arf2-6* that remained in the rosette condition, i.e., showed an increased delay of senescence after irradiation.

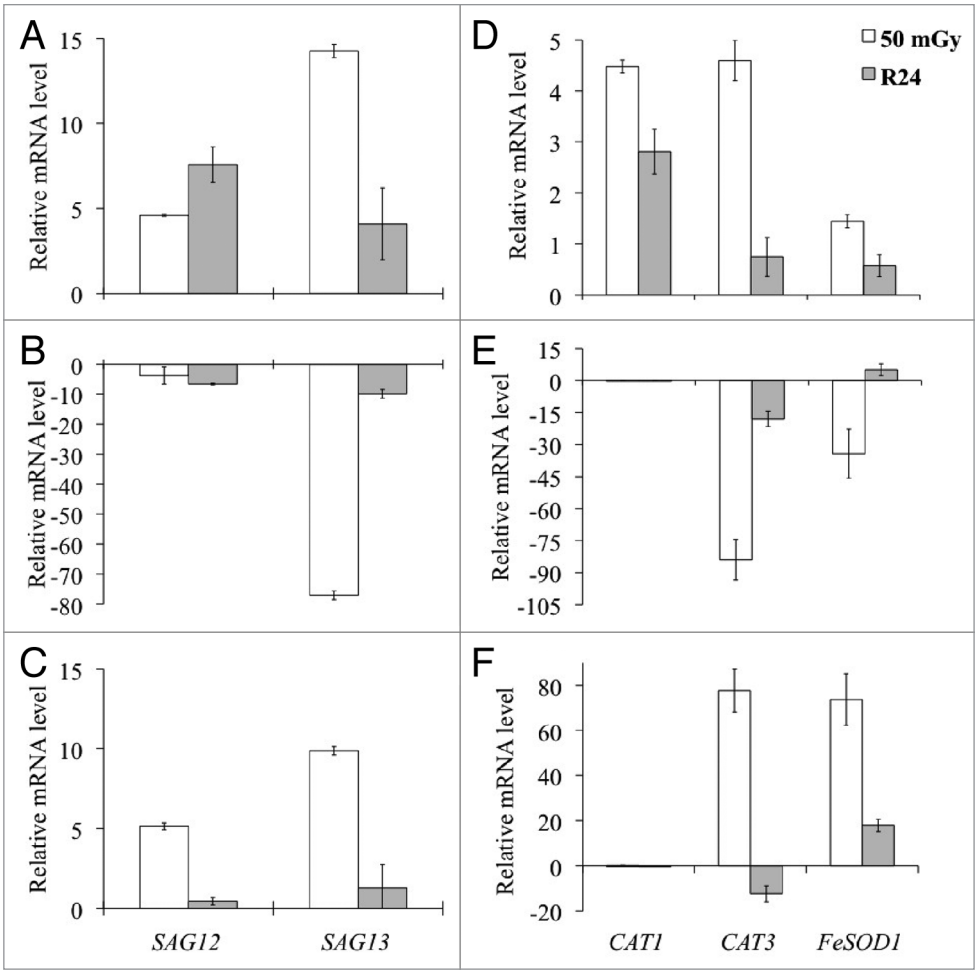

Figure 3. Effect of neutron irradiation on senescence and oxidative stress related genes. Relative mRNA transcript of genes bound to the senescence (*SAG12 and SAG13*) measured after the treatment with 50 mGy absorbed neutron dose (A–C), and 24-h after the recovery from the treatment (R24, grey bars). Measurements were carried out on 35-d-old wild-type Col (A), *aux1* (B) and *eir1* (C) plants. Relative mRNA transcript of oxidative stress related genes *CAT1, CAT3* and Fe*SOD1* (D–F). For normalization and calibration see Figure 2.

Discussion

After 3 hours of neutron irradiation, the genes chosen by us from the *ARF* family (*ARF1, ARF2* and *ARF19*), which have been shown to control significant aspects of plant morphogenesis and growth,[28,34-36] were notably downregulated, whereas of the genes chosen from the *AUX/IAA* family (*AUX/IAA3, AUX/IAA6, AUX/IAA7*), which also are considered to be involved in significant processes,[25,34,37] only *AUX/IAA7* was clearly upregulated. These general effects appear negative in the wild-type plants, since a downregulation of the genes *ARFs* can lead to the reduction of the pool of ARF proteins and to the inhibition of the processes controlled by them. In addition, the ARFs proteins, when bound to the AUX/IAAs ones, whose transcription is upregulated at least in AUX/IAA7, cannot activate the auxin response genes as has been reported extensively.[38-41] This, at least, can be said on the basis of the knowledge we have today on the functions of these two gene families. On the other hand, we found a significantly different situation when the transcription of the same groups of genes was studied in the background of the two auxin transport mutants *aux1* and *eir1*. In these cases, in fact, the effect was frequently reversed, because the *ARF*s resulted upregulated in the mutants, whereas *AUX/IAA7* was upregulated in *aux1*, but downregulated in *eir1*. *AUX/IAA7* is a gene, which characterizes the *axr2* mutant, it is connected with the action of auxin, and in its mutated form induces dominant strong pleiotropic modifications of plant development, i.e., reduced plant size, suppression of gravitropism in shoots and roots, small anomalous flowers, and an extended life cycle.[30] On the other hand, *AUX/IAA3* and *AUX/IAA6* characterize the mutants *shy2* and *shy1*, whose most significant characteristic is the suppression of the mutation in *hy2*, which induces elongated hypocotyls in white and red light.[29,42,43] These two genes, however, did not show significant variations in transcription both in the wild-type and in the auxinic mutants. Furthermore, no particular phenotype is displayed by *ARF1*, which was the first of the family to be described,[35] even though its action seems particularly connected to that of the gene *ARF2*.[44] The latter, on the contrary, appears to have many functions. It is a suppressor of the mutation hookless and a gene involved in the control of senescence. As said above

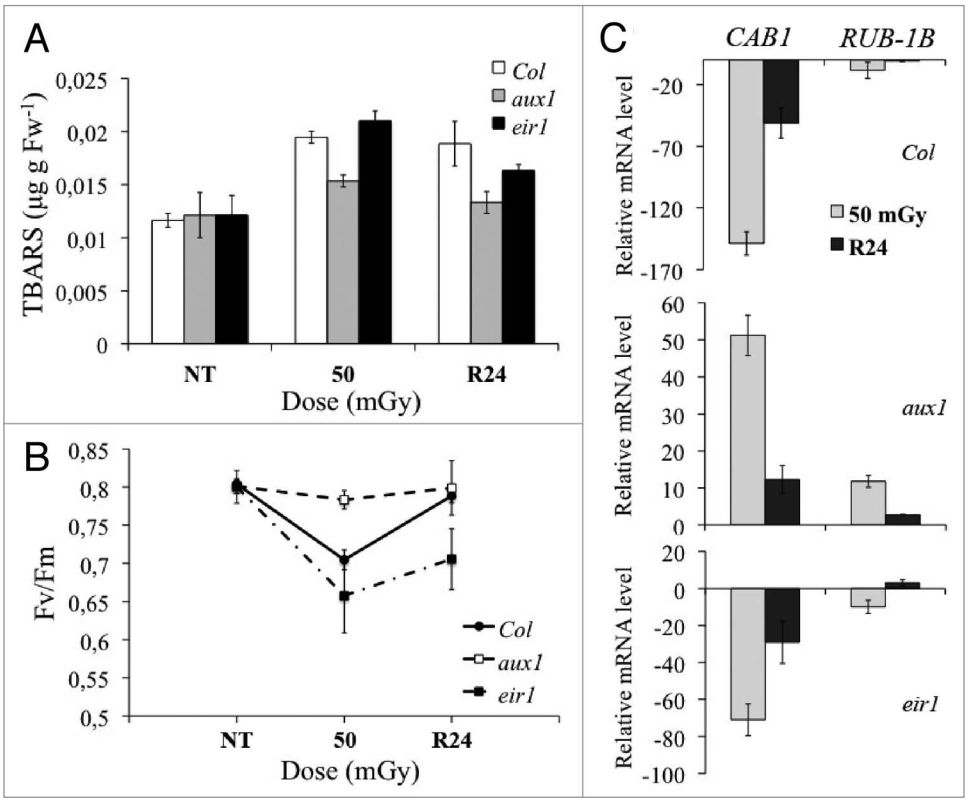

Figure 4. Neutron induced physiological alterations in plants. The physiological effect of neutron radiation on 35-d-old Arabidopsis plants. (A) The content of thiobarbituric acid reactive substances (TBARS) in wild-type Col (white bars), *aux1* (gray bars) and *eir1* (black bars) plants, before and after irradiation with neutron at 50 mGy and after 24 h recovery. (B) Chlorophyll fluorescence (Fv/Fm ratio) measured in control conditions after irradiation with neutrons at 50 mGy, and after recovery from stress (R24). Measurements were done on dark adapted leaves of Col (black circles), *aux1* (white squares) and *eir1* (black squares) plants. (C) Quantitative mRNA transcript level of photosynthetic apparatus related genes *CAB1* and *RUB-1B* after neutron irradiation at 50 mGy (gray bars), and 24-h after the treatment (black bars) measured in the wild-type Col plants, and in the auxin mutants *aux1* and *eir1*. Expression levels are calibrated with respect to the relative mRNA amount in the untreated control plants. Note: bars represent means ± SD (n = 5–7); NT, untreated plants; 50, 50 mGy of neutron irradiation; R24, 24 hours of recovery after the treatment.

its mutation confers juvenility to the plants, i.e., extended life cycle.[44,45] *ARF19* is a gene that has been connected to the action of both auxin and ethylene,[46] which complements *ARF7/NPH4*, involved in phototropism, it resulted downregulated in the wild-type, but upregulated in the mutant *eir1*.[24,46] This result could be an indication that the gene is involved in controlling auxin transport and stress conditions promoted by ethylene. Additional impairment of gene transcription connected to auxin was also seen in the transcript of the auxin transport genes *AUX1* and *EIR1*, both downregulated in the wild-type after irradiation, as well as reciprocally in the mutants (data not shown). What is apparent, therefore, is that the disturbances in the transport of auxin induce significant variations in the expression of the genes *ARF*s and *AUX/IAA7* in the mutants *aux1* and *eir1*.

A specific objective of our research was consequently analysing the expression of genes target of senescence in the irradiated plants, since it is already well known that microgravity and the space environment induce earlier aging, as well as the production of oxidative processes in animals and humans.[47,48] In this part of the research what was found (**Fig. 3A–C**) is that the genes specifically involved in senescence *SAG12* and *SAG13*,[49,50] were upregulated in the wild-type. However, again in this case, as in that of the auxin activated genes, a reverse process was seen in the mutant *aux1*, because both *SAG12* and *SAG13* were downregulated, whereas in *eir1* the effect was like that in the wild-type. This result supports an involvement of auxin transport in the acceleration of senescence seen in the wild-type. Furthermore, in the wild-type, the genes for the catalase *CAT1* and *CAT3* and for Fe*SOD1*, which are involved in the elimination of free radicals, increased their transcription after irradiation. Result that is in line with the acceleration of the stress and senescence processes induced by radiation.[21] However, the genes controlling the oxidative processes were downregulated in *aux1* and, since plants with low catalase activity show less severe stress symptoms,[51] from these data again emerges a clear difference between the wild-type and the two auxinic mutants (at least for *aux1*). These observations, considering that the two mutants show only modest defects, apparently not serious for the life of the plants, lead us to think that they could be grown in space more successfully than the wild-type plants, and that a defect in auxin transport in roots can be advantageous in some cases.

Furthermore, an increase of TBARS, i.e., of thiobarbituric acid (**Fig. 4A**), revealed, especially in the wild-type, damage to the plasmatic membrane, a process bound to senescence.

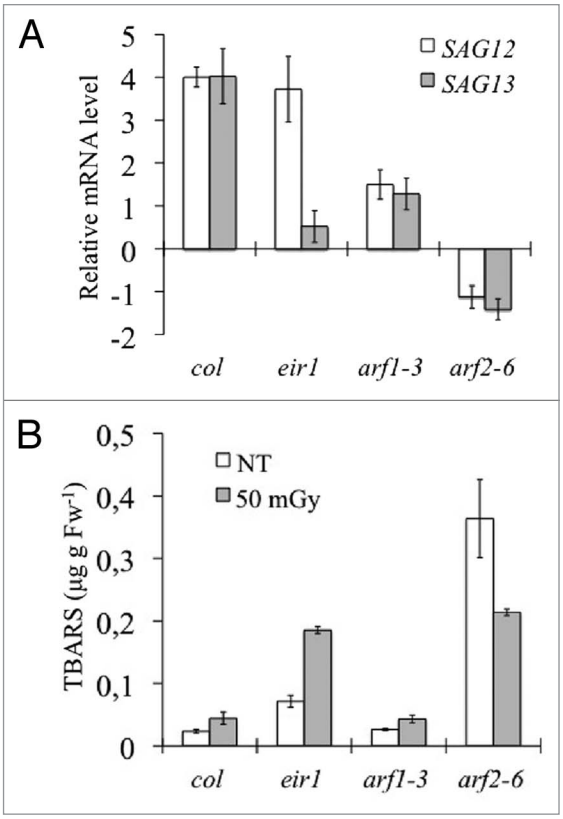

Figure 5. Late effects of neutrons on Arabidopsis plants. Determination of the late effects of neutron radiation on wild-type (Col) Arabidopsis plants, the auxin transport mutant *eir1*, and the auxin response factors *arf1-3* and *arf2-6*. (A) Quantitative qPCR analysis of mRNA expression levels for the senescence activated genes *SAG12* (white bars) and *SAG13* (gray bars) after irradiation of the plants (absorbed dose 50 mGy). Gene expression is shown as log2 fold change of mRNA level, normalized with *ACTIN8* gene, and calibrated with respect to the mRNA level of the respective control plants. For log2 > 0 the gene is upregulated, log2 < 0 is downregulated, and log2 = 0 is unchanged compared to the control level. (B) Quantification of thiobarbituric acid reactive substances (TBARS) in the wild-type (Col), and the mutants *eir1*, *arf1-3* and *arf2-6*, measured on not-treated control conditions (white bars) and after irradiation with neutrons at a dose of 50 mGy (gray bars). Note: The measurements were done 20 days after the irradiation. Bars represent means SD (n = 4–7); NT = untreated plants.

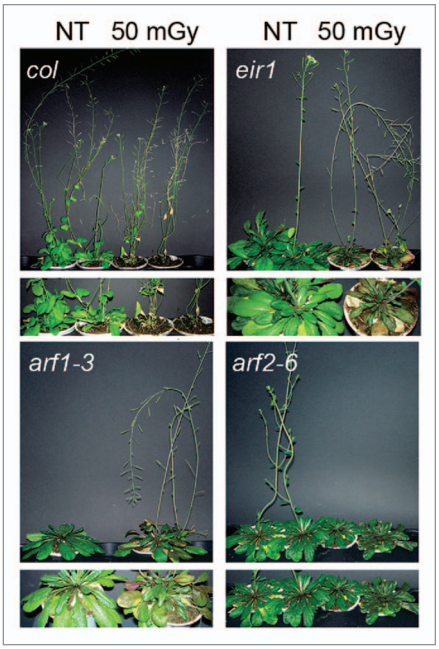

Figure 6. Alteration of the senescence mechanism induced by neutrons. Phenotypical effect of neutrons (at 50 mGy of absorbed dose) on plant senescence 4 weeks after the treatment. Each image represents a comparison between control (NT = untreated plants) and treated (50 mGy) 15-d-old seedlings of the wild-type Col, the auxin transport mutant *eir1*, and two senescence mutants *arf1-3* and *arf2-6*. The lower pictures represent an enlarged detail of rosettes show immediately above. Note: all pictures are representative of a cultivar of at least 30 plants coming from three independent experiments.

The maximum photochemical efficiency of Photosystem II (PSII), determined from the ratio of variable Fv to maximum Fm fluorescence Fv/Fm = (Fm - F0)Fm, was consistently lowered after the course of the neutron irradiation experiments in the wild-type and *eir1* (**Fig. 4B**). This indicates possible damage of the photosynthetic process in the chloroplasts, and suggests that neutron irradiation induces lipid peroxidation associated with the damage and degradation of PSII. In fact, measurement of two genes connected with photosynthesis (**Fig. 4C**) i.e., *RUBS-1B* and *CAB1*, chosen to check the general effect of irradiation on the process, showed in the case of *CAB1* and in the wild-type, as well as in *eir1*, a general decline in transcription, whereas in *aux1* upregulation was seen. This indicates that in *aux1* again the irradiation has opposite effects with respect to the wild-type and frequently also with respect to *eir1*. No large variation in transcription was seen by contrast in the case of *RUBS-1B*.

Regarding the recovery over a short period of time, as mentioned previously, we measured a remarkable return to pre-treatment status in all the genes considered (**Figs. 2–4**), whereas in the case of recovery over a long period of time some effects of irradiation persisted. In fact, after 20 days from the irradiation (**Fig. 5**) both *SAG12* and *SAG13* showed still a consistent upregulation in the wild-type, whereas in the mutants *arf1-3* and *eir1* (as regards *SAG13*) the transcription values were low, and especially in *arf2-6* the senescence genes were clearly downregulated, showing juvenility. However, the data relative to the level of TBARS (**Fig. 5B**) showed a value particularly high in *arf2-6*, a fact that indicates that some negative effects of the irradiation persisted even in the most juvenile of the mutants.

The data collected four weeks after the irradiation, on the other hand, showed an accelerated senescence in the adult plants (**Fig. 6**), with the exception of *arf2-6*, in which case the juvenility was even increased by the irradiation. This gene, controlled by auxin in its activity, as said, is already known as a trigger of juvenility when mutated, probably as a consequence of the fact that it seems to control the switching between different and progressive morphological stages in the plant.[24,44,45] Should further testing reveal that the plants mutated in *ARF2* are healthy and reproductive, the manipulation of this gene could become a useful technique to apply in the production of plants suited to space flights.

Table 1. List of qPCR primer pairs used for the gene expression experiments

Gene name	Primer ID	Sequence
ARF1	ARF1 Fw1	5'-ATGCCTGTGCTGGACCTCTTGTAAC-3'
	ARF1 Bw1	5'-CCTCGGAAAATGTGCCTAAAATGCC-3'
ARF2	ARF2 Fw1	5'-CTCGTCGTCGGTATTCAAGCGTC-3'
	ARF2 Bw1	5'-ATCTGTTGTTCTGCCGCCTGGTTCG-3'
ARF19	ARF19 Fw1	5'-CCTCCTGTGGGAAGTCTTGTGGTTTAC-3'
	ARF19 Bw1	5'-GCTCCAACCTGTGGTAAGCAAGTG-3'
IAA3	IAA3 Fw1	5'-AAACAGAGCTGAGGCTGGGATTA-3'
	IAA3 Bw1	5'-AGAAACCCGACAACCCAAGCA-3'
IAA6	IAA6 Fw1	5'-TTCGATTGGGTCTTCCAGGAGATA-3'
	IAA6 Bw1	5'-ATCTTGCTGGAGACCAAAACCA-3'
IAA7	IAA7 Fw1	5'-AAGCAGTTGAGAGTCCTGCCAAAT-3'
	IAA7 Bw1	5'-CCGTCATATTGTTGATCATTGATGC-3'
SAG12	SAG12 Fw1	5'-GATGAAGGCAGTGGCACACCAACCG-3'
	SAG12 Bw1	5'-GACATCAATCCCACACAAACATACACA-3'
SAG13	SAG13 Fw1	5'-CTGGTGGCTCTAAAGGCATCGGGTC-3'
	SAG13 Bw1	5'-CTTATGTTGTCGCTCGCCCATTCGC-3'
CAB1	CAB1 Fw1	5'-TGAAGGCTACAGAGTCGCAGGAA-3'
	CAB1 Bw1	5'-GCTCTCTTTCTCCTCTCACACTCAC-3'
RUB-1B	RUB-1B Fw1	5'-CATCACAAGCAATGGGGGAAGAG-3'
	RUB-1B Bw1	5'-AACCTTCAGTTTTTTCACTTT-3'
CAT1	CAT1 Fw1	5'-CTGCTCTGGAAATCGTGAGAAGTGC-3'
	CAT1 Bw1	5'-AGAAACCAAACCGTAAGAGGAGCATA-3'
CAT3	CAT3 Fw1	5'-CACTCAGAGACATCGCCTTGGACCG-3'
	CAT3 Bw1	5'-CGTGGGTGAGACGTGGCTCCGATAG-3'
FeSOD1	FeSOD Fw1	5'-TCCAGAACCGAAGACCAGATTACAT-3'
	FeSOD Bw1	5'-CTTGACACACACAAAACGCACACAC-3'
ACT8	ACT8 Fw1	5'-ATGAAGATTAAGGTCGTGGCA-3'
	ACT8 Bw1	5'-TCCGAGTTTGAAGAGGCATC-3'

Furthermore, of the two mutants involved in auxin transport, *aux1* was particularly interesting in that its reaction to the aging and stressing agents (i.e., neutron irradiation) was almost opposite to those in the wild-type. This observation could indicate that, by manipulating the transport of auxin, it might be possible to increase the resistance of the plants to stress. Perhaps the transport of auxin may also be one of the keys to senescence in plants.

Materials and Methods

Plant material and growth conditions. Seeds of *Arabidopsis thaliana* plants, ecotypes Ws (Wassilewskjia) and Col (Columbia), as well as the mutants *aux1*, *eir1*, *arf1-3* and *arf2-6*, were provided by the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK). Before plating, the seeds were sterilized for 10 minutes in a solution containing 50% commercial bleach and 0.01% SDS, followed by four washes with sterile distilled water. Seeds were plated in Petri dishes, on a growth medium made up of 1.5% agar, 1% sucrose, and 0.5X MS basal medium enriched with Gamborg's vitamins and adjusted to pH 5.7 with NaOH and then transferred to 7 cm pots on a sterile mixture made up of 40% sand, 35% turf and 25% soil. The plants were grown by keeping them at first in short day (8/16 h light/dark photoperiod) to promote rosette growth, and after three weeks in long day (16/8 h light/day photoperiod). Growth chamber conditions were: 150 μmol m^{-2} s^{-1} light intensity, 23/19°C day/night air temperature, relative humidity ca. 65%.

Neutron exposure facility and experimental set-up. For all the neutrons irradiation experiments Arabidopsis 35-d-old plants (wild-type Ws, col, and the mutants *aux1*, and *eir1*, *arf1-3* and *arf1-6*) were used. Treatments were carried out at the Frascati Neutron Generator (FNG), a research center of the National Agency for New Technologies, Energy and the Environment (ENEA) in Rome, Italy. To generate the neutron field a deuterion beam was accelerated up to 300 keV inside an accelerating pipe and focused onto a target plate containing tritium. A nuclear fusion reaction occurs between deuterium and tritium ions, resulting in the production of α particles and neutrons with a kinetic energy of 14 MeV. The produced particle fluxes are constant on a generic spherical surface surrounding the neutron source. A wood panel (**Fig. 1**) was placed perpendicular to the direction of the accelerating pipe, and the iso-flux curves described on the panel were represented by circumferences having as a center the intersection point between the panel itself and the horizontal axis of the accelerating pipe. The plants were placed along three circumferences, each corresponding to a different total neutron dose: 30, 50 and 75 mGy. During each radiation session the plants were irradiated in the dark at 23°C and 60% relative humidity for an uninterrupted period of ~3 hours at a constant rate. The absorbed doses in this experimental configuration were calculated by using the Monte Carlo N-Particle transport code (MNCP). Several plants used as control were housed in a separate room completely shielded against the neutron radiation, and kept at the same environmental conditions of the irradiated samples.

After the treatments, three samples from five plants (control and irradiated) of each ecotype or mutants were collected and stored at -80°C for further analysis. Leaves from both irradiated and control plants were detached, immediately immersed in RNALater solution (Sigma, CA, USA), and stored for further molecular analysis.

Late effects of neutrons on Arabidopsis plants were assayed by growing seedlings of the wild-type Col and the mutants *eir1*, *arf1-3* and *arf2-6* for two weeks in Petri dishes; then plants were irradiated with neutrons and recovered, transferring them to the

growth chamber, together with the control plants, for 4 more weeks. Three weeks after the recovery period, leaf samples of both irradiated and control plants were collected and stored at -80°C.

Finally, at the end of the recovery period, images of the plants were recorded using a digital camera. For each experiment, only results obtained with the 50 mGy absorbed irradiation dose are presented. Only insignificant qualitative or quantitative differences were noted in the data collected at the other two irradiation doses (30 and 75 mGy).

Chlorophyll fluorescence measurement and lipid peroxidation assay. Damage to the photosynthetic apparatus after neutron radiation was assessed by measuring the photosynthetic efficiency of the photosystem II (PSII), using a portable chlorophyll fluorometer MINI-PAM (Waltz, Effeltrich, Germany). The chlorophyll fluorescence, given as the Fv/Fm ratio, was measured under pre-stressed conditions and one hour after the neutron irradiation after 30 minutes of dark adaptation.

Lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) content, according to Heath & Packer.[52] Three different replicates from at least three plants (control and treated) were used for each ecotype. Leaf material (0.1 g) was homogenized in 1.2 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 *g* for 30 min and 0.5 ml of the supernatant was added to 1 ml 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated in a boiling water bath for 30 min, and then quickly cooled on ice. Absorption of the solution was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. For calculation of the content of MDA-TBA complex the molar extinction coefficient of 155,000 was used.

Quantitative *real time* qPCR analysis. Total RNA was extracted from 50 mg of each leaf sample using the TRIZOL reagent (Invitrogen, CA), following the manufacturer's instructions. Two μg of total RNA was reverse transcribed using the Super Script III (Invitrogen) reverse transcriptase to synthesize the first-strand cDNA mixture, which was used for the polymerase chain reactions (PCR). For each point of the experiments at least three leaves (for total RNA extraction from 35-d-old plants), harvested from 3 different plants or 100 seedlings (for total RNA extraction from 15-d-old plants), were used for all ecotypes and mutants. *Real time* quantitative polymerase chain (qPCR) reactions were performed using an Mx3000P® (Stratagene, CA) system following the protocol described in Fortunati et al.[53] Conditions for all qPCR reactions were: 10 min at 95°C and 35 three-temperature cycles (30 sec at 95°C, 45 sec at 57°C and 1.2 min at 72°C). For the complete list of primers used in qPCR reactions see **Table 1**. All qPCR data were analyzed using the MxPro v 4.0 software (Stratagene).

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