

Effect of darkening the cotyledons on the growth and curvature of the sunflower hypocotyl: evidence of hydraulic signalling

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Abstract

When both cotyledons of light-grown sunflower seedlings (*Helianthus annuus* L.) were darkened by covering them with aluminium foil, the resulting increase in the rate of elongation of the hypocotyl was closely proportional to the associated reduction in seedling transpiration and to the increase in xylem water potential (ψ_{Px}). Covering only one cotyledon induced curvature away from that side. This response was associated with a higher ψ_{Px} in the vascular bundles connected directly with the covered cotyledon than in those connected with the illuminated cotyledon. The water content of peripheral tissues of the hypocotyl below the covered cotyledon was also higher than that of similar samples from below the illuminated cotyledon. These results are consistent with the hypothesis that the effect of darkening the cotyledons on the growth and curvature of the hypocotyl is mediated by hydraulic signalling, characterized by transmission to the hypocotyl of an increase in ψ_{Px} resulting from the reduction in cotyledon transpiration.

Key words: Cotyledons, *Helianthus annuus*, hydraulic signalling, hypocotyl, transpiration, water potential.

Introduction

In a study of phototropism in the sunflower (*Helianthus annuus* L.) Lam and Leopold (1966) showed that darkening both cotyledons by covering them with aluminium foil increased the rate of elongation of the hypocotyl. Covering only one cotyledon restricted this response to the side of the hypocotyl below the covered cotyledon,

causing the hypocotyl to curve away from that side. Similar results have been reported from other experiments with sunflowers (Bruinsma *et al.*, 1975) and several other species (Black and Shuttleworth, 1975; Shuttleworth and Black, 1977). There is, however, no general agreement as to the mechanism involved. In the study by Lam and Leopold (1966), a bioassay of diffusates from the cut ends of excised hypocotyls indicated that more auxin (indoleacetic acid) was translocated into the hypocotyl from darkened than from illuminated cotyledons, and this was suggested as the possible cause of the curvature of the hypocotyl away from the side below the covered cotyledon. This postulated mechanism is a modification of the classical Cholodny–Went theory (Went and Thimann, 1937), which attributes phototropic curvatures to the light-induced translocation of auxin from the illuminated to the shaded side of the shoot. Although the validity of this theory has been questioned (Firn and Digby, 1980), a forum recently conducted to assess the current status of the theory, as applied to both phototropic and gravitropic responses, showed that it still had considerable support, but generally in a modified form (Trewavas, 1992). The majority of participants suggested that, while the effect of tropic stimulation on the lateral distribution of auxin may not adequately account for the associated changes in growth, tropic curvatures may be controlled by the interaction between auxin and other factors. Specific suggestions included possible interactions with lateral gradients of Ca^{2+} , tissue sensitivity to auxin, or growth inhibitors.

The need for a broader interpretation of the Cholodny–Went theory is also suggested by more recent studies of phototropism of the sunflower hypocotyl. Experiments in which auxin distribution was measured by physico-

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chemical methods of analysis failed to detect any difference in the auxin content of the illuminated and shaded sides of phototropically stimulated hypocotyls of the sunflower (Bruinsma *et al.*, 1975; Feyerabend and Weiler, 1988). It was suggested (Bruinsma and Hagesawa, 1989) that differences in the growth-promoting activity of extracts from opposite sides of phototropically stimulated organs, as determined by bioassays that are not specific for auxin, may be caused by gradients of growth inhibitors. Xanthoxin was identified as a possible inhibitor involved in the phototropic curvature of the sunflower hypocotyl (Thompson and Bruinsma, 1977; Franssen, 1980). However, although Franssen and Bruinsma (1981) reported that xanthoxin accumulated preferentially on the illuminated side of phototropically stimulated sunflower hypocotyls, Feyerabend and Weiler (1988) were unable to detect any asymmetry in its distribution. Attempts to inhibit hypocotyl elongation by exogenous applications of xanthoxin were unsuccessful (Franssen, 1980).

The present investigation is based on previous evidence that water may play an important role as a limiting factor in the growth and phototropic curvature of the sunflower hypocotyl. In experiments with dark-grown seedlings, increasing the relative humidity from approximately 50–100% caused a 2–3-fold increase in the rate of elongation of the hypocotyl (McIntyre, 1980). It was also shown that, when the seedlings were stimulated phototropically by unilateral blue light, the shaded side of the hypocotyl had a higher water content than the illuminated side, even when phototropic curvature was physically prevented. In further investigations (McIntyre and Boyer, 1984), the promotion of hypocotyl elongation by increased humidity was correlated with reductions in transpiration and with increases in the water potential (ψ_w) and turgor pressure (ψ_p) of the growing region of the hypocotyl. A linear relationship between transpiration and leaf water potential (ψ_L) has been reported from experiments with light-grown sunflowers (Boyer, 1977) and various other species (Hailey *et al.*, 1973; Neumann *et al.*, 1974; Bunce, 1978). It has also been shown that changes in ψ_L produced by the effect of light on transpiration can be rapidly transmitted to the stem (McBurney and Costigan, 1982, 1984), causing closely correlated changes in stem diameter (Klepper *et al.*, 1971) and elongation (Woodward, 1981). Thus, the purpose of the present investigation was to test the hypothesis that a similar mechanism may play a role in the effect of darkening the cotyledons on the growth and curvature of the sunflower hypocotyl.

Materials and methods

Plant culture technique

Experiments were conducted with the same inbred line of *Helianthus* (CM 90 RR) used in previous investigations

(McIntyre, 1980; McIntyre and Boyer, 1984); another inbred line (CM 38) was included for comparison in one experiment. Seeds were soaked in water overnight, then dehulled and germinated on moist filter paper in darkness at 25°C for 24 h. After being grown for a further 24 h in shallow trays of vermiculite, seedlings were selected for uniformity of root and hypocotyl length and transplanted into 7.5 cm diameter plastic pots. Three seedlings were planted per pot, and thinned to one per pot in a further selection for uniformity 2–3 d after transplanting. Illumination was provided by cool-white fluorescent tubes at a photon flux density of 165 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 14 h daily, a day/night temperature of 25/30°C, and 50–60% RH. The pots were placed in trays containing a shallow layer of distilled water to minimize fluctuations in the ψ_w of the vermiculite, and were leached daily with 100 ml of 10^{-1} Hoagland's solution (Hoagland and Arnon, 1939).

Experimental treatment

The effect of covering the cotyledons on the growth, ψ_{PX} and curvature of the hypocotyl was investigated by covering either one or both cotyledons with aluminium foil, the edges of which were folded so as to ensure complete exclusion of light. These treatments were applied 5 d after the seedlings were transplanted into the pots, and when the hypocotyls were 2–3 cm long.

Measurements

The length of the hypocotyls, from the cotyledonary node to the junction with the root, was measured to the nearest mm with a ruler. Their curvature after 4 h was measured with a protractor from projected images of photographic negatives. The transpiration rate of seedlings with covered or illuminated cotyledons was determined by weighing the pots after sealing the drainage holes with paraffin wax and covering the vermiculite with aluminium foil, which was sealed around the rim of the pot with vaseline, and around the base of the hypocotyl with lanolin.

The ψ_{PX} of the hypocotyl was measured using the pressure chamber technique (Scholander *et al.*, 1965). The chamber was lined with wet blotting paper, and the seedling was wrapped in a double layer of moist cheesecloth immediately before cutting the hypocotyl to minimize errors due to water loss from the shoot after excision (Turner and Long, 1980). The hypocotyl was cut 3–4 cm below the cotyledonary node. In comparing the ψ_{PX} of plants with both cotyledons either covered or illuminated, the cut end of the hypocotyl was observed at $\times 12$ magnification under a dissecting microscope during pressurization. The pressure was increased at 0.02 MPa s^{-1} . The balance pressure was defined as the pressure at which sap had appeared at the cut end of all of the six vascular bundles visible in the transverse section (Fig. 1A).

In comparing the ψ_{PX} of vascular bundles connected to the covered and illuminated cotyledons on the same plant, the vascular anatomy of the hypocotyl was first examined in serial transverse sections of excised hypocotyls fixed and stored in FAA (formalin/acetic acid/50% ethanol; 5:5:90, by vol.) The sections were cut by hand, stained in a 0.5% solution of safranin and stored in a 50% aqueous solution of glycerin. The subsequent ψ_{PX} measurements were confined to the pair of vascular bundles on opposite sides of the hypocotyl, since these were directly connected with the mid-rib of opposite cotyledons (see below). Since preliminary measurements showed that the difference in ψ_{PX} between the bundles connected to the illuminated and covered cotyledons was quite small, the accuracy of the measurements was increased by using a more sensitive pressure gauge graduated in 0.002 MPa divisions and

with a maximum range of 0.4 MPa. Before excising the hypocotyl, a black line was drawn down the side beneath the covered cotyledon with a felt-tipped pen. The ink was visible on the epidermis when the cut end of the hypocotyl was examined under the microscope, thus enabling the two vascular bundles supplying the covered cotyledon to be distinguished from those on the opposite side. During the ψ_{PX} measurements, the pressure was increased at 0.002 MPa s^{-1} until sap had appeared at the cut ends of both bundles on the covered side. This usually occurred in both bundles at almost the same time. After the balance pressure had been recorded, pressurization was resumed and continued until the same end point was reached by the two bundles supplying the illuminated cotyledon.

A comparison was also made of the water content of samples of peripheral tissue taken from below the illuminated and covered cotyledons. The cotyledon treatment and environmental conditions were as described above. Prior to sampling, the side of the hypocotyl below the covered cotyledon was identified by marking it with a thin line using a ball-point pen. The hypocotyl was then severed from the roots and a segment 2 cm long, and 0.5–2.5 cm below the cotyledonary node, was excised from the apical half. A cutting device, made by taping a pointed scalpel blade to either side of a spatula, was used to make parallel cuts 1.1 mm apart in the segment on the side below either the covered or illuminated cotyledon. A tissue sample of this width was then stripped from the segment with fine forceps. The sample, comprising the epidermis and two to three layers of cortical cells, was immediately weighed to $\pm 1 \mu\text{g}$ on a microbalance. While the first sample was being weighed (approximately 30 s), the hypocotyl segment was kept in a high humidity by placing it on a piece of aluminium foil in a 5 cm diameter Petri dish lined with wet filter paper. The other side of the segment was then sampled. The order in which the opposite sides were sampled was alternated with each replicate to avoid any time factor effect. In the last of four replicate experiments, the sampling technique was modified by stripping the samples from the intact plant after delimiting the ends of the sample with shallow transverse cuts 2 cm apart. The purpose of this modification was to eliminate any possible effects of hypocotyl excision on the lateral redistribution of water. The method was otherwise the same as in the previous replicates. All samples were dried at 80°C for 24 h and their dry weight determined.

Replication and statistical analysis

All experiments were repeated at least once with similar results. The statistical significance of the difference between treatment means was determined by Student's *t*-test.

Results

Covering both cotyledons reduced seedling transpiration rate by 62% (Table 1). This effect was associated with a 57% increase in the ψ_{PX} of the hypocotyl, and a 62% increase in the length of the hypocotyl during the 2 d treatment period. When the experiment was repeated, the corresponding percentages for these effects were 55%, 59% and 62%, respectively. When only one cotyledon was covered, curvature of the hypocotyl away from the side of the covered cotyledon was initiated after about 3 h. The mean curvature ($\pm \text{se}$) after 4 h in two replicate

Table 1. Effect of covering both cotyledons on seedling transpiration rate, and associated effects on the ψ_{PX} and growth of the hypocotyl

Treatment of cotyledons	Transpiration ($\text{g H}_2\text{O plant}^{-1} \text{ 2 d}^{-1}$)	Hypocotyl	
		ψ_{PX} (MPa)	Growth rate (mm 2 d^{-1})
Illuminated	$5.07 \pm 0.51\text{a}$	$-0.21 \pm 0.009\text{a}$	$7.8 \pm 0.68\text{a}$
Covered	$1.94 \pm 0.22\text{b}$	$-0.09 \pm 0.004\text{b}$	$20.7 \pm 1.44\text{b}$

All data were recorded 48 h after treatment, and are mean values $\pm \text{se}$ ($n=10$).

Within each column, means followed by different letters are significantly different at the 1% level.

experiments ($n=10$) were 19.8 ± 2.1 and 19.3 ± 2.0 , respectively.

Examination of serial transverse sections of the hypocotyl showed six well-defined vascular bundles in sections cut between 3.0 and 3.5 cm below the cotyledonary node (Fig. 1A). Two of the bundles (Fig. 1; 1, 2) were connected directly with the mid-rib of the cotyledon on that side, while the two opposite bundles (Fig. 1; 3, 4) were connected to the midrib of the other cotyledon. At a higher level (Fig. 1B, C) each of these bundles branched

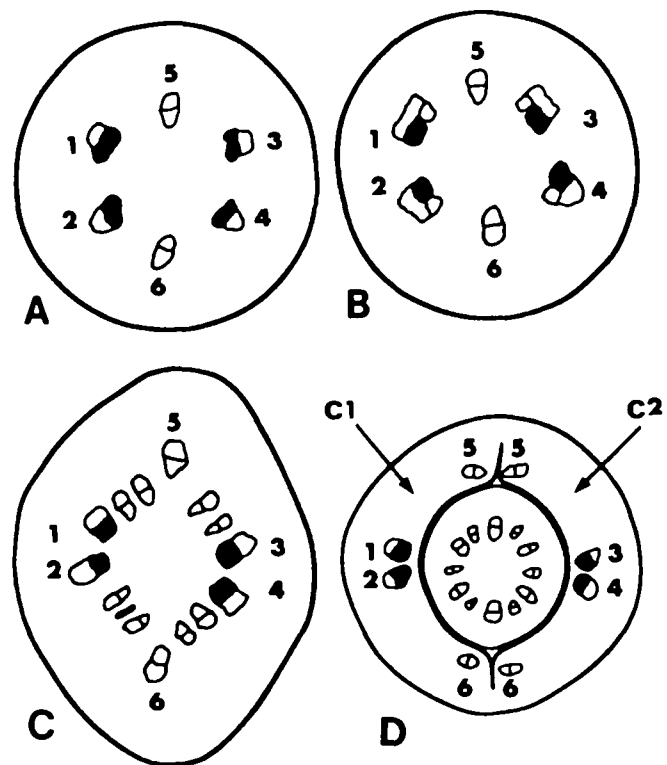


Fig. 1. Diagrammatic illustration of the distribution of the vascular bundles in transverse sections of the hypocotyl of the sunflower seedling at 30 mm (A), 25 mm (B), 1 mm (C), and 0 mm (D) below the cotyledonary node. The xylem of the vascular bundles used for ψ_{PX} measurements (A; 1, 2 and 3, 4) is shaded. Note that these bundles are directly connected to the midrib of opposite cotyledons (D; C1, C2). Magnification: A–C, $\times 17$; D, $\times 11.5$.

Table 2. Comparison of the ψ_{PX} of vascular bundles supplying illuminated and covered cotyledons on the same plant in two sunflower cultivars

Cultivar	Experiment number	ψ_{PX} (MPa) of vascular bundles supplying	
		Illuminated cotyledon	Covered cotyledon
CM 90 RR	1	$-0.159 \pm 0.008a$	$-0.147 \pm 0.008b$
	2	$-0.174 \pm 0.007a$	$-0.159 \pm 0.007b$
CM 38	1	$-0.249 \pm 0.005a$	$-0.235 \pm 0.005b$
	2	$-0.261 \pm 0.009a$	$-0.239 \pm 0.010b$

The location of the vascular bundles used for ψ_{PX} measurements is shown in Fig. 1A. In each experiment, means followed by different letters are significantly different at the 1% level. ($n=10-14$).

to produce additional bundles which formed a ring in the stem just below the cotyledonary node (Fig. 1;D). The path of these axial bundles above the node was not investigated, but it may be assumed that they provided the vascular connections to the first pair of true leaves, which varied from 0.5–1.5 cm in length, and also to the younger leaves at the shoot apex.

As noted above, measurements of the ψ_{PX} of the vascular bundles supplying the illuminated and covered cotyledons on the same plant were restricted to the pair of bundles on either side of the hypocotyl (Fig. 1; 1, 2 and 3, 4). The two bundles located between the cotyledons (Fig. 1; 5, 6) could not be used since both branched just below the node, the two branches entering opposite cotyledons as lateral veins (Fig. 1D). The measurements showed that the ψ_{PX} of the bundles supplying the covered cotyledon was higher than that of those supplying the illuminated cotyledon (Table 2). All the differences were small, varying from 0.012–0.02 MPa and from 5.6–8.5% over the four experiments, including both sunflower cultivars. The ψ_{PX} of the bundles in cv. CM 90 RR were lower than for cv. CM 38 in both experiments.

The water content of samples of peripheral tissue from the region of the hypocotyl below the covered cotyledon was higher than from below the illuminated cotyledon in all four replicate experiments (Table 3). This difference

Table 3. Dry weight and water content of peripheral tissue from below the illuminated and covered cotyledons on the same plant

Experiment number	Cotyledon treatment	Hypocotyl sample	
		Dry weight (mg)	Water content (% dry weight)
1	Illuminated	$0.333 \pm 0.016a$	$849 \pm 34a$
	Covered	$0.338 \pm 0.022a$	$916 \pm 42b$
2	Illuminated	$0.246 \pm 0.016a$	$774 \pm 63a$
	Covered	$0.273 \pm 0.016a$	$852 \pm 34b$
3	Illuminated	$0.398 \pm 0.042a$	$791 \pm 38a$
	Covered	$0.428 \pm 0.033a$	$852 \pm 29b$
4	Illuminated	$0.404 \pm 0.030a$	$690 \pm 41a$
	Covered	$0.483 \pm 0.043b$	$767 \pm 49b$

Means followed by different letters are significantly different at the 5% level ($n=9-12$).

ranged from 7.7–11% over the four experiments, and was consistently significant at the 5% level. The dry weight of the samples from below the covered cotyledon was also higher than from below the illuminated cotyledon in all the experiments. This difference was only significant at the 10% level in experiments 2 and 3, but reached the 1% level in experiment 4.

Discussion

These results are consistent with the hypothesis that hydraulic signalling plays a major role in the effect of darkening the cotyledons on the growth and curvature of the sunflower hypocotyl. This conclusion is supported by the similarity between the reduction in transpiration induced by covering the cotyledons and the associated increases in the ψ_{PX} and rate of elongation of the hypocotyl (Table 1). It also agrees with previous investigations cited in the Introduction, which provided similar evidence that systemic, growth-inhibiting effects of leaf illumination are mediated by the transmission to the stem of transpiration-induced reductions in leaf ψ_L . The sensitivity of this mechanism was well illustrated by Woodward (1981) who showed that, when plants of *Circaea luteciana* were growing in partial tree cover, rapid reductions in their ψ_L in response to brief periods of sunfleck exposure, were followed within 2 min by reductions in stem elongation. However, the postulated role of transpiration in the present investigation would seem to disagree with results reported by Black and Shuttleworth (1974), who found that enclosing illuminated cotyledons of *Cucumis sativum* in transparent (polyethylene) envelopes did not promote hypocotyl elongation. It was therefore concluded that the increased elongation induced by covering the cotyledons with opaque material e.g. aluminum foil, could not be attributed to any 'interference with gas exchange'. It seems likely, however, that the temperature of the illuminated cotyledons, when enclosed in transparent plastic, would be significantly increased and that, if the envelopes were not completely sealed, this temperature effect may have counteracted any transpiration-reducing effect of the increase in ambient humidity. Unfortunately, no data were given on the effect of the plastic covers on transpiration.

In the present investigation, we postulated that, when both cotyledons were covered, the increase in the rate of hypocotyl elongation was caused by the associated increase in ψ_{PX} . On this hypothesis, one might expect that the asymmetrical growth which causes curvature of the hypocotyl when only one cotyledon is covered (Lam and Leopold, 1966) would be correlated with an asymmetry in ψ_{PX} distribution. Evidence of this effect was provided by the measurements of the ψ_{PX} of the vascular bundles (Table 2), which showed that the ψ_{PX} of the two bundles supplying the covered cotyledon was higher than that of

the bundles supplying the illuminated cotyledon. The magnitude of this difference, which ranged from 0.012–0.02 MPa, is likely to have been reduced by the occurrence of some degree of equilibration between the bundles when the hypocotyl was excised, and during subsequent pressurization. The difference may also have been reduced by the influence of the ψ_{PX} of the axial bundles which were connected with the measured bundles above the point at which the ψ_{PX} measurements were recorded (Fig. 1B, C). However, while the measured difference in ψ_{PX} between the bundles supplying the opposite cotyledons may thus be considerably less than was present in the intact plant, it does provide evidence that differential illumination of the cotyledons causes a lateral asymmetry in ψ_{PX} distribution within the hypocotyl.

Further evidence of this effect was provided by the comparison of the water content of the peripheral tissues of the hypocotyl from below the illuminated and covered cotyledons. In contrast to the measurements of the ψ_{PX} of the vascular bundles, which had to be made at least 3 cm below the cotyledonary node to facilitate the use of the pressure chamber technique, the samples of peripheral tissues were taken from 0.5–2.5 cm below the node, and thus included the region of maximal elongation rate of the sunflower hypocotyl (Beck, 1941). These measurements showed that the water content of the peripheral tissues from below the covered cotyledon was considerably higher than from below the illuminated cotyledon on a dry weight basis. When considered in relation to the effect of the same cotyledon treatment on the ψ_{PX} of the vascular bundles, this result is consistent with the hypothesis that covering either one or both cotyledons promotes growth and curvature of the hypocotyl by increasing the ψ_w gradient, and hence the transport of water, from the xylem to the peripheral cell layers. A similar growth-promoting effect of increases in ψ_{XY} can account for the rapid growth response of the sunflower hypocotyl to root excision (McIntyre and Boyer, 1984), the triggering effect on axillary bud growth by stem decapitation in beans (*Phaseolus vulgaris* L.) (McIntyre and Damson, 1988) and by leaf excision in milkweed (*Asclepias syriaca* L.) (McIntyre and Hsiao, 1990), and the induction of systemic increases in leaf thickness by localized leaf injury in a wide range of species (Boari and Malone, 1993). The diversity of these responses is evidence of the important role of ψ_{PX} and hydraulic signalling in the regulation of plant development (McIntyre, 1987).

In the present investigation, the samples of peripheral tissues from below the covered cotyledon had not only a higher water content than those from below the illuminated cotyledon, but also had a higher dry weight (Table 3). Evidence of a similar effect of differential illumination on dry weight distribution was found in previous experiments, in which the shaded side of the hypocotyl of dark-grown sunflowers stimulated photo-

tropically with unilateral blue light, had a higher dry weight than the illuminated side (McIntyre, 1980). In that investigation, it was postulated that this effect may have been caused by the more favourable water status of the tissues on the shaded side, and their consequently greater capacity for growth and nutrient accumulation. This explanation may account for the similar effect of differential cotyledon illumination in the present investigation. It would also be consistent with data by Kutschera (1991), who showed that the increased rate of hypocotyl elongation when sunflowers were transferred from light to darkness was associated with an enhanced accumulation of soluble sugars in the region of elongation. There is also evidence that the supply of assimilates via the phloem, and correlated effects on the osmotic potential (ψ_s) and turgor (ψ_p) of the growing cells, are important factors in the control of hypocotyl elongation in sunflowers (Kutschera, 1994), castor bean (Meshcheryakov *et al.*, 1992) and of stem growth in peas (Schmalstig and Cosgrove, 1990). It seems likely that, in the present investigation, an increase in solute accumulation contributed significantly to the ψ_w gradient between the xylem and peripheral tissues, and thus to the promotion of hypocotyl elongation and to the phototropic response. Additional solutes may also have been provided by the accumulation of root-derived nutrients in the xylem caused by the suppression of transpiration from the covered cotyledon (Russell and Shorrocks, 1959). While this response would reduce the ψ_s of the xylem sap, an effect that would not be shown by the pressure chamber measurements recorded in the present investigation, it is likely that some of these solutes would be absorbed from the xylem by adjacent tissues in the growing region of the hypocotyl (Pate, 1980), where they would contribute osmotically to water uptake and cell elongation.

In conclusion, while the present results are consistent with the postulated role of hydraulic signalling in the effect of cotyledon shading on the growth and curvature of the sunflower hypocotyl, they have also raised some important questions for further investigation. Perhaps the most obvious need is for a critical study of the effect of cotyledon shading on the gradients of ψ_w , ψ_p , and ψ_s between the xylem and the epidermis in the growing region of the hypocotyl. Techniques that would be suitable for such an investigation have already been well established (Meshcheryakov *et al.*, 1992; Nonami and Boyer, 1993).

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