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Kinetin-serine regulation of photosynthetic pigments and some antioxidant enzymes during dark induced senescence in spinach leaf discs

Somveer Jakhar* and D. Mukherjee

Plant Physiology and Biochemistry Laboratory, Department of Botany, Kurukshetra University, Kurukshetra 136119, India.

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Interesting observation of earlier investigation on regulation of leaf senescence in pigeon pea with serine prompted us to undertake studies on kinetin (Kn)-serine interaction in senescent leaf discs of *Spinacia oleracea* L., cv. S-23, over time, in the dark to determine their effectiveness individually, and in combination, on photosynthetic pigments, total soluble proteins, lipid peroxidation and activities of the senescence associated enzymes- protease, lipoxygenase (LOX), guaiacol peroxidase (POD) and superoxide dismutase (SOD). Serine and Kn each individually, and in combination, minimized loss in chlorophyll a and b in all days and carotenoids at day 6. Kinetin and serine retarded protein loss and also curtailed protease activity. POD activity increased in untreated leaf discs up to 6-day; SOD activity decreased in control as leaf discs senescence advanced. Senescence increased POD activity and promoted chlorophyll degradation. Serine along with Kn was able to minimize increases in POD and maintained slightly higher level of SOD activity in all days when compared with the control. Although, combined treatment of serine and Kn could lower malondialdehyde content slightly at day 2, LOX activity was reduced. Serine and Kn can scavenge ROS by regulating antioxidant enzymes during leaf senescence.

Key words: *Spinacia oleracea*, chlorophyll, lipid peroxidation, lipoxygenase, superoxide dismutase.

INTRODUCTION

When detached leaves are placed in dark several changes characterizing senescence such as loss in chloroplast pigments, proteins; and increase in lipid peroxidation and membrane permeability resulting in injury are triggered, all of which lead to decreased photosynthetic output (Nooden et al., 1997; Prochazkova et al., 2001). Among the plant growth regulators, ABA and ethylene accelerate senescence symptoms (Smart, 1994). Exogenous application of cytokinins inhibits degradation of chlorophyll and photosynthetic proteins (Richmond and Lang, 1957; Badenoch-Jones et al., 1996). Low light intensities, or dark, result in reduced expression of light-dependent genes and disappearance of photosynthetic proteins and chlorophyll (Wingler et al.,

*Corresponding author. E-mail: somveerjakhar@hotmail.com.

1998). Light is an important factor initiating, and/or modulating, senescence rate in attached and detached plant systems (Biswal and Biswal, 1984; Ono et al., 2001). Higher photon flux density may cause photo-oxidative damage and induce leaf senescence (Prochazkova and Wilhelmova, 2004). Complete deprivation of light induces senescence in individual leaves of *Arabidopsis* ecotype Landsberg *erecta* but may inhibit leaf senescence when applied to the whole plant (Weaver and Amasino, 2001).

Regulation of many plants developmental processes by light are affected by plant growth regulators, and increased endogenous cytokinins may inhibit leaf senescence under certain conditions (Wingler et al., 1998). There is evidence that this could be mediated by an increment of antioxidant defenses (Synkova et al., 2004). Kinetin application delays senescence and is effective in minimizing chlorophyll and protein loss (Thimann, 1980; Mukherjee and Ponmeni, 2004; Mukherjee and Jakhar, 2009). Experiments with pea (*Pisum sativum* L. cv. Alaska) seedlings and oat (*Avena sativa* L. cv. Victory) leaf segments showed the mode of action of cytokinins, and the role of amino acids in regulation of senescence (Shibaoka and Thimann, 1970; Martin and Thimann, 1972a, b, 1973; Satler and Thimann, 1983; Veierskov et al., 1985).

Two most important markers of leaf senescence, viz. chlorophyll degradation and massive hydrolysis of protein occurring in the detached first leaves of *Avena* were found to be vigorously promoted by L-serine (Shibaoka and Thimann, 1970). Selecting various protein amino acids also showed that cysteine, threonine, alanine and glycine exhibited weaker action in promoting senescence whereas arginine did not favor the action of L-serine when both were present. Using three different concentrations of kinetin viz. 0.05,0.5 and 5.0 ppm along with L-serine at 0.5, 5.0 and 50 X 10^{-3} M concentrations, Martin and Thimann (1972a) clearly indicated how serine could overcome the action of kinetin and promote senescence in oat leaves.

Along with the decline in chlorophyll and protein content, increment was observed in α-amino nitrogen content. That was the basis for further investigation to determine how leaf senescence was promoted by L-serine (Veierskov et al., 1985). Antagonism was established between kinetin and L-serine; and amino acids L-alanine, L-cysteine and others. However, investigations carried out in our laboratory with spinach (*Spinacia oleracea* L.) and pigeon pea (*Cajanus cajan* L. cv. UPAS-120) leaves, serine alone, and in presence of kinetin, minimized protein degradation and accumulation of free amino acids (Mukherjee and Jakhar, 2009; Mukherjee et al., 2011). There were less degradation of chlorophylls and carotenoids in leaf discs in the presence of serine, and also when serine and kinetin were both present. Spinach (*S. oleracea* L.) was used as a test plant because being a leafy vegetable, we wanted to find out whether serine can alter activities of some antioxidant enzymes and membrane damage. An experiment was undertaken to determine how antioxiodant enzymes and lipoxygenase behave in the presence of serine and kinetin to regulate senescence in spinach leaf discs as these aspects have not been investigated earlier.

MATERIALS AND METHODS

Leaf discs were cut using cork borer from senescent spinach leaves cv. S-23 (procured from CCS Haryana Agricultural University, Hisar, India). Leaf discs, 0.75 cm^2 area, were placed in Petri dishes (9 cm diameter). Sixty to 65 leaf discs were placed on moistened filter paper (Whatman No. 1) In Each Petri dish having 5 ml of test solution and maintained in complete darkness at 25 ± 2°C. Applied treatments were carried out as follows:

1) 0.38 µM kinetin (Kn),
2) 5 mM DL-serine,
3) 0.38 µM Kn + 5 mM DL-serine,
4) Phosphate buffer, pH 5.2 as control and
5) Double distilled water (DDW) as control.

For each treatment, leaf discs were maintained in 5 Petri dishes. Sampling and estimations were carried out at 0, 2, 4 and 6-day after placement in the Petri dishes to determine amount of chloroplast pigments, protein and MDA contents, and enzymatic activities of peroxidase, lipoxygenase and superoxide dismutase (SOD).

Estimation of chlorophylls and carotenoids

Leaf sample (200 mg) was ground in chilled 80% acetone (AR grade) with 20 mg of $CaCO_3$ and centrifuged at 3000 g for 5 min. Absorbance of the filtrate was recorded at 645 and 663 nm for chlorophylls and at 480 and 510 nm for carotenoids depending on respective peaks in their absorption spectra using a UV-Visible spectrophotometer (Specord-205, Analytic-Jena, Germany). Chlorophyll (Chl) amount was estimated with the formula of Arnon (1949). Carotenoid level was calculated by the method of Holden (1965).

Estimation of total soluble protein

Total soluble proteins were estimated according to the method described by Bradford (1976) using Coomassie Brilliant Blue G-250. Fifty milligrams of fresh leaf tissue (earlier stored in a freezer) was dropped in boiling 80% ethanol (EtOH) on a water bath for a minute. The tissue along with EtOH was cooled to room temperature and homogenized. The extract was centrifuged at 10,000 g for 5 min. The residue was re-extracted with 5% perchloric acid followed by centrifugation at 10,000 g for 5 min. 5 mL of 1N NaOH was added to the residue and maintained in warm water (40-50°C) with regular shaking for 30 min. The clear supernatant was used for further analysis.

Estimation of protease activity

The procedure for protease extraction was a slight modification of that described by Yomo and Varner (1973) and Ihnen (1976). Samples, 200 mg, were homogenized in 100 mM phosphate buffer (using equimolar KH_2PO_4 and Na_2HPO_4), pH 7.2, and the final volume

volume raised to 25 mL. Casein (1%) (Sigma, St. Louis, U.S.A.) was prepared by dissolving 1.0 g casein in 2.0 mL of 0.1 N NaOH and the final volume made to 100 mL with 100 mM phosphate buffer, pH 7.6. To 1 mL of casein, 1 mL of enzyme extract was added and incubated for 3 h at 37°C. The reaction mixture was pH 7.5. After incubation, 1 mL of 16% TCA was added to all reaction sets and centrifuged. The residue was discarded. Of the resultant 3 mL filtrate, 0.5 mL was used to estimate protease activity by the ninhydrin method (Yemm and Cocking, 1955) as modified by Reimerdes and Klostermeyer (1976).

Measurement of peroxidase (POD) activity

Total peroxidase activity was measured by the method of Maehly (1954). Plant material (0.1 g) was homogenized with ice cold distilled water and centrifuged in a Remi centrifuge at 6000 g for 10 min. The supernatant was used as the enzyme source and final volume of the extract raised to 10 mL with ice cold double distilled water. The reaction set was prepared by mixing 2 mL each of enzyme source; phosphate buffer (pH 7.0); guaiacol (20 mM), and H_2O_2 (10 mM) in sequence. A blank set was prepared by mixing 2 mL of enzyme source; 2 mL of phosphate buffer (pH 7.0) and 4 mL of double distilled water. Blank, and reaction sets were kept undisturbed at room temperature exactly for 10 min, then the absorbance was recorded in a spectrophotometer at 420 nm. Protein was estimated from the same extract following the procedure of Bradford (1976).

Measurement of lipoxygenase (LOX) activity

The LOX activity was estimated according to Doderer et al. (1992). Two hundred milligrams of leaf sample was homogenized in ice cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 µM EDTA in a pre-chilled pestle and mortar. The homogenate was transferred to tubes and centrifuged at 4°C in a refrigerated centrifuge (Compufuge, CPR-24, Remi, New Delhi, India) for 15 min at 15000 g. The supernatant was transferred to 30 mL test tubes for use as the enzyme extract. 5 mL of distilled water with 50 µL Tween 20 was added to 35 µL linolenic acid (substrate), pH 8.5-8.6. The final pH was adjusted to 9 by adding 0.2 M NaOH drop by drop until the linoleic acid dissolved completely. The pH was adjusted to 6.5 by adding 0.2 M HCl. To this solution, 0.1 M phosphate buffer, pH 6.5, was added and the final volume of substrate raised to 100 mL with the same buffer. Blank set was prepared in a cuvette with 2.95 mL of substrate solution. In the reaction set, 0.05 mL of the enzyme extract was added to the cuvette containing 2.95 mL of the substrate solution at zero time. Absorbance was noted at 234 nm for every minute upto 5 min. The amount of protein was estimated by the method of Bradford (1976).

Measurement of superoxide dismutase (SOD) activity

Fifty milligrams of fresh leaf tissue was crushed in 2 mL of 0.1 M EDTA- phosphate buffer, pH 7.8, containing K_2HPO_4 and EDTA and the final volume raised to 100 mL with double distilled water (DDW). This was centrifuged at 15000 g and the resultant supernatant used as crude extract. The reaction mixture was prepared by adding 0.1 mL of crude extract followed by 0.9 mL of DDW, 0.5 mL of 300 mM Na_2CO_3 (pH 10.2), 0.5 mL of 378 µM p-nitrobluetetrazolium chloride (NBT), 0.5 mL of 78 mM L-methionine and 0.5 mL of 7.8 µM riboflavin. The final reaction mixture was 3 mL. The reaction was carried out in test tubes at 25°C for 15 min under 100 µmol photon $m^{-2}s^{-1}$ PFD from fluorescent lamps. The initial rate of reaction, measured by the difference in increase in absorbance at 560 nm in the presence, and absence, of extract was proportional to the amount of enzyme. The unit of SOD activity was obtained as that amount of enzyme which under the experimental conditions caused a 50% inhibition of the reaction observed in the absence of enzyme (Giannopolitis and Ries, 1977).

Measurement of lipid peroxidation

The level of lipid peroxidation in samples was measured by estimating the malondialdehyde (MDA) present (Heath and Packer, 1968). Leaf samples (0.2 g) were homogenized in 3 mL of 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 15000 g for 15 min. To 1.0 mL aliquot of the supernatant, 2.0 mL of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) was added. The mixture was heated at 95°C for 30 min in a water bath and then cooled in an ice bath. After centrifugation at 10000 g (Remi) for 10 min the absorbance of the supernatant was recorded at 532 nm. The value for nonspecific absorption of each sample at 600 nm was recorded and subtracted from the absorbance recorded at 532 nm.

Statistical analysis

A mean of three readings was taken in every replication. In biochemical estimation, three aliquots were used for each replication. Statistical analysis was done using Statistical Packages for Social Sciences (SPSS) version 16.0. One-way ANOVA was used to test whether there was a significant difference in various estimations.

RESULTS

As the senescence of leaf discs progressed over a period of 6-day, the reduction in total chlorophyll and carotenoids was 45 and 47.24%, respectively (Table 1). Application of serine alone and in combination with Kn (0.38 µM), reduced the loss as compared to the control. The Chla : Chl b ratio increased as leaf discs senesced in the control (Table 1). Serine application minimized loss of total Chl and carotenoids by about 4.5 and 7% respectively. The combined action of Kn and serine at a highest rate reduced pigment loss. Total soluble protein content and total crude protease activity over 6 days in control and treated leaf discs varied due to treatment (Tables 2 and 3). In untreated control (buffer) and treated leaf discs, protein content gradually declined and protease activity increased from 0 to 6 days. For crude protease activity, a sharp increase was noticed not only in the control leaf discs but also in the sets having serine and kinetin when applied alone and in combination (Table 2). However, the degree of rise was lower with Kn and serine as compared to the control. Combined treatment of serine + Kn was most effective in minimizing protein degradation and reducing protease activity.

Peroxidase (POD) and superoxide dismutase (SOD) activities varied due to treatments. The POD activity gradually increased over 6 days regardless of treatment; the magnitude of increments between 2-4 and 4-6 day stages were greater in the control. Application of serine and kinetin reduced POD activity.

Table 1. Interaction effect of day and treatment on the Chl. a, Chl. b, total Chl., and carotenoids (mg g^{-1} dry wt) in excised leaf discs of *Spinacea oleracea* L. incubated in the dark.

Day	X Treatment	Chl.a	Chl.b	Total Chl.	Carotenoid
0	None	1.251^{a}	0.382^{a}	1.633^{a}	0.477^{a}
	DDW	0.533^{cd}	0.196^{bcd}	0.729^{d}	0.257^{b}
	Phosphate buffer	0.597^{bc}	0.208^{bc}	0.805^{bcd}	0.235^{bcd}
2	Serine	0.627^{b}	0.212^{bc}	0.839^{bc}	0.243^{bcd}
	Kinetin	0.629^{b}	0.221^{bc}	0.850^{bc}	0.249^{bcd}
	Serine + Kinetin	0.631^{b}	0.231^{b}	0.862^{b}	0.252^{bc}
	DDW	0.423^{ef}	0.149^{ef}	0.572^{ef}	0.237^{bcd}
	Phosphate buffer	0.453^{de}	0.179^{cde}	0.632^{e}	0.220^{cd}
4	Serine	0.565^{bc}	0.193^{bcd}	0.758^{d}	0.225^{bcd}
	Kinetin	0.579^{bc}	0.199^{bcd}	0.778^{cd}	0.236^{bcd}
	Serine + kinetin	0.590^{bc}	0.201^{bcd}	0.791^{bcd}	0.216^{d}
	DDW	0.310^{e}	0.075^{e}	0.385^{h}	0.120^{g}
	Phosphate buffer	0.330^{e}	0.113^{f}	0.443^{gh}	0.124^{fg}
6	Serine	0.360^{fg}	0.139^{ef}	0.499^{fg}	0.145^{efg}
	Kinetin	0.379^{efg}	0.148^{ef}	0.527^{f}	0.249^{ef}
	Serine + kinetin	0.393^{efg}	0.160^{de}	0.553^{f}	0.171^{e}

Means followed by different letters are significantly different at 0.05% level, using DMRT. *Each value indicates mean of three replicates.

Table 2. Interaction effect of day and treatment on protein (mg mg^{-100} dry wt), protease (µM lysine equivalent mg^{-100} dry. Wt. h^{-1}) and peroxidase activity (mg mg^{-100} dry wt) in excised leaf discs of *Spinacea oleracea* L. incubated in the dark.

Day	X Treatment	Protein	Protease	Peroxidase
0	None	18.76^{a}	4.36^{a}	2.08^{a}
	DDW	15.23^{d}	22.46^{h}	3.12d^{efg}
	Phosphate buffer	15.98^{cd}	21.36^{hi}	3.09^{efg}
2	Serine	16.99^{bc}	19.30^{ij}	3.00^{efg}
	Kinetin	17.16^{abc}	18.00^{j}	2.86^{fg}
	Serine + kinetin	17.96^{ab}	17.46^{j}	2.67^{gh}
	DDW	9.36^{h}	46.76^{d}	3.89^{c}
4	Phosphate buffer	9.98^{gh}	42.46^{e}	3.69^{cde}
	Serine	11.36^{fg}	36.41^{f}	3.40^{cdefg}
	Kinetin	12.64^{ef}	35.40^{f}	3.10^{efg}
	Serine + kinetin	13.46^{e}	32.47^{g}	2.76^{gh}
	DDW	2.39^{i}	66.36^{a}	4.93^{a}
	Phosphate buffer	3.46^{ij}	60.36^{b}	4.61^{ab}
6	Serine	3.97^{ij}	58.30^{b}	4.01^{bc}
	Kinetin	4.56^{i}	54.31^{c}	3.86^{cd}
	Serine + Kinetin	4.76^{i}	47.19^{d}	3.56^{cdef}

Means followed by different letters are significantly different at 0.05% level, using DMRT. *Each value indicates mean of three replicates.

The order of effectiveness of treatments in lowering POD activity was serine + kinetin > kinetin>serine. SOD activity increased during first two days in control and treated discs. From day 2 to day 6, a decrease in SOD occurred. Effectiveness of serine and kinetin individually, and together, occurred at day 2 and 4 in leaf discs.

Changes in MDA content due to lipid peroxidation and lipoxygenase (LOX) activity rose on specific days irrespective of treatment (Table 3). The MDA increment from day 2 to 4 was higher than LOX activity in the control. The MDA value decreased slightly due to treatment of serine and kinetin only up to day 2, but not thereafter. Both serine and kinetin arrested the rise in LOX activity. The additive effect of these compounds was greater than each alone.

DISCUSSION

Presented data have indicated that very rapid degradation of chlorophylls and carotenoids in leaf discs held in water control can be effectively reduced even by phosphate buffer (Table 1). Further, effectiveness of serine and Kn in lowering total Chl. was more evident when they were applied together (Table 1). Relatively greater retention of Chl a, Chl b and carotenoids have been revealed in spinach leaf discs also by the

Table 3. Interaction effect of day and treatment on Superoxide dismutase (SOD) (units min^{-1} mg^{-1} protein), MDA content (nmol g^{-1} dry wt) and Lipoxygenase (LOX) activity (µmol min^{-1} mg^{-1}) in excised leaf discs of *Spinacea oleracea* L. incubated in the dark.

Day	X Treatment	SOD	MDA content	LOX
0	None	4.23^{bc}	0.00367^{d}	0.167^{i}
	DDW	4.68^{ab}	0.01296^{c}	0.296^{fg}
	Phosphate buffer	4.70^{ab}	0.01310^{c}	0.270^{gh}
2	Serine	4.86^{ab}	0.01260^{c}	0.206^{hi}
	Kinetin	4.97^{a}	0.01036^{cd}	0.189^{i}
	Serine + Kinetin	5.21^{a}	0.00987^{cd}	0.200^{hi}
	DDW	3.31^{e}	0.03476^{b}	0.379^{e}
	Phosphate buffer	3.32^{e}	0.03400^{b}	0.356^{ef}
4	Serine	3.59^{de}	0.03390^{b}	0.340^{ef}
	Kinetin	3.76^{cde}	0.03390^{b}	0.300^{fg}
	Serine + Kinetin	3.98^{cd}	0.03381^{b}	0.204^{hi}
	DDW	1.07^{f}	0.05237^{a}	1.067^{a}
	Phosphate buffer	1.17^{f}	0.05210^{a}	0.949^{b}
6	Serine	1.19^{f}	0.05160^{a}	0.720^{c}
	Kinetin	1.26^{f}	0.05150^{a}	0.624^{d}
	Serine + Kinetin	1.28^{f}	0.05103^{a}	0.480^{hi}

Means followed by different letters are significantly different at 0.05 % level, using DMRT. * Each value indicates mean of three replicates.

application of both Kn and serine like earlier studies in pigeon pea (Mukherjee and Ponmeni, 2000) and in spinach leaves (Mukherjee and Jakhar, 2009). The retention in the amount of chlorophylls by Kn ttreatment has been also observed by other workers (Paranjothy and Wareing, 1971; Hukmani and Tripathi, 1994).

Alteration in POD activity increased in untreated leaf discs. In treated discs POD activity increased to a lesser extent as compared to the control. The combined treatment reduced the activity of POD. Grover and Sinha (1985) reported increase in POD activity as leaf senescence continued in detached pigeon pea and chickpea leaves. Further, a decline in POD activity in leaves occurred after flowering; and control leaves had lower activity of this enzyme than leaves from deflowered plants at all stages (Grover et al., 1985). When leaves were induced to senesce by detaching and incubation in water, POD activity increased. The POD activity increased during senescence of detached leaves or leaf discs (Parish, 1968; Jakhar and Mukherjee, 2006). However, Srivastava et al. (1983) found no difference in POD activity between young and mature leaves of barley (Hordeum vulgare L.). In *Festuca aurundinaceae* Schreb leaves the increment in POD activity was responsible for cessation of growth (MacAdam et al., 1992). Activity of guaiacol-dependent POD increased during the entire ontogeny of bean cotyledons (Wilhelmova, 1998). Serine and Kn could not completely prevent senescence-related increase in protease and POD in complete darkness. During an investigation on chlorophyll metabolism on chlorophyll metabolism in tobacco leaves, Kato and Shimizu (1985) concluded that peroxidase could also degrade chlorophyll with a number of phenolics including guaiacol and p-coumaric acid. Moreover, this process of chlorophyll bleaching was inhibited by peroxidase inhibitors.

The MDA concentration, and specific activity of POD, tended to increase and SOD decreased as senescence progressed. Serine individually and in combination with Kn, produced slightly higher SOD activity during senescence. Decrease in reactive oxygen species (ROS) may be due to the increase in SOD activity in serine and Kn treated plants; which are an intrinsic part of plant senescence and inhibit the process of oxidative deterioration (Thompson et al., 1987). Antioxidative enzymes SOD, CAT and POD are the main protective enzymes engaged in removal of free radicals and activated oxygen species (Blokhina et al., 2003; Devi and Prasad, 2005). A decrease in SOD activity occurring during senescence (Droillard and Paulin, 1990) can be taken as a supporting marker for the progress of senescence. Huang and Liu (2002) reported that stress decreases SOD activity which can be mitigated by exogenously applied cytokinin.

Although a sharp increase was noticed in MDA content during 0 to 6-day in leaf discs in dark, effect of serine and kinetin was very little; that too was visible only at 2-day. There were no changes in leaf discs in the presence of serine and kinetin. An increment in LOX activity occurred in leaf discs over six days. Serine and Kn lowered the activity considerably; further reduction was brought about by a combination of serine and Kn. Similarly, treating intact pea leaves with either cytokinin or α-tocopherol resulted in lower LOX activity relative to controls (Leshem, 1988). The enzyme is responsible for lipid peroxidation by forming lipid hydroperoxides and superoxide radicals. The LOX increased in controls over 6 days. The elevated LOX activity is a common feature of senescent plant organs (Grossman and Lesham, 1983; Thompson et al., 1987) and flower petals (Rouet- Mayer et al., 1992).

However, reports are also available where a decline in LOX activity has also been reported in detached wheat and rye leaves (Kar and Feierabend, 1984) and soybean cotyledons (Peterman and Siedow, 1985). Since lipid peroxidation is mediated by ROS (Arora et al., 2007), kinetin may either be directly scavenging ROS and decreasing lipid peroxidation, or modulating activity of antioxidant enzymes.

Kinetin and serine delayed senescence in cut leaf discs of spinach by maintaining protein content and lowering protease and POD activity, keeping a higher activity of SOD enzyme and reducing oxidative stress such as lipid peroxidation and LOX activity.

Conflict of Interests

The author(s) did not declare any conflict of interests.

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