MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE COOL TEMPERATURE HINDRANCE OF EARLY FIBER ELONGATION OF COTTON USING OVULES CULTURED *IN VITRO* AS A MODEL SYSTEM

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Reaching this point of my career while writing this dissertation, I am reminded of the many emotions I have felt as a result of my efforts to obtain the degree of Ph. D.. I have felt excitement, fear, frustration, relief, happiness, and regret. I also experienced excitement at the prospect of coming to a new place with the opportunity to receive a higher degree and fear of the challenges that awaited me. Frustration stemming from many months of effort without results was followed by relief and happiness when publishable results were finally obtained. I regret that I have put my education in front of the needs of my family who remained in China and was not able to spend more time with them. There is happiness because after years of struggling and bewildering I will finally receive this degree, which many people believe requires too much work and dedication for the rewards received. I have been challenged because I am a foreigner to this country and I have had to adjust to a different culture and learn a new language.

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ABSTRACT

Cool temperatures limit cotton yield and fiber quality. The effect is especially severe in major temperate cotton-producing regions such as the Texas High Plains. Cotton (*Gossypium hirsutum* L. cv Acala SJ-1) ovules cultured *in vitro*, were used to study the effects of cool temperatures on fiber initiation and elongation. Ovules, which produce normal fibers as *in planta*, were cultured 1 day before or on the day of anthesis under constant 15°C or 34°C or 12 hr/12 hr cycling at 15/34°C or 34/15°C. The similar initiation patterns observed by scanning electron microscopy (SEM) *in vivo* and *in vitro* confirm previous evidence that cultured ovules are valid models for fiber development research. SEM and ruler measurement data indicated that there are three stages of fiber elongation as distinguished by different temperature responses: initiation, early elongation to attain a length of 0.5-3 mm, and later elongation to attain the genetically determined potential length. Initiation and early elongation were delayed 2-3 days by the low cycling temperature, but later elongation occurred at similar rates under cycling and constant 34°C regimes. The results provide direct evidence to confirm hypotheses derived from field data. Further comparative analyses included determination of peroxidase activity and of profiles of newly synthesized proteins labeled with ^{35}S-methionine to elucidate mechanisms underlying the initial delay and eventual acclimation in the elongation rate. Peroxidase activity was developmentally delayed and constantly depressed under 15/34°C and 15°C compared to 34°C. One and two dimensional gel fluorographs of proteins labeled at the 34°C side showed that the 15/34°C cycling caused a developmental delay in the change of particular proteins, which correlated temporally with the temperature-induced difference in timing of fiber initiation and early elongation, but no new proteins were detected by the labeling at the warm side of the cycle. However, 15°C specifically enhanced and suppressed the synthesis of some proteins, reduced total protein synthesis, and caused differences in mRNA populations. Other labeling experiments demonstrated that a 15°C-induced unique 12 kD protein was degraded at 34°C; this protein may aid fiber elongation recovery under the cool cycling regime. In contrast, only a few high molecular weight proteins synthesized at 34°C showed slight reduction in intensity upon shifting into 15°C. Therefore, degradation of proteins synthesized at 34°C by 15°C is not major cause of initial elongation delay. We concluded that the primary effect of 15°C is to inhibit and delay the synthesis of a set of proteins that may be important in normal fiber development.

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ABBREVIATIONS

cpm	counts per minute
CA	cold acclimation
dac	day after culture
dpa	day postanthesis
GA	gibberellic acid
IAA	indole-acetic acid
IEF	isoelectric focusing
MW	molecular weight
OD	optical density
1D or 2D	one or two-dimensional gel electrophoresis
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Trichloracetic Acid
^{35}S-a.a.	^{35}S-methionine/cysteine

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Being the outermost layer, the plant cell wall has to carry out several strategic roles, including constraint of expansion of the pressurized protoplast within. Therefore, cell walls regulate the rate and direction of cell expansion and, ultimately, plant growth. It is usually the wall that controls how fast and in which direction the cell will expand. Primary cell walls are laid down by cells that are still growing, and it is these primary walls that control cell growth. Secondary walls are synthesized inside primary walls by some cells to facilitate specialized functions that require high wall strength. The plant cell wall is composed of cellulosic microfibrils embedded in an amorphous matrix of non-cellulosic substances consisting mainly of pectic and hemi-cellulosic polysaccharides, proteins, and sometimes, lignin. During growth of the cell, polymers of the wall interact and change, and the resulting alteration in the properties of the wall can be correlated with a variation in its function. Since cotton fibers grow relatively synchronously, they are a unique tool for fundamental research in botany, especially suitable for studies of basic processes such as cell differentiation, cell expansion, the structure and mode of synthesis of cell walls, and the function of cell walls during growth (2). Furthermore, the economic importance of the cotton fiber crop and remaining problems with its efficient production under natural environments provide the impetus to determine the critical factors that control its development.

1.1 Chronology of Cotton Fiber Development

A mature cotton fiber is a single elongated epidermal cell of the ovule with a thickened secondary wall. The development of the cotton fiber can be divided into several stages: fiber cell determination, initiation, elongation, secondary wall thickening, and maturation (22, 51, 100). Fibers developing *in vitro* on ovules cultured according to the method of Beasley (10, 11) have been valuable models for research on fiber development because they develop similarly to the *in vivo* condition (67), although few fibers reach complete maturity and length as observed *in vivo*.

The early development of fibers consists of two integrated steps designated as spherical expansion above the ovular epidermis and elongation (99). The events of initiation and elongation are overlapping on the individual ovule and are not unambiguously distinct from one another for an individual fiber. To facilitate understanding of the

following discussion, which will concentrate on the initiation and early elongation phase of fiber development, some terminology is defined as follows:
(1) determination refers to the process by which some epidermal cells on the cotton ovules become fiber primordial cells (also called pre-fiber cells) that have the physiological capacity to become fibers under appropriate conditions; (2) initiation refers to the protrusion (out-growth) of pre-fiber cells on the surface of the ovule; and (3) elongation means the continued growth of the initiated fiber cells.

1.1.1 Fiber Determination

Ovule epidermal cells are closely packed, cuboidal, rich in cytoplasm, and contain a large nucleus (52). The natural time of fiber cell determination is believed to be between -3 and -2 days pre anthesis (dpa), when potential fiber cells of the ovule epidermis acquire the physiological capacity to elongate in response to indole-3-acetic acid (IAA) and/or gibberellin (GA). The evidence for this is that ovules cultured *in vitro* from -3 to -1 dpa, but not before, produce fibers upon addition of exogenous hormones, and that the amount of fiber production and the numbers of fiber-producing ovules increase with the ovule age (37, 40). The determination event is associated with changes in protein profiles as determined by silver-stained two-dimensional (2D) gel electrophoresis (37, 39). The prefiber cells could be ultrastructurally discriminated from other epidermal cells as early as 16 hour (h) preanthesis by cell and nuclear enlargement and by an increase in cytoplasmic density due to the release of phenolic substances from the vacuole (81). In non-differentiating fiber cells, the phenols are retained in the vacuole. The phenolic compounds are o-diphenols that presumably inhibit IAA oxidase to allow an intracellular auxin level high enough to initiate fiber differentiation (75). An increased number of ribosomes and rough endoplasmic reticulum in differentiating fiber cells suggests a greater capacity for protein synthesis at anthesis (81).

Fiber cells receive nutrients from the outer layer of the seed coat underlying the epidermis, which is several cells thick and supplied with vascular tissue. Fiber cell determination and initiation are clearly not induced by the pollination-fertilization process but are a part of the total flower-formation mechanism, accompanying expansion of the petals and elongation of the style and anthers before anthesis (22). Although all epidermal cells (except the stomatal guard cells and cells comprising the micropyle) are potential fibers, not all the cells differentiate into fiber initials (5, 99). Fiber density is about 3300 per mm^2, and the ratio of fiber-forming cells to the total numbers of epidermal cells is

about 1:3.7 at anthesis. Fibers do not occur in a regular pattern (5, 99). The mechanisms controlling fiber cell differentiation remain mysterious, but are interesting for theoretical and practical reasons.

1.1.2 Initiation

The majority of fibers are initiated by the morning of the day of anthesis (22, 100). The initiation is characterized by a spherical expansion above the ovular epidermis, marking the onset of fiber growth (5, 99). Expansion of lint fibers starts at the crest of funiculus and spreads around the lateral circumference of the ovule, with a few hours delay at the chalazal end, and continues to 4 - 5 days at the extreme end of the micropylar region, presumably along a hormone gradient (99). The foot of a fiber initial is associated with the vascular system, which suggests that transport of phytohormones and nutrients may be involved in fiber initiation. Furthermore, the distribution of the vascular system around the ovules suggests a possible role in formation of the gradient of initiation on the ovule surface (37). Alternatively, there may be an inhibitor of fiber initiation/elongation in the micropylar region, although a possible candidate, ABA, was equally detected in both the chalazal and micropylar ends of the ovule (37). The gradient in the pattern of initiation is highly correlated with the variation in length of mature fibers on individual ovules. The fibers that initiate early are destined to become lint (2-3 cm in length), whereas epidermal cells that initiate elongation in subsequent waves from 5 to 10 dpa result in short fuzz (2-5 mm) fibers (3, 52). The time of fuzz fiber initiation corresponds to the time that epidermal cell division ceases (100). Fuzz fibers have little commercial value and adhere to the seed during ginning. Both the range in time of the initiation and extent of fuzz formed vary with *Gossypium* species and cultivars. The physiological and biochemical restrictions leading to fuzz fiber differentiation are unknown. Attempts to culture ovules from the ligon lintless mutant, which has only dense fuzz fibers (55), to induce lint fiber growth have not succeeded. However, ovules from a conditional fiberless mutant of cotton *G. arborerum* L. permit fiber growth if cultured at 30°C and below, but not at 32°C (8). Apparently, there are genetic controls regulating fiber determination and initiation that are not related simply to addition of phytohormones to the culture medium.

1.1.3 Elongation

After initiation, the fiber initials enter the elongation phase immediately. The transition to the elongation phase starts slowly as the blunt tipped cells begin to grow

toward the micropylar end (5, 99). At 1-2 dpa the fibers twine into groups and their tips become tapered, apparently because the rate of longitudinal growth exceeds the rate of diametric expansion, which is restricted by the orientation of cellulose microfibrils transverse to the long axis of the cell (for review, see Seagull, 1986) (97). The elongation continues for 24-28 d to reach a final length of 2-3 cm for a fiber with approximate diameter of 20-50 um (22). The growth rate *in situ* reaches a maximum around the 8th to 10th dpa, diminishes during maximum secondary wall thickening, and is almost zero at 30 dpa (57, 96). Prominent nucleoli were not observed in nuclei after 10 dpa. The amount of ribosomes that are synthesized in very early stages of fiber development may determine the degree of elongation as well as the thickness of the fiber (82). Elongation is driven by a negative water potential generated by a high vacuolar concentration of potassium malate (23). Growth occurs throughout the length of the fiber, but is more rapid at the tip (91). There is considerable overlap between later stages of elongation and the secondary wall thickening phase (67, 96), with the degree of overlap being related to cultivars (56) and the temperature regime under which the fiber is developing (45). Potential fiber length is a genetic attribute, but realized fiber length is the product of the rate of elongation per day and the total period of elongation.

1.1.4. Secondary Wall Thickening and Fiber Maturation

Secondary wall synthesis is generally considered to begin at 16 dpa (2, 100), with a range of at least 12 to 18 dpa, varying with cultivars and temperature regime (45); it completes around 40-50 dpa (51, 67, 96). While elongation via primary wall synthesis is occurring at a high rate, the contribution of cellulose to the weight/fiber length is small. A sharp increase in the rate of cellulose synthesis occurs around 16 dpa and is followed by a greater increase in rate around 28 dpa (67). The fibers at early stages of secondary wall deposition continue to elongate primarily by tip growth, and the cellulosic microfibrils shift to a helical orientation along with the distinct increase in birefringence typical of secondary walls (67). The entire period of secondary wall formation, which typically lasts until day 40-50, is marked by the deposition of almost pure cellulose in the wall.

By 50-70 dpa, depending on environmental conditions, the ovary wall splits and opens along locular suture lines, leaving seeds and fibers exposed in the cotton boll (5), which causes the fibers to dry and collapse as spiral twisted ribbons. The degree of secondary wall thickening and the angle of spirals relate to micronaire and fiber strength, respectively, and thereby to the quality of the fiber. The quality of fiber is important to the

spinning and weaving performance and is determined by several fiber parameters: staple length, tensile strength, and fineness. Thus, good quality cotton should be long, strong, and fine. Independent genetic control of fiber elongation and secondary wall deposition makes it possible to dissociate them so as to alter either elongation or dry weight increase of fibers (56). These fiber properties also are greatly influenced by environment and environment-genotype interactions (33, 34, 35, 56, 104). This provides a window of opportunity to improve fiber traits through selection for high quality varieties, by cultivation practices, and by targeted manipulation of the cotton genome.

1.2 Plant Growth Substances Regulate Fiber Initiation and Elongation

Numerous studies and considerable evidence indicate that hormones play a decisive role in fiber development [for a detailed review, see Beasley (3) and (57)]. These studies were greatly facilitated by development of the ovule culture system (10, 11). *In vivo*, pre-fiber cells appear to be in a latent state awaiting an elongation stimulus (40) or an inhibitor repressor presumably provided by the phytohormones synthesized during anthesis or included in *in vitro* culture medium. Fertilized ovules cultured at 1-2 dpa can grow fiber without growth substances, but fiber production is markedly increased by the addition of GA (4, 10). The unfertilized ovules, on the other hand, require addition of IAA and /or GA to the basal medium to produce fiber, with IAA providing greater fiber development. Hence, auxin is the major relevant hormone produced during the process of fertilization, whereas GA is probably derived mostly from sources external to the ovule (11). The effects of simultaneous additions of IAA and GA are approximately additive and occasionally synergistic on fiber production (11). Differences in optimal hormonal treatment exist between fiber initiation and elongation. GA can induce the initiation of fiber, but can not stimulate as much elongation as when an auxin also was included in the medium (8). Auxin alone induces the initiation of fewer fiber initials per ovule than GA, but affords a greater subsequent elongation (57). The necessity of GA during initiation was shown clearly by the demonstration that exposure to GA for 1 day before transfer to IAA was sufficient to induce equal fiber production compared to continuous culture (12). Later reports suggest that IAA may be of greater importance to fiber elongation and wall thickening than gibberellins, with initiation and ovule growth being predominantly determined by GA [for review see (2, 57)]. Other primary stimuli for fiber initiation and elongation provided by fertilization are excluded since only auxin and GA are required for fiber growth on unfertilized ovules cultured in a defined mineral medium (11). However,

the data do not exclude the possibility that these exogenous growth regulators induce other essential ones to be synthesized endogenously. While promoting the program of elongation, IAA and GA are not necessarily involved in the determination step because only epidermal cells that have differentiated into fibers can respond to the influence of IAA and GA (37).

ABA counteracts the effects of IAA and GA (11) and inhibits *in vitro* fiber growth, in part by interfering with malate metabolism but only when it is applied during the first 4 days of culture (24). Ethephon, an ethylene-releasing growth regulator, inhibits normal ovule and fiber development and induces callusing (50). Ethylene causes excessive ovular callus formation in combination with GA and reduces the percentage of ovules forming fibers in response to IAA (7). These past studies suggest that phytohormones probably function as signal or regulatory molecules to trigger a series of physiological and biochemical events that finally lead to fiber initiation and elongation. However, it is well known that separate phytohormones have overlapping functions and that the range of physiological activities of a given phytohormone is usually extensive. The regulation of fiber development is not likely mediated by the independent role of any single hormone, but by subtle, coordinated interactions among several of them and possibly other unidentified substances.

1.3 Responses of Cotton Fiber Growth to Low Temperatures

In addition to phytohormones, many other physical and physiological factors are known to affect fiber initiation and elongation. For example, osmotic pressure, mainly due to the accumulation of K^+ and malate, is the driving force for fiber elongation (23), and exogenously supplied CO_2 inhibits callus formation and promotes fiber growth especially when parental plants are under stress (111). In the field, plants are subjected to a wide range of environmental conditions and each environmental factor has a minimum and maximum level beyond which a plant cannot survive (63). Cold temperature in the range of 0-15°C is one of the such important factors governing the distribution and productivity of cultivated plants, particularly limiting agricultural use in temperate regions of plants native to subtropical or tropical regions (18).

Cool temperatures hinder cotton growth and development from seed germination through boll maturation. The effects are particularly severe on the Texas High Plains because of the sharp temperature drops below 20°C during most nights of the growing season (see (32) for review). Although somewhat species and genotype dependent, 15°C

is generally the minimum temperature required for vegetative growth and fiber elongation (78). Temperatures below 20°C prolong the elongation period, decrease overall elongation rate, and thus can reduce final fiber length if the growing season is limited. The rate and amount of cellulose synthesis are similarly affected (32, 33, 34, 35). Corresponding results have been obtained recently for fibers elongating on ovules cultured after fiber initiation under low cycling temperature regimes (34/22°C or 34/15°C, 12 h/12 h cycle) (45), supporting the validity of using cultured ovules as a model system for studying the effects of temperature on fiber development. Temperature, like GA, increased both the number of ovules producing fiber (in response to IAA) and the amount of fiber produced per ovule, with 34°C being optimum (6). The ability of unfertilized ovules to produce fibers in response to IAA at temperature below 28°C compared to 34°C controls was markedly increased by including NH_4^+ in NO_3^- based medium and increasing IAA concentration. Improved fiber production by increase in temperature or inclusion of NH_4^+ and extra GA presumably occurs via mechanisms that promote IAA uptake in each case (6). The effects of these changes on fiber development are independent of the availability of reduced nitrogen as a general substrate for growth (9).

The cool temperature-induced decrease in overall elongation rate could be due to a similar reduction throughout the time-course or to more specific inhibition at certain developmental stages. The temperature sensitivity of higher plants is not constant throughout their life cycle (63). Based on data from field plants of several varieties grown with controlled night temperatures, Gipson and co-workers suggested that the initial stages of fiber elongation up to 15 dpa were extremely sensitive to cool temperature, whereas the later stages were more temperature independent (34, 35). They suggested that this early sensitivity was due more to delay in onset than to subsequent decrease in rate of fiber growth. Stewart (100) reanalyzed some of these data to support the contention that cool temperatures delay onset of fiber initiation, but that the subsequent early stages of elongation (before a fiber was 6 mm long) were less temperature-sensitive than later stages. In agreement with the original interpretation of Gipson and co-workers, the time-course of lengthening of *in vitro* fibers on ovules cultured at 1 dpa (after initiation occurs *in vivo*) suggested that early elongation until the fiber was about 3 mm long (which occurred from day 6-12, depending on temperature regime) was more temperature-sensitive than later stages (45). Even under 34/15°C cycling, the rate of elongation eventually recovered to that of the control, although the length on a given day was always reduced due to the early delay. Compensating for the early developmental delay, the elongation period was

prolonged so that the same final length was attained under 34°C or 34/15°C cycling (45). None of the previous data allowed clear discrimination of cool temperature effects on fiber initiation compared to early elongation because cool temperatures were not applied controllably and comparatively at different stages, and fiber measurements with a ruler did not begin until 5-6 dpa at the time when fibers were at least 1 mm long. Therefore, how low temperature affects fiber initiation or the onset of the elongation were not determined until the research described here.

1.4 Plant and Cell Responses to Low Temperatures

1.4.1 Change in Protein Profiles

Exposure of many plants to low, non-freezing temperature results in increased freezing tolerance, a processes known as cold acclimation (CA) (38, 41). Numerous studies have demonstrated that a variety of changes occur during CA, including alterations in membrane protein (71) and lipid (74) compositions, increased soluble sugar (sucrose) and protein, and appearance of new isoenzymes ((41, 105), for review) and messenger RNAs (44). However, yet to be determined is whether any of the these responses have specific roles in protecting plants from damage by cold and freezing temperatures as opposed to being part of the stress response causing a metabolic adjustment to low temperature. Although evidence is increasing that altered gene expression, as measured by transcript abundance, during CA is correlated with the degree of cold or freezing tolerance (31, 46, 64, 72, 110), relatively few cold-regulated (COR) proteins have been assigned particular functions. Certain COR-polypeptides from spinach and cabbage have cryoprotective properties in *in vitro* assay (42). The level of COR genes in alfalfa correlated positively with freezing tolerance (110). Another COR gene, *kinl*, isolated from *Arabidopsis* encodes a polypeptide similar to type I fish antifreeze proteins (58). One of the cDNAs isolated from low temperature treated tomato fruit was a thiol protease, which is speculated to degrade cold-denatured proteins (94, 95). Recently, a group of COR-proteins from *Arabidopsis* showed a common feature of heat stability, and cDNA clones were identified that encoded polypeptides with a sequence homology to certain boiling-stable LEA (late embryogenesis abundant) proteins that are responsive to ABA and water stress (31, 64). Elucidation of the molecular mechanism involved in generating cold tolerance is a major effort in CA research.

To date, most work has been dedicated to species resistant to chilling but sensitive to freezing, and few data are available for any species sensitive to non-freezing, low

temperatures (18). The resistance of cotton seedlings to 5°C, which develops rhythmically as well as being induced by low temperatures (below 25°C), coincides with an increased level of polyunsaturated fatty acid (66, 88). Enrichment in polyunsaturated fatty acids is associated with enhanced membrane fluidity under cool temperature (63, 74). In cultured ovules cycled to 15°C, respiration recovered immediately upon rewarming, while the rate of cellulose synthesis required several hours for full recovery to the control 34°C level (90). This result suggests that activities of enzymes responsible for cell wall synthesis are inhibited by low cycling temperature. Because of its major commercial value and interesting botanical feature as an outgrowth of a single epidermal cell of an ovule, it is important to determine the mechanisms by which cotton fiber development is affected by and/or can acclimate to cool temperature. Information is just becoming available on the physiological and biochemical aspects of the responses of cotton fibers to low temperature (45, 90).

1.4.2 Peroxidase versus Fiber Development under Low Temperatures

As an integrated part of plant response to stress, activities of many key enzymes are regulated. Peroxidase activity, for instance, was increased by ethylene (27), wounding (60), and cold temperature (77). Obviously, activities of some enzymes are mediated during fiber development and elongation adaptation. Both peroxidase and IAA oxidase activities were greatly reduced around the day of anthesis and sharply increased during secondary wall thickening (85, 86, 103), which may well relate to the requirement for IAA to achieve fiber initiation and elongation (10, 11). In addition, peroxidase has been suggested to regulate IAA levels by acting as an IAA oxidase in cotton plants (27), and ethylene induced an increase in peroxidative IAA oxidase in cotton (73). All these results indicate that peroxidase may play important roles in cotton fiber development.

Peroxidases (EC, 1.11.1.7) are a class of metalloprotein enzymes containing porphyrin-bound iron which, in the presence of hydrogen peroxide, catalyze the oxidation of a variety of organic compounds. This family of isozymes is widely distributed in the plant and animal kingdoms (106) and has been proposed to mediate several plant processes such as host defense (62), cross-linking of hydroxyproline-rich glycoprotein monomers in the cell wall (28, 30), cross-linking of pectic polysaccharides with phenolic acids in the cell wall (29), lignification (61), phenol oxidation, auxin oxidation (47), and the regulation of cell elongation (36). The phenolic cross-links between matrix polymers could reduce the extensibility of growing cell walls and control the rate of cell growth. This mechanism

would require a negative correlation between wall peroxidase activity and growth rate. These varieties of function are consistent with the existence of numerous peroxidase isozymes, and some isozymes also might be good markers to evaluate stress effects on cell wall development (49).

Cotton peroxidase is ubiquitous in various tissues: young and mature leaves, stems, petioles, roots and bolls. Different peroxidase isozymes show a pattern of tissue-specific expression, with a major one occurring in each of the organs examined (109). Cotton ovule and cell suspension cultures release peroxidase into the surrounding media (49, 68), presumably reflecting isoforms that are normally wall-bound and/or secreted. Both cytoplasmic and wall-bound forms of peroxidase are associated with developing ovules and fibers, and their activities and expression are developmentally regulated (68, 85, 103). Expression of peroxidase mRNA determined by northern analysis indicates that ovules, fibers, and leaves of cotton share at least one isoform in common, but the level of expression varies with the tissue (our unpublished data). Recently, a cotton leaf and cotyledon peroxidase was cloned from upland cotton (89); undoubtedly, the use of these cDNA clones will aid in determining the potential *in vivo* function and regulation of these isozymes. Nothing was known before the research described here about the regulation of cotton ovule and fiber peroxidase activities under cool temperature.

1.5 The Purpose and Scope of the Research

As reviewed above, cool temperatures pose one of the greatest obstacles to maximum production of a valuable cotton crop in temperate regions because the resulting immature fibers cannot command a premium price for the grower or provide optimal spinning properties for the processor (32). Finding ways to produce more uniformly mature cotton fibers in temperate growing regions is the major need of the cotton production and textile industries. To meet this demand, a pre-requirement is to elucidate the cellular mechanisms through which low temperatures hinder elongation and fiber wall deposition and through which adaptation occurs. Several mechanisms might underlie the adaptation of fiber elongation to cool temperatures. First, cellulose synthesis for cell wall thickening may be reduced in favor of primary elongation as part of the adjustment mechanism. This may require a fundamental shift in metabolism to ensure primary wall extension during a stress. Lower micronaire value, an indication of poor cell wall development, is often caused by reducing night temperature below 25°C (32) and by severe drought and excess soil moisture (80). Second, though constant 15°C is extremely

detrimental to elongation, it may not be so as part of a cycling regime if only part of a 24 h period is used for elongation, even under constant warm temperature. Finally, exposure to 15°C could trigger adaptive mechanisms that allow faster elongation during the 15°C or the 34°C part of the cycle.

To begin to understand the underlying mechanisms of cool temperature responses, cotton ovule cultures (10) were used as a model system. The validity of using cultured ovules as model system has been established by several lines of evidence: the basic similarity of fiber developmental phases in *in vitro* cultures and field grown plants (67); the observation that, as in the field, fiber elongation *in vitro* was hindered by temperatures less than 34°C (unless additional auxin or ammonium was added) (6); and the demonstration that cycling temperatures cause similar changes in fiber length and weight accumulation *in vitro* as occur in the field (45). Also, the *in vitro* ovule culture differs from general cell suspension culture in the aspects that it supports normal fiber development and embryogenesis as *in vivo* (10, 11, 101). Furthermore, this system offers several advantages over field study by: 1) providing a much more convenient and easily manipulatable system than fibers developing on whole plants (10), thereby allowing techniques such as radio-labeling of protein and nucleic acid in developing fibers; 2) permitting monitoring of the effects of both physical and physiological factors on the very early stages of fiber development including initiation; and 3) leading to direct information on the effects of low temperature at the cell and tissue level without complexity arising from the whole plant, such as reduced assimilate synthesis and translocation that would reduce sugar available for fiber development and cellulose synthesis. Our current understanding of fiber development and the experiments herein described would not have been possible without the availability of this culture system and the experimental manipulatability and repeatability afforded by this system (4, 10, 11, 12).

The goal of this research project was to characterize in upland cotton the cool temperature inhibition of fiber initiation and early elongation with microscopical, biochemical, and molecular methods. Plants, including cell cultures, often show a specific response to environmental stresses (heat, cold and salt) by inducing new adaptive proteins, or increasing or decreasing the amount of proteins already present (26, 41, 58). Since existing cotton cultivars developed by selective breeding respond somewhat differently to cool temperature stress, one can be optimistic that further improvements could be made through targeted molecular changes. For example, the response to low temperature might possibly be improved by causing putative adaptive proteins to be expressed constitutively.

However, adaptive proteins related to this particular response and their genes first must be identified. So far, little research has been directed toward determining the mechanism of this response to low temperature despite its adverse economic consequence and fundamental importance to understanding the regulation of cell differentiation, expansion, and cell wall deposition. The three basic parts of the study listed below were carried out under control and cycling temperature conditions. They are described in detail in the following sections:

(1) Morphological characterization by scanning electron microscopy and fiber measurement.

(2) Molecular characterization by analyzing changes in protein synthesis around fiber initiation and elongation.

(3) Biochemical characterization by analysis of changes in specific enzyme activity.

CHAPTER 2
EFFECTS OF COOL TEMPERATURE ON FIBER INITIATION AND ELONGATION CLARIFIED BY ANALYSIS OF *IN VITRO* CULTURES

2.1 Overview

Both field and *in vitro* studies suggest that the early stage of fiber development is more temperature sensitive than the later stages (32, 45). These results are not complete or precise due to the variability in outdoor temperatures and the analysis of elongation being limited by ruler measurements that did not begin until about 5 dpa when fibers were >1 mm long. The early events of fiber initiation and elongation have been described previously using SEM (5, 99), but no correlation was made with the effects of environmental stress. It should be noted that the pre-fiber cell determination stage cannot be studied *in vitro*, because this step will have occurred before ovules can be successfully cultured between -2 and 2 dpa. Previous *in vitro* studies did not include analysis of the initiation step because ovules were cultured 1 to 2 dpa after initiation occurred *in vivo*. The field data indicate that this distinction is important (35, 104), although unequivocal interpretation is difficult because of the inability to determine effects on fiber initiation and elongation separately from the available data (100). The lack of common terminology to describe the early stages of fiber development also has contributed to confusion in comparison of previous reports. Missing information would, therefore, include which stage of fiber growth before 5 dpa is most sensitive to low temperature, how inhibition of early stages affects later elongation, and how low temperatures and *in vitro* culture might affect the polarity or gradient pattern of fiber initiation (99). To determine cool temperature effects on these early events directly and precisely, a logical first step was to examine with scanning electron microscopy (SEM) the initiation event under low temperature compared to constant high (optimal) temperature controls. Cycling temperatures, rather than constant cool temperatures, were chosen as experimental conditions because they are most commonly experienced by field crops in temperate regions (45). We have overcome several limitations of prior field analyses of cool temperature effects on fiber elongation by controlling temperature regimes absolutely in culture incubators, culturing ovules before fiber initiation occurs *in vivo*, and using SEM to separate cool temperature effects on fiber initiation from those on subsequent elongation. These SEM data combined with ruler measurements can provide a complete picture of cool

temperature effects on all stages of fiber elongation. This chapter is greatly related to a journal article previously published (112).

2.2 Materials and Methods

2.2.1 Plant Growth and Ovule Culture

Plants of upland cotton *Gossypium hirsutum* L. were grown in a greenhouse with natural light and temperature control for approximately 30°C days and 22°C nights. Cycling temperatures in this range are optimum for good growth and flowering of cotton (79). The results shown in this report are for cv. Acala SJ-1, although some tests also were performed on other cultivars including Paymaster 404, Coker 312, and one from Yugoslavia as described below.

Ovaries from the greenhouse-grown plants were collected in the morning (8-9 am) one day before flowering or on the day of flowering. After surface sterilization of the ovaries, ovules were dissected and floated on a basal medium (10, 12) containing 22 g/l glucose, 1 mg/l NAA, and 0.2 mg/l GA at pH 5.0. (Ovules harvested two and three days before flowering also were tested, but their fiber initiation was too erratic to allow reliable comparison of temperature treatments.) Ovules were cultured in the dark under different temperature regimes, including 34°C constant, 15°C constant, and 34°C/28°C, 34°C/22°C or 34°C/15°C cycling temperature (12 h/12 h). Although by our convention the high temperature is placed first, the ovules were initially exposed to a 12 h period of cool temperature in the cycling regimes except otherwise noted. The same convention is also applied to other chapters in the dissertation. The control condition of 34°C has been previously shown to be optimum for growth of cultured ovules (12, 45). For comparison with *in vitro* experiments, ovules were also harvested from greenhouse-grown plants at an appropriate age and examined with SEM to confirm previously published results.

The time-dependence of the results is indicated by days after flowering using the terminology "days post-anthesis (dpa)," which is a common designation for timing of fiber development (22). Days after culture (dac) is also sometimes indicated to provide an additional parameter for comparison of results derived from ovules cultured 1 day preanthesis (-1 dpa) or on the day of anthesis (0 dpa).

2.2.2 Ruler Measurement of Fiber Length

The rate of later elongation of fibers on ovules cultured -1 dpa was determined by ruler measurements of fibers at 3 d intervals from 5 to 44 dpa (6 to 45 dac). Fibers were

straightened in a water stream and length from the edge of the fiber halo to the chalazal end of the ovules was measured (35). A dissecting microscope was used to measure fibers less than 2 mm long. Fibers on 30 ovules were measured on each day for two replicate experiments. The 60 data points for each day were averaged, and the standard deviation of the data points was determined.

2.2.3 Preparation of Ovules for SEM

For comparative SEM of 2 or 4 temperature regimes, ovules from 2 or 4 ovaries were divided equally at the time of culture between 2 or 4 flasks so that any variation due to the source ovary would be equalized between tests. The numbers of ovules in each flask was 25 - 35, a variation that did not affect the experimental results. Each comparative test was repeated 2 - 6 times with similar results. The number of replicates for individual experiments is indicated in each figure legend.

For SEM analysis, ovules from paired flasks were collected, stained in 0.05% Toluidine blue O for 2 min, and rinsed with medium twice before fixation. During subsequent manipulations, the staining aided in distinguishing the underside of the floating ovule where fiber development was suppressed in the early stages of culture. Fixation in 2% glutaraldehyde (0.05 M cacodylate buffer, pH 7.2, 24 h, 4°C) was followed by dehydration in an ethanol series and critical point drying. All the ovules from one flask were mounted on an aluminum stub with carbon tape (underside of ovules downward) and sputter coated with gold/palladium. Specimens were examined with an Hitachi-S-570 SEM at 15 kV. Micrographs of representative ovules were taken on Kodak Plus-X 35 mm film. In choosing representative ovules, those that showed clearly inferior development compared to others in the same experiment were ignored; about 20% inferior ovules is a common feature of this culture system (40). Of the ovules that developed fibers, the extent of development was very similar and a representative picture is shown.

2.3 Results

2.3.1 Time-course of fiber elongation *in vitro*

By 5 dpa (6 dac), control ovules grown under 34°C constant were >2 mm long (Fig. 2.1). Fibers cycled at 34°C/15°C did not reach a similar length until 8 dpa (9 dac), indicating that exposure of -1 dpa ovules to cool temperatures caused about a 3 d delay in fiber initiation and/or early elongation. The overall rates of early elongation until fibers were about 2 mm long were 0.38 mm/day and 0.21 mm/day for 34°C constant and

34°C/15°C cycling, respectively. However, the measurement of the 34°C/15°C fibers on day 6 indicates that this overall early elongation rate at 34°C/15°C is biphasic with a rate of 0.091 mm/day until 5 dpa (6 dac) and an elongation rate of 0.44 mm/day between 5 dpa (6 dac) and 8 dpa (9 dac).

The initial delay in attainment of fibers about 2 mm long under 34°C/15°C cycling was perpetuated throughout the time-course of fiber lengthening (note average 3 d separation between curves in Fig. 2.1) and reflected in the prolonged elongation period required to reach the control length of about 20 mm. After the fibers were about 2 mm long under both temperature conditions, similar overall rates of elongation occurred. For 34°C constant, the data points between 5 dpa (day about 2 mm length attained) and 32 dpa (day final length attained) can be fitted by a straight line ($y=-0.221 + 0.657x$; $r^2=0.981$) reflecting an average later elongation rate of 0.657 mm/day. Similarly for 34°C/15°C, the data points between 8 dpa (day about 2 mm length attained) and 35 dpa (day final length attained) can be fitted by a straight line ($y=-3.426 + 0.688x$; $r^2=0.989$) reflecting an average later elongation rate of 0.688 mm/day.

Figure 2.2 shows a plot of fiber elongation rate (mm/day) against fiber length for both temperature conditions in which each point indicates the average elongation rate over the previous three days. The abscissa of fiber length follows the tradition of Stewart (100) in using fiber length as an indicator of similar physiological age, which differs from temporal age under cycling temperature conditions (45). This plot indicates that the only substantial variation in average rate of later elongation under the two temperature conditions occurred when fibers were greater than 5 mm but less than 14 mm long. With the exception of that variation, fibers of similar length had similar elongation rates under both temperature regimes.

2.3.2 SEM comparison of fiber initiation and early elongation *in vitro* and *in vivo*

Fiber initiation, indicated by bulging of the epidermal cells, and early elongation on cultured ovules (-1 dpa) followed the same basic patterns as previously described *in vivo* (99). There was a gradient of initiation from the funiculus point of the chalazal end to the micropylar end, with fibers on the extreme chalazal and micropylar ends initiating last (Fig. 2.3a). As fibers began to elongate, a transition from blunt to tapered tips occurred and the polarity of fiber growth was oriented toward the micropyle (Fig. 2.3b). Fiber initiation was sometimes more uneven *in vitro* than *in vivo*, and variation was enhanced by cool

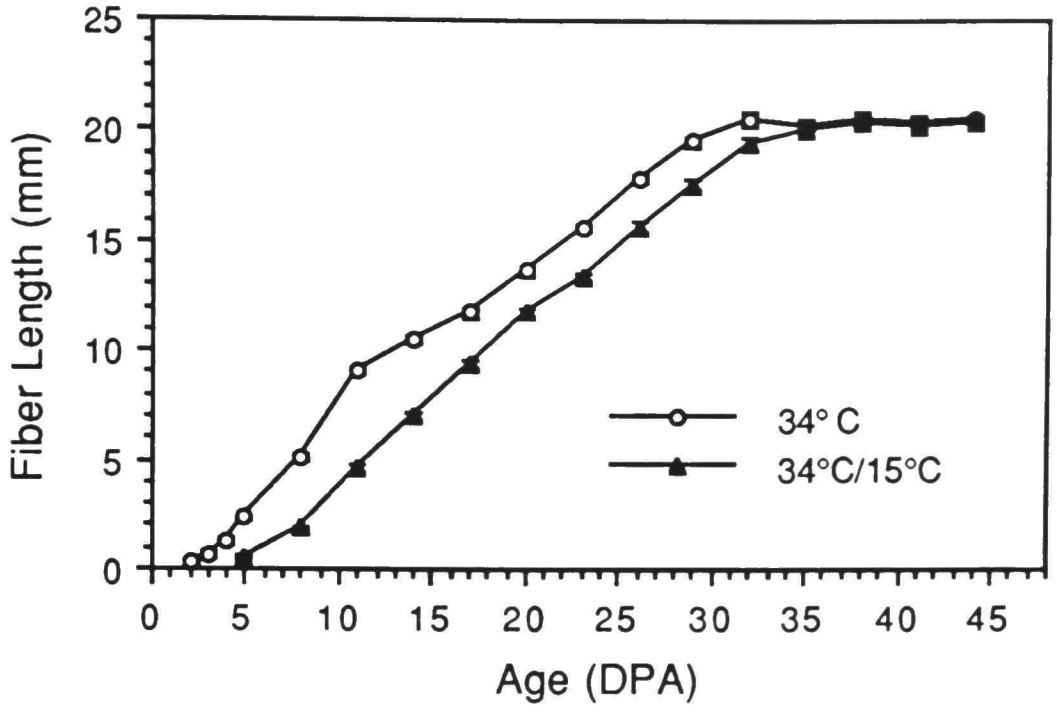


Fig. 2.1. Accumulation of fiber length versus time in -1 dpa ovules cultured at 34°C constant or 34°C/15°C cycling. Each data point is the average of fiber length measured by a ruler on 60 ovules in 2 separate experiments (bars = standard deviation).

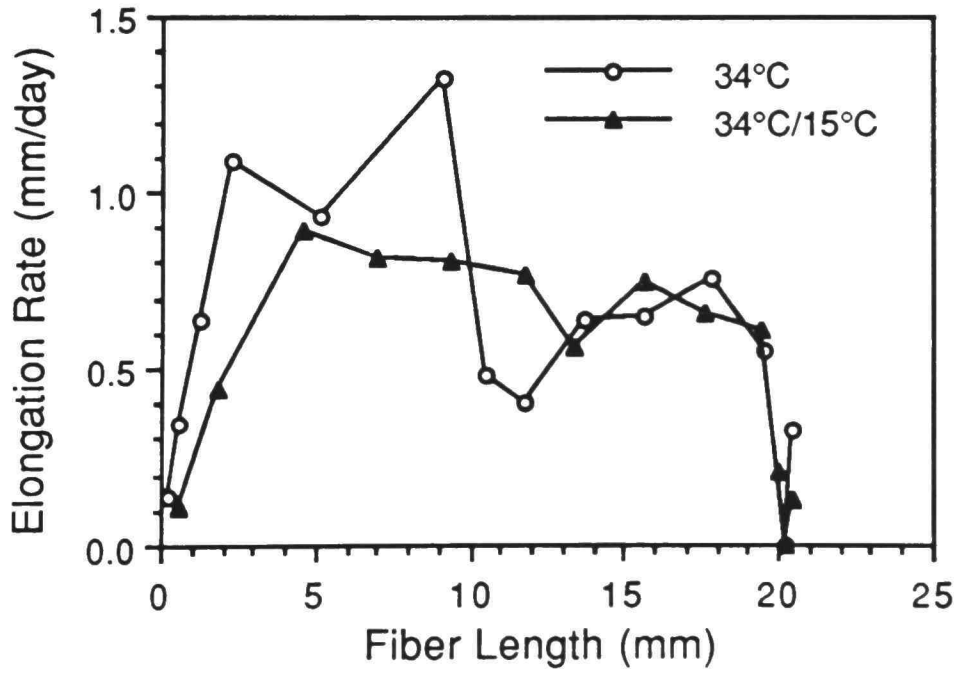


Fig. 2.2. Elongation rate versus fiber length at 34°C constant or 34°C/15°C cycling calculated from the data in Figure 1. Each data point indicates the average elongation rate for the previous three days of growth for a fiber of a particular length.

temperature cycling *in vitro*. Fiber initiation and early elongation appeared morphologically the same under all temperature conditions tested (for examples, compare Figs. 2.3 a-d with Figs. 2.3 e-h), although differences in timing were observed (see below).

2.3.3 Effects of cool temperature on fiber initiation and early elongation

Even though ovules were excised and cultured on -1 dpa, fibers initiated on the normal day of anthesis (0 dpa; 1 dac) under 34°C (Fig. 2.3a). (Initiation was not observed 12 h after culturing, but the majority of the fiber cells had initiated by 24 h.) By 1 dpa (2 dac), many control fibers had made the transition to tapered tips (Fig. 2.3b) and elongation was rapid on the following two days (Fig. 2.3 c,d). In contrast, ovules cultured under 34°C/15°C cycling showed little evidence of fiber initiation until 1 dpa (2 dac; Fig. 2.3 e,f), reflecting a 1 d delay compared to the control condition. Once initiation had occurred, the cycles of cool temperature continued to retard the rate of early elongation (Figs. 2.3 g,h). The 3 dpa (4 dac) fibers under 34°C/15°C (Fig. 2.3h) looked similar to the longest fibers at 1 dpa (2 dac) under 34°C constant (Fig. 2.3b), indicating about a 2 day delay in early elongation induced by 34°C/15°C cycling.

Data are not shown for the following observations because the electron micrographs did not differ significantly from those included. Fibers on 34°C/28°C cycled ovules showed almost identical timing of development to 34°C controls. Fibers on 34°C/22°C cycled ovules showed delays in development similar to those induced by 34°C/15°C cycling, although the rate of fiber elongation after initiation appeared intermediate between 34°C constant and 34°C/15°C cycling. Similar temperature dependencies for fiber initiation and early elongation were also observed in other cultivars of *Gossypium hirsutum* L.: cv.. Coker 312, cv. Paymaster 404, and a reputedly cold-tolerant Yugoslavian variety (Dr. Brad Waddle, retired cotton breeder, Univ. Arkansas, personal communication).

To determine whether cool temperature effects on early elongation depended on the delayed initiation caused by 34°C/15°C cycling of ovules harvested -1 dpa, ovules were harvested on 0 dpa after initiating fibers *in vivo* and cultured at 34°C constant or 34°C/15°C cycling. By 1 dpa (1 dac) fibers on 34°C constant ovules had begun to elongate (Fig. 2.4a) and elongation progressed rapidly for the next two days (Fig. 2.4 b,c). In contrast, fibers on ovules grown under 34°C/15°C cycling retained the rounded shape of newly initiated fibers on 1 dpa (1 dac; Fig. 2.4d) and had just begun to elongate on 2 dpa (2 dac; Fig. 2.4 e). Fibers on 34°C/15°C ovules at 3 dpa (3 dac; Fig. 2.4f) appeared similar to those on

34°C ovules at 2 dpa (Fig. 2.4e), indicating that cycling temperature caused about a 1 day delay in early fiber elongation if cycling commenced just after initiation occurred *in vivo*.

The ability of 15°C to slow but not halt fiber initiation and early elongation was demonstrated by culturing ovules at 15 °C constant after harvest on -1 dpa (Fig. 2.5 a-c) or 0 dpa (Fig. 2.5 d-f). Note that, in contrast to previous figures, these micrographs were taken 5, 10, and 15 days after culture. By 15 dpa, fibers on 15°C constant ovules had not surpassed the normal 2 dpa stage of development. Constant 15°C often caused great variability in the elongation of individual fibers on the same ovule (Fig. 2.5f). Fibers on ovules exposed to this prolonged cool temperature stress elongated rapidly upon transfer to 34°C constant (data not shown).

2.4 Discussion

The similarity of patterns of fiber initiation *in vivo* and *in vitro* is another indication that cultured ovules can be used as valid models for investigating factors that control *in vivo* fiber development (45). The gradient of fiber initiation has been hypothesized to relate to a gradient in hormone concentration that declines from the chalazal end (99) or to the transport of hormones through the vascular system that begins at the funiculus, circles the chalazal end, and declines to the micropyle (37). Since ovules float *in vitro* with both ends equally exposed to the aqueous environment and exhibit the same pattern of fiber initiation as *in vivo*, they may also take up and transport exogenous hormones through the vascular system rather than only by simple diffusion. Alternatively, other unidentified factors might control this gradient. The timing of fiber initiation *in vitro* was not changed by exposure of -1 dpa ovules to hormones in the culture medium, even though by -2 dpa epidermal cells have differentiated into pre-fiber cells and attained full capacity to continue to develop *in vitro* (40). This observation supports the hypothesis that there is a developmental link between fiber initiation and time of anthesis *in situ* that cannot be broken by culture manipulations that separate the ovules from all other tissues and expose them to the exogenous hormones that are required to sustain *in vitro* fiber development (40). By -1 dpa the ovules must have acquired an internal clock that allows initiation only on the normal day of anthesis.

Our results show that cycling to cool temperatures of 22°C or 15°C delays fiber initiation and early elongation. Cycling to 15°C from -1 dpa onwards caused about a 1 d delay in fiber initiation and about a 2 d delay in early elongation as demonstrated by SEM. The sum of these two delays is consistent with the 3 d increase in the overall elongation

period evident in Fig. 2.1. This difference in extent of delay of initiation and early elongation suggests that cool temperatures have independent effects on these two processes. This hypothesis is supported by the observations that, even if initiation is allowed to occur *in vivo*, 34°C/15°C cycling and 15°C constant still retard subsequent elongation.

However, once the fibers reached about 2 mm in length, the rates of subsequent elongation were similar under 34°C constant and 34°C/15°C cycling. The early developmental delay, not continuously lower rates of elongation, account for the extra time required to reach the control length under 34°C/15°C cycling compared to 34°C constant. Similarity between rates of later elongation (after the fibers were 2 to 3 mm long) under 34°C constant and 34°C/15°C cycling was reported in a previous study of ovules cultured at 2 dpa (when initiation and early elongation had occurred *in vivo*) (45). However, in that study, 12 days of culture were required for 34°C/15°C cycled ovules to attain 2.5 mm in length in contrast to about 4 days for the 34°C constant condition, indicating an overall developmental delay of about 8 days. The shorter 3 d developmental delay observed in the current study may relate to the earlier time of exposure to 15°C (-1 dpa compared to 2 dpa, leading to 72 h difference). Perhaps alternative gene expression leading to adaptation to the cool temperature can occur more quickly during the early part of the developmental program for fiber initiation and elongation.

All of these *in vitro* results are consistent with those obtained from field experiments on the Texas High Plains using *G. hirsutum* cv. Acala 1517 BR-1 or BR-2 in which a 15°C night temperature caused a 4 to 5 d delay in lengthening of fibers to the point of being ruler-measurable and attainment of maximum elongation rate compared to 25°C (35). Similarly, an initial lag of 3 to 10 days in attainment of 2 mm length was observed in cotton fibers grown under different field temperature regimes in India (104). The same general pattern of temperature response was observed *in vitro* in four cultivars of *G. hirsutum* tested in the current study, suggesting that this effect is common to many commercial cultivars of cotton. This observation is especially interesting because Coker 312, Paymaster 404, and the Yugoslavian cultivars are all more cool-temperature tolerant than Acala SJ-1 in some aspects of growth or fiber development, yet they do not show substantial differences in the cool temperature response of fiber initiation and early elongation.

Our refined analyses of the cool temperature response suggests that fiber elongation has three phases, as distinguished by a differential response to 34°C/15°C cycling, fiber

initiation, early elongation to attain about 2 mm in length, and later elongation to attain the genetically determined potential length. Fiber initiation is delayed 1 day by 34°C/15°C; early elongation to attain 2 mm length is delayed about 2 additional days; and later elongation is not hindered in rate compared to 34°C constant. Since all phases of fiber elongation involve apparently similar processes, e.g., expansion of the plasma membrane and cell wall, it is interesting to speculate why their temperature dependencies are different. This is especially true since the phases of slower elongation (initiation and up to 2 mm long) are more sensitive to cool temperature than the later phase of faster elongation. Other work in this laboratory has shown that glucose uptake and rates of cellulose synthesis and respiration are all decreased by 34°C/15°C cycling (90), but these processes would need to occur during all phases of fiber initiation and early elongation. It is possible that the first days of fiber development are critical in establishing basic physiological machinery that can work in a more temperature independent manner once in place. This possibility is consistent with the observation that the greatest incorporation of ^{3}H-uridine in cotton fibers occurs prior to 6 dpa and cell division still occurs up to 6 dpa (13), and the more recent suggestion based on similarity of stage-specific mRNA populations that gene transcription ceases early in fiber development (65). Although constant 15°C is extremely detrimental to elongation (Figs. 2.5 a-f), it might not be detrimental as part of a cycling regime if only part of the daily period was normally used for elongation or if only part of the day were necessary to establish the physiological frame-work for turgor-driven elongation that proceeded at other times. Alternatively, exposure to 15°C could trigger adaptive mechanisms that allow faster elongation during the 15°C or the 34°C parts of the cycle than in the constant 34°C controls, either of which could equalize the overall rate of elongation.

Consistent with field studies, 34°C/15°C cycling causes the elongation period to be prolonged to compensate for the initial developmental delay in passing through the initiation and early elongation phases so that the same fiber length is finally attained if the growing season is long enough. However, a population of short fibers would be observed if a growing season with cool nights was ended early and abruptly, for example, by an early frost. In the absence of water and nutrient stress, final crop yield is highly dependent on the accumulated heat units during fruiting and fiber maturation (78). In this study, in addition to observing a delay in fiber initiation and early elongation, we observed a decrease in the number of highly elongated fibers on cycled ovules (results not shown). Similarly, ovules cultured from field grown plants late in the season produced fewer fibers than those from greenhouse plants (W. Xie and J.M. Stewart,unpublished results). Taken

together those data indicate that environmental conditions around the time of fiber initiation are critical to cotton productivity and fiber quality.

Fig. 2.3. SEM of -1 dpa ovules (pre-fiber initiation) cultured at 34°C constant (a-d) or 34°C/15°C cycling (e-h) for 1 (a, e), 2 (b, f), 3 (c, g), and 4 (d, h) days (0 to 3 dpa). The magnification is indicated by the bar in h. The experiment was repeated 8 times during 2 years with similar results.

34/15°C 34°C

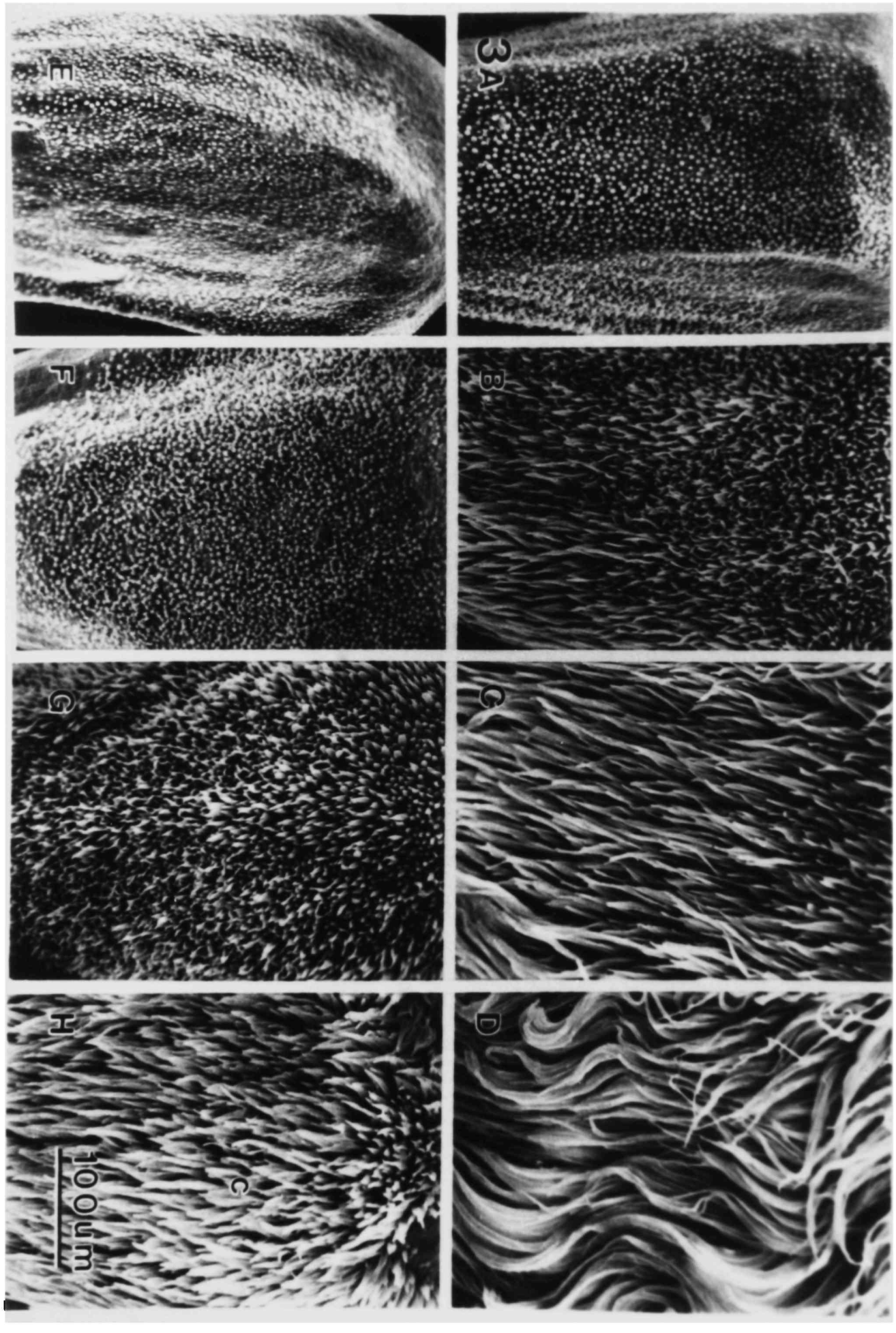
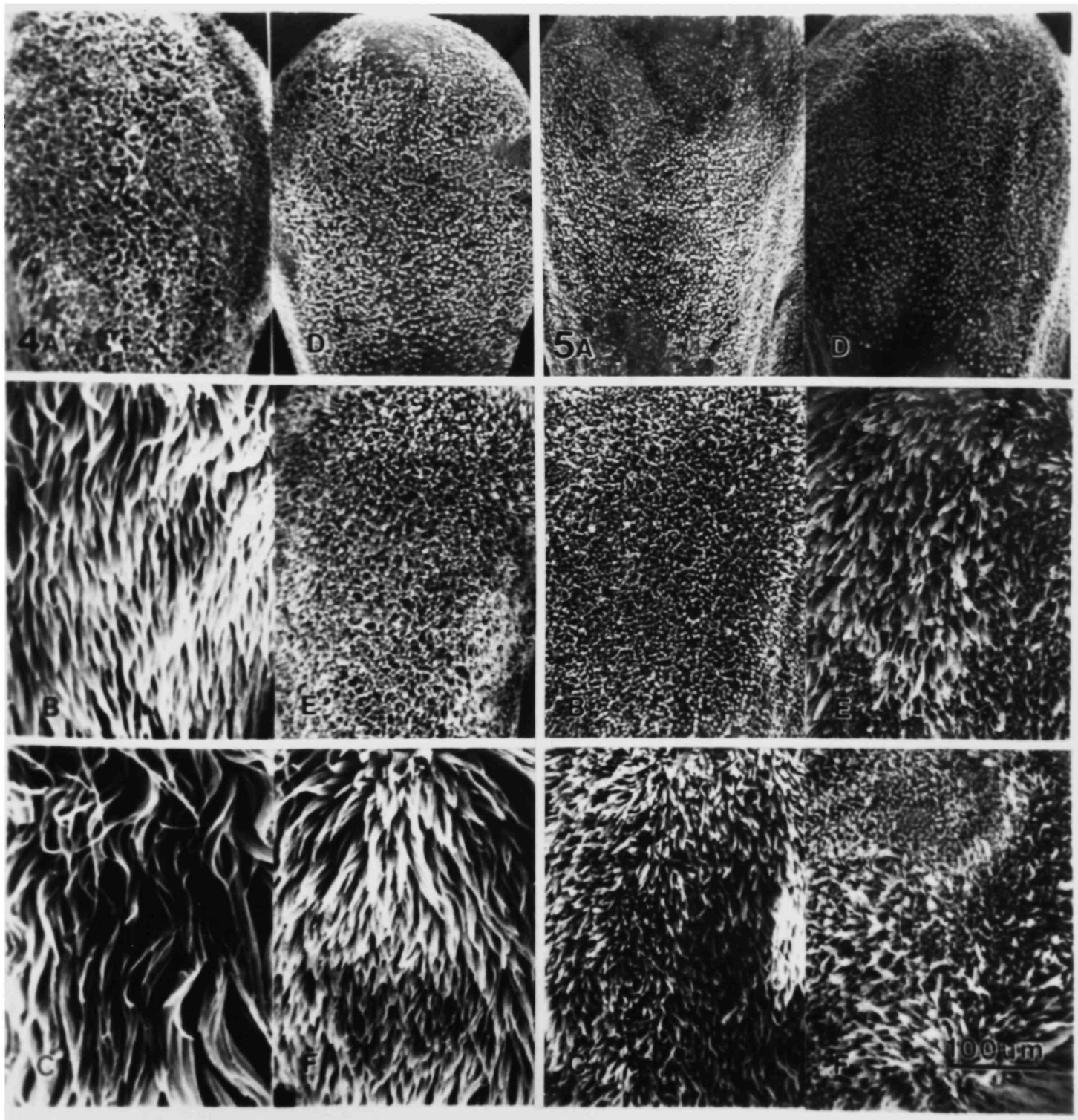


Figure 2.4 (Left) SEM of 0 dpa ovules (on which initiation would have occurred *in vivo*) at 34°C constant (a-c) or 34°C/15°C cycling (d-f) for 1 (a, d), 2 (b, e), and 3 (c, f) days (1 to 3 dpa). The experiment was repeated 3 times with similar results.

Figure 2.5 (Right) SEM of -1 dpa (pre-fiber initiation; a-c) and 0 dpa (initiation *in vivo*, d-f) ovules cultured for 5 (a, d), 10 (b, e), and 15 (c, f) days at 15°C constant. The experiment was repeated 3 times with similar results.

34°C Constant 34/15°C 15°C Constant



CHAPTER 3
LOW TEMPERATURE-INDUCED CHANGES IN PROTEIN SYNTHESIS DURING FIBER INITIATION AND EARLY ELONGATION ON COTTON OVULES CULTURED *IN VITRO*

3.1 Overview

The morphology and physiology of fiber grown *in vitro* have been well characterized, but protein synthesis in response to low temperature has not been examined during fiber development. Both field and *in vitro* studies suggested that early elongation of cotton fiber was highly temperature sensitive, whereas later elongation became adapted to cool night or cycling temperatures (35, 45). The low cycling temperatures caused an 8 d delay in recovery of elongation rate to control levels when ovules were cultured at 2 dpa after fiber initiation (45), but only a 3 d delay if ovules were cultured at -1 dpa before fiber initiation (Chapter 2). This variable time required for adaptation may suggest different effects of cool temperatures on different cell processes, but in either case cellular and metabolic adjustments have likely been made to allow normal fiber elongation under the adverse condition (45, 112). Based on results from low temperature responses in a number of plant species (14, 19, 20, 42, 64, 110), if such an adjustment in cellular metabolism occurred, it would quite likely be accompanied by changes in gene expression and protein profiles induced by the cycling temperature.

Cool temperature induced changes in gene expression should be interpreted against the background of normal developmental changes, and some related studies have been done. A detailed study of the protein constituency of ovules and early developing fibers from -6 dpa to 17 dpa by 2D gel electrophoresis showed that major proteins had a similar pattern from -3 dpa to 2 dpa (39). There are three possible interpretations of this result. First, it is possible that new proteins required for fiber initiation/elongation were induced before -3 dpa by endogenous phytohormones or other factors. This possibility is inconsistent with other studies that showed changes in protein profiles between -3 and -2 dpa (37) and, probably, with the requirement of unfertilized -3 dpa ovules for two exogenous hormones to begin fiber elongation *in vitro*. Second, it is also possible that no new proteins are required. Some constitutive proteins may simply be activated by phytohormones after fertilization resulting in initiation of the process of fiber elongation and its continuation. Hence the activities of hormone-responsive enzymes could differ between the pre- and post-fertilized ovules. However, how those enzyme activities are

affected by endogenous hormone levels and environmental or thermal stress is unknown. Third, it is also possible that the silver-staining technique of 2D gel electrophoresis did not detect newly synthesized proteins, which would be especially likely if new proteins were minor species with regulatory roles and/or were present for only a short time. The phenolic smearing on their gels (37) also makes it difficult to be sure of detecting subtle changes.

One of the major goals in our research is to determine the physiological and biochemical basis of cool temperature effects on fiber development with the hope that specific molecular changes can be planned for improvement of fiber quality. Therefore, in this study, 1D and 2D gel electrophoresis and fluorography were used to determine possible changes in protein profiles induced by low cycling temperature during fiber initiation and early elongation. In particular, we investigated whether 15°C could induce or repress synthesis of particular proteins correlated with the initial delay of fiber elongation or its eventual recovery in the *in vitro* system.

3.2 Materials and Methods

3.2.1 Ovule Sources and Cultures

Cotton ovules (cv. Acala SJ-1) at -1 dpa were cultured in a modified BT medium (10, 11) with randomization of ovules to reduce effects of possible variation among ovaries as described previously [(112) or see Chapter 2]. Ovules were incubated for 1-13 days under constant 34°C or 15°C, 34/15°C and/or 15/34°C (12 h/12 h) cycling, and other temperature regimes from 5°C to 40°C as specified in each experiment. Ovules at -2 dpa were used in particular experiments. In other experiments, ovules at different ages were collected from flowers tagged on greenhouse-grown plants (natural day light and approximately 32/22°C day/night regime).

3.2.2 *In Vivo* Protein Labeling

Ovules were transferred to a sterile Petri dish containing 3 ml culture media pre-equilibrated to the experimental temperature. Protein labeling was immediately performed without injuring the ovules by adding 50-100 uCi L-^{35}S-amino acids (77% L-^{35}S methionine and 18% L-^{35}S cysteine; >1000 Ci/mmol, New England Nuclear) to the media at the beginning of the cycle. The radiolabeling period lasted 1-24 h dependent upon the experiments. Uptake was terminated by collecting and quickly rinsing the ovules 5 times with non-radiolabeled culture medium. Ovules were then frozen in liquid N_2 and ground to a powder immediately or stored at -80°C until proteins were extracted for analysis.

3.2.3 Extraction of Total Proteins

Cotton ovules were ground into fine powder with a cold mortar and pestle in liquid nitrogen. Total soluble protein was extracted by further homogenizing with 3-5 ml extraction buffer consisting of 0.2 M borate buffer pH 7.6 (54), 5 mM DTT, 10% glycerol, and 0.5% (w/v) protease inhibitor stock that contains 2% PMSF (phenylmethyl sulfonyfluoride), 12% N-carbobenzoxy-L-phenylalanine and 20% 1,10-phenanthroline (by w/v in pure EtOH). After spinning at 500 g to remove debris, the supernatant was collected and extracted once with 1 vol. of extraction-buffer-saturated, distilled phenol. The aqueous phase and the white material at the interface were removed and the phenol layer was remixed twice with 1 vol. of extraction buffer and spun at 10,000 rpm (12,060 g), 10 min, 4°C (Sorvall SS-34 Rotor) to re-separate the phases. Proteins were precipitated from the phenol by the addition of 2.5 vol. cold 0.1 M ammonium acetate in methanol overnight at -20°C, then pelleted by centrifugation at 10,000 rpm (12,060 g) for 20 min. The supernatant was discarded and the protein pellet was transferred to a microfuge tube and washed twice with 0.1 M ammonium acetate in methanol, vacuum dried, and dissolved in 50-100 µl 1D sample buffer (62.5 mM Tris-HCI pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% ß-mercaptoethanol, and 0.125 % Bromophenol Blue) (59) or 2D UKS buffer (9.5 M urea, 5 mM K_2CO_3, 1.25% SDS, 5 mM dithiothreitol (DTT), 6% Triton-X-100, 2% Ampholyte pH 3-10) (21). Insoluble substances were removed by spinning at top speed in an Eppendorf microcentrifuge. Protein extracts were stored at -80°C until use. This method precipitates extraction buffer-phenol-soluble protein and avoids excessive smearing of the gels.

Crude or phenol-extracted, radiolabeled protein was quantitated by aliquoting 5 µl of sample onto a GF/C glass filter (Whatman, Maidstone, England). The filters were rinsed with 10% trichloracetic acid (TCA) and cold acetone, air-dried, added to 3 ml of Scintiverse BOA scintillation cocktail (Fisher Scientific), and counted in a Beckman LS 7500 Scintillation Counter. For fluorography, equal amounts of protein based on TCA insoluble radioactive counts (cpm) were loaded for all treatments unless sufficient protein was not available. In this case, adjustment of exposure time was made to equalize final cpm x h as specified later in the corresponding figure lengend. Each sample consisted of 60 kcpm and 500 k or 1,000 kcpm for 1D and 2D gels, respectively. Non-radioactive protein was extracted similarly and quantified by the Biorad assay (16). IEF gels were loaded with 120 ug protein and the final 2D gels were silver stained (15).

3.2.4 Gel Electrophoresis

Protein gels were run at least twice for each treatment within an experiment and each experiment was repeated twice to ensure the reproducibility of the results. Two-dimensional gel electrophoresis was carried out according to O'Farrell (76) with the modification that protein samples were loaded on the acidic side of the IEF gels (25). IEF gel solution {9.2 M Urea, 3.8% (w/v) acrylamide, 0.2% Bis, 4% Ampholyte (4 parts pH 5-8 : 1 part pH 3-10), 3% Triton X-100} (21) was poured into a glass capillary tube (14 cm high and 1.5 mm i.d.) and polymerized for 2 h at room temperature. The degassed electrode buffers were 0.1 M NaOH for the catholyte and 8.6 mM H_3PO_4 (1 ml 85%/L) for the anolyte. Isoelectric focusing was at constant 500 V for 14 -16 h, and 800V for 3 h, all at 4°C. After extrusion, each IEF gel was incubated for 15 min with shaking at 22°C in 3 ml modified equilibration buffer (5 mM DTT substituted for 10% ß-mercaptoethanol) (76) and then subjected to electrophoresis on a 12% acrylamide, 1.5 mm thick gel or stored at -80°C until use. A set of 4 gels could be run simultaneously in a single gel apparatus.

The 1D (12%, 0.75 mm) or 2D (12%, 1.5 mm) slab gels were run at constant current of 20 mA/gel at 4°C. Gels were fixed in acetic acid for 20 min, infiltrated in 15% 2,5-diphenyloxazole (PPO) in acetic acid for 1.5 h, soaked in ice water for 30 min, vacuum dried on a slab gel drier (Hoefer Sci. Ins., SE1160, S.F. CA), and fluorographed using Fuji X-ray films at -80°C with an intensifying screen. Each SDS-PAGE gel was run with a single lane of molecular weight markers: Phosphorylase B, 92.5; Albumin, 69; Ovalbumin, 46; Carbonic Anhydrase, 30; and Cytochrome C, 12.3 kD.

3.2.5 Analysis of the Gel Fluorographs

Exposed films were compared manually by use of back-lighting and tracing spots or spot constellations onto clear acetate sheets. The acetate could then be overlaid on another film for comparison and all results described are consistent with the original films. This method is not as sensitive as computer-assisted methodologies, particularly in regard to detection of quantitative differences, and this study can be extended in the future by reanalysis of the films. To make the photographic reproductions of the original films, they were placed on a light box and photographed with T-Max 100 film with an exposure time that was kept constant for each experiment. All gels to be directly compared were photographed on the same roll of film and printing was done under standardized conditions (F 5.6 lens stop, 1/2 contrast filter, 15 second exposure and 2 min developing time). The photographs were printed on Kodak Polycontrast III paper. Representative spots only

were marked on the gel photographs. Clarity was still reduced somewhat in the photographic reproductions, so some quantitative changes that were observed during the analysis of original films are not clearly obvious in the prints. Therefore, comparison described here emphasizes only qualitative changes.

3.2.6 RNA Isolation and *In Vitro* Translation

Total RNA was isolated with hot borate buffer from ovules of -1 dpa that had been cultured 1 d under 34°C or 15°C following the protocol of Dr. Thea Wilkins (personal communication, UC-Riverside CA). Poly (A^+) RNA was purified based on the method of Sambrook et al.(93). Translation was performed with the rabbit reticulocyte system by the manufacturer's instruction (Promega, Madison, WI) and the products was analyzed by 2D gel as described above.

3.3 Results and Discussion

3.3.1 Optimum Temperature for Protein Synthesis

Effects of different temperatures on ^{35}S-methionine uptake into ovules and incorporation into protein were determined. Ovules of -1 dpa harvested from greenhouse plants were incubated 10 h in 28°C, then labeled for 3 or 12 h at the following temperatures: 5, 15, 22, 28, 34 and 40°C. One-dimentional gels of crude protein extracts showed that 40°C both induced and suppressed bands at low molecular weight (MW) (Fig. 3.1 arrows = 40°C induced; arrowhead= 40°C suppressed) compared to 28° and 34°C. At both time points, only a few high MW bands were detected at 5°C. No difference in the band pattern was detected between 28°C and 34°C. At 3 h, temperature below 22°C reduced the rate of synthesis of a band at 13 kD (closed triangle) and induced a new band at 12 kD (open triangle). The 12 kD band that was present at 3 h, but not after 12 h labeling, must be transiently synthesized upon shifting from 28°C to these lower temperatures. A 19 kD band at 40°C behaves similarly (i.e., induced at 3 h and not present at 12 h), and this band probably is analogous to a heat-shock protein induced by 40°C in cotton seedlings (87). Therefore, different proteins are induced in cotton in response to specific temperatures.

Based on total cpm of crude protein extracts, the uptake of ^{35}S-a.a. decreased in the order of 28°, 34°, 40°, 22°, 15°C, 5°C (Table 3.1). The effect of optimum temperature, 28°C, on ^{35}S-a.a uptake and protein synthesis can be seen also easily when comparing cpm counts from the extracts of ovules labeled for 3 and 12 h in 5°C and 15°C with those from

ovules labeled for half hour in 28°C then 2.5 h in 5°C and 15°C (Table 3.1). The half hour incubation in 28°C increased the radioactivity of the extracts 3 times and 50% respectively, in subsequent 5°C and 15°C incubations. The protein profiles corresponding to these two treatments are also shown in Fig. 3.1 (lanes a &b). These results are consistent with previous data (36; Rao and Owen, unpublished data), and with field studies showing that fiber elongation proceeds only above 15°C and that night temperature below 25°C reduced its rate (34). Similarly, SEM observations and ruler measurements made on *in vitro* fibers showed that temperatures below 28°C (e.g., 34/15°C and 34/22°C) were detrimental, while both 34/28°C cycling and 34°C constant allowed optimum fiber elongation [(45); data not shown in Chapter 2]. Therefore, since extra ^{35}S-a.a. uptake at 28°C does not result in faster fiber elongation, 34°C was used as a control for consistency with previous studies (45, 90).

Table 3.1 Effects of different temperatures on ^{35}S-methionine uptake into cotton ovules (cpm/µl crude protein extract).

Temp °C	5	15	22	28	34	40
3 h	4,934	10,121	11,179	40,310	21,958	16,509
12 h	15,048	26,657	31,023	101,060	40,916	37,862

Other data: 1) cpm from ovules labeled for 0.5 h in 28°C and 2.5 h in 5°C or 15°C are 14,068 and 15,466, respectively.

3.3.2 Total Protein Profiles around Fiber Initiation and Early Elongation *in vivo*

Silver stained gels from -3 , -2 -1, 0, 1, 2 and 3 dpa ovules harvested from greenhouse-grown plants were analyzed to determine background information about protein profiles. The data points were chosen to span the determination, initiation, and early elongation stages of fiber development (99). There was no difference in the 1D gel patterns among these dates (gels not shown). Analysis of 2D gels (only examples are shown) of -3, 0 and 3 dpa (Fig. 3.2) revealed that several soluble proteins were increased (arrows), decreased (circles) or completely absent (boxes) on 3 dpa as compared to 0 and -3 dpa. Fibers would have been elongating between 1-3 dpa. Protein profiles on -3 and 0 dpa were very similar, indicating a lack of major detectable soluble protein changes associated with initiation. This, in agreement with a previous study (39), may indicate that

fiber cell determination (commitment to elongation) occurs earlier than -3 dpa or that non-extracted proteins or minor protein species are critical regulators.

The silver-staining technique allows detection of accumulated total proteins, but it may not be sufficiently sensitive to detect minor changes in protein synthesis around initiation, especially changes in low abundance regulatory proteins. Therefore, 2D gel fluorographs were produced after labeling -3, -1, 0 and 3 dpa ovules at 28°C each with 50 uCi ^{35}S-a.a. (Fig. 3.3). Protein profiles on all days were similar to those detected by silver staining in that some protein spots (diamonds in Fig. 3.2 and Fig. 3.3) became less intense on 3 dpa. Protein synthesis was reduced on 3 dpa as reflected by lower cpm counts of radioactivity incorporated into protein per ovule (1/3 -1/2 of other ages, Table A.1). However, the more sensitive labeling method did reveal other differences. Two proteins of 92 kD were reduced (circle) and a 12 kD protein was increased (arrow) on other days compared to -3 dpa. On 3 dpa, an intense band of about 25 kD on a 1D gel (data not shown) and a 2D gel and other new proteins (arrows Fig. 3.3) were induced or greatly increased compared to 0 dpa or previous days. Both the silver-staining and fluorography confirm that protein synthesis is developmentally regulated during early elongation, but that few detectable changes occurred between the fiber determination, initiation and early elongation stages. Similar protein profiles from -3 to 2 dpa in a previous report suggest a plateau in protein expression when the first round of initiation is completed, but before major elongation has begun (39). These authors speculated that proteins putatively responsible for fiber initiation/elongation were synthesized before initiation. In partial agreement with this previous study, our radiolabeling results reveal synthesis of age-specific proteins only between 0 to 3 dpa. These results also indicate that temperature-induced changes should be detectable against the rather constant control profile during the initiation/early elongation period and that fluorography is the most sensitive available detection method.

3.3.3 Low Cycling Temperatures Caused a Developmental Delay in Changes in the Protein Profiles

Cotton ovules of -1 dpa, after being cultured for 1-13 days at 34°C or 15/34°C (the initial exposure temperature was 15°C), were labeled with 50 uCi/treatment ^{35}S-a.a. for 12 h at 34°C. The 15/34°C ovules were subjected to the 15°C side of the cycle for 12 h before labeling, which commenced immediately upon shifting to 34°C. Total low cycle time was cumulative with 12 h for each day of culture of 15/34°C ovules. The high side of the cycle

was used to determine if previous exposure to 15°C affected subsequent synthesis of protein in 34°C.

The 1D gel clearly illustrates a developmental delay in the synthesis of specific low molecular weight bands induced by 15/34°C (12 h/12 h) cycling. That is, shifts in protein patterns look the same under both temperature conditions, but later days under 15/34°C are most similar to early days under 34°C. Several specific protein bands demonstrated this trend (Fig. 3.4). For example, the bands at about 10 kD and 20 kD (arrowheads), which appeared intense on 3 dpa and thereafter at 34°C, only became evident on 5 and 10 dpa under the cycling. A band at 15 kD (arrow) disappeared on 1 dpa at 34°C and 2 d later at 3 dpa under the cycling. A 40 kD band disappeared on 5 dpa and re-appeared on 10 dpa in 34°C, but disappeared for the first time on 10 dpa in 34/15°C (arrow). The temporal difference in the appearance and disappearance of these bands was consistent with the SEM data and ruler measurements showing a faster pace and earlier end of fiber development under constant 34°C (Chapter 2). Their involvement in cellular low temperature sensitive processes is also suggested, since synthesis of other proteins seemed unaffected qualitatively.

Extension of the time course to 2D gel fluorographs from 0 to 10 dpa (34°C) or 13 dpa (15/34°C) (Fig. 3.5) confirmed the results seen in 1D gels, indicating a developmental delay in protein patterns induced by 15/34°C. The results are summarized below. First, both quantitative and qualitative differences were observed in protein profiles between 34°C and 15/34°C ovules especially when compared at the same early age. For example, some proteins present at -1 or 0 dpa in 34°C were not present or very faint under cycling until 1 dpa (Fig. 3.5 a & b, squares). Similarly, 2 species that appeared strong until 3 dpa under the cycling (gel not shown), almost disappeared at 5 dpa in 34°C but not until 7 dpa in 34/15°C (Fig. 3.5c, big square). Proteins at this same location reappeared at later days under both temperature conditions (Fig. 3.5d), which indicates developmental regulation. A transition in protein population occurred between 2-10 dpa *in vivo*, and proteins for secondary wall thickening were presumably synthesized from 6-10 dpa *in vivo* (39). Second, within each temperature regime, protein profiles were similar except for quantitative changes before 2 dpa (Fig. 3.5 a & b) and after 7 dpa for 34°C and 10 dpa for 15/34°C (Fig. 3.5d). The overall protein population was dramatically reduced at 5 dpa in 34°C and 7 dpa in 34/15°C (Fig. 3.5c), but both high and low molecular weight species were recovered 2-3 d later (Fig. 3.5d). This abrupt decrease in protein species coincides with attainment of a 2 mm long fiber and subsequently similar rates of elongation under

both temperatures (at 5 or 8 dpa, respectively). Third, synthesis of some proteins was not affected by the low temperature cycle (examples are indicated by circles and squares in Fig. 3.5), or, after the initial suppression compared to 34°C, even appeared more intense in 15/34°C than in 34°C at 7 and 10 dpa (indicated by diamonds in Fig. 3.5d). The recovered synthesis of these labeled proteins at 15/34°C in the later days may result from turnover of protein or from use of unused labeled amino acid pools instead of increased ^{35}S-a.a uptake or change in the rate of synthesis. More importantly, however, it may reflect the cellular adjustment that allows rapid elongation to proceed under the cool temperature.

In contrast to a previous study showing that unique and more abundant proteins were synthesized under cycling in cultured ovules and fibers at 18 dpa (Rao and Owen, unpublished data), no new proteins were detected in cycled ovules of any age when labeled at 34°C (Fig. 3.5) at both the 1D and 2D levels although similar total TCA-insoluble counts were recorded under both regimes (Table 3.2). The adverse effects of previous exposure to 15°C in protein synthesis observed here and the more than 3 h delay in full recovery of cellulose synthesis after entry from 15°C into 34°C in the cycled fiber (90) both indicate that the previous 12 h in 15°C can impose a metabolic stress on ovules and fibers that extends to the warm side of the cycle. Though we cannot determine whether the effect of each cycle is independent or cumulative, the developmental delay in the protein synthesis seems to favor a continuous effect. Our results provided direct evidence of the molecular basis underlying the early delay in initiation and later adaptation in elongation under low temperature. Presumably, at least some of the suppressed proteins that appear later under 15/34°C are required for rapid elongation. Proteins that are candidates for this role include those at 10, 12, 20 and 92 kD.

Table 3.2 Time course of total ^{35}S-methionine incorporation into protein under constant 34°C and 34/15°C (cpm x 10^5/µl phenol extract)

	Ovule Age (dpa)						
T°C	-1	0	1	2	3	5	10
34°C	1.14	1.21	1.06	0.89	0.63	0.48	0.48
34/15°C	1.28	1.17	1.20	1.12	0.73	0.92	0.52
%*	112	97	113	126	116	192	108

* The percent cpm in 34/15°C compared to 34°C.

3.3.4 Effects of 15°C on Synthesis of Particular Proteins

Because no unique proteins were detected when 15/34°C ovules were labeled on the 34°C side of the cycle, the possibility that 12 h of 15°C exposure may not be sufficiently low or long enough to induce new proteins was addressed by labeling experiments at constant 15°C. The results are shown in Fig. 3.6 and 3.7. Ovules of -1 dpa were cultured 2 h in 34 °C or 15°C and then labeled for 1 (not shown), 3 (a), 6 (b, h), 9 (c, i), 12(d, j) and 24 h (e, k) at the culture temperatures. Also, -1 dpa ovules were cultured 24 h in 34 °C (f), 15°C (l), 34/15°C (g), or 15/34°C (m), then labeled for 12 h in their initial culture temperatures: either 34 °C or 15°C. A 2 h pre-incubation under the labeling temperatures was used to avoid the interfering effects of previous temperatures because 50 ml medium requires 2 h to reach equilibrium upon shifting in either direction between 34°C and 15°C (45). Also in our pilot labeling experiments in which the ovules were transferred to 3 ml pre-equilibrated medium and labeled immediately, transient synthesis of particular proteins was observed.

Both 1D (Fig. 3.6) and 2D (Fig. 3.7) gel fluorographs clearly showed differential regulation in protein synthesis by 15°C or 34°C, especially among species of low molecular weight. Constant 15°C enhanced protein bands of 12 and 14 kD (Fig. 3.6, arrowheads) and suppressed 6 other bands (arrows; 10, 15, 16, 20, 30, and 69 kD). The enhanced 12 kD band could indicate an early developmental stage that was passed quickly at constant 34°C because the 12 kD band was faintly observed until 9 h (lane a-c) in 34°C. The 6 suppressed bands may also reflect the 15°C-induced developmental delay instead of a 34°C-specific response since they appeared in the ovules that were labeled 12 h in 15°C after 24 h culture in 15/34°C cycling (lane m). The previous 12 h exposure to 34°C would speed up the development. This supports the previous results from cycling experiments, and suggests that protein synthesis in the cool side is benefited from the warm side of the cycle. The differential regulation of these proteins is further substantiated by their presence in either cycling condition in a separate experiment of 24 h labeling (data not shown). Among these 6 bands, the 10, 15 and 20 kD are of the same molecular weight as those inhibited by the cycling temperature. This suggests either that their synthesis is more sensitive than the other bands to 15°C, or that the synthesis of other bands recovers more rapidly late in 34°C side of the cycle. These proteins may be the candidates that play important roles in later adaptation of fiber elongation. A group of low MW proteins was the most sensitive to low temperature. The appearance of several small proteins (11-38 kD) were reported also in

cold acclimated alfalfa seedlings (70). In several cases, heat-stable COR proteins of high MW were associated with freezing tolerance in spinach (53) and *Arapidopsis* (64).

Additionally, analyses of 2D gels of proteins labeled for 1-24 h under constant 34°C or 15°C (only 12 and 24 h labeling as examples are presented in Fig. 3.7) clearly illustrated that proteins synthesized by ovules of the same age were different under 34°C and 15°C constant, which could be explained primarily by the 15°C-induced developmental delay, or, vice versa. These gels indicate that 14, 16, 20 and 92 kD proteins could be associated with initiation and early elongation since they were intense at 24 h in 34°C at the time fiber initiation occurs (circles and diamonds, Fig. 3.7). A 12 kD protein (square) seems to be unique to 15°C because it never appeared in 34°C culture across the time course, but was detectable after 3 h in 15°C and thereafter. This protein was not present during 12 h labeling at 34°C after a 34/15°C cycle, but appeared upon labeling at 15°C after 1 cycle in 15/34°C (gels not shown). On the other hand, 34°C induced several unique protein species of low MW (circles in Fig. 3.7). A 13 kD spot (indicated by square) was detectable during 3-12 h and disappeared at 24 h labeling in 34°C, whereas the protein was present across the time course in 15°C (Fig. 3.7), indicating that this particular protein was developmentally regulated. Consistent with the result from cycling conditions, the changes observed were mainly quantitative and the majority of proteins were synthesized rapidly in 15°C by 3 and 24 h (Fig. 3.7). This indicated that a base population of protein was established and maintained even under 15°C.

In contrast to the cycling result, constant 15°C greatly reduced total protein synthesis. The cpm counts in ovule extract labeled at 15°C were 38.6-17.9% of the control 34°C over the time course, averaging only a quarter of those in 34°C (24.57%, Table 3.3). In a similar experiment, total cpm counts in crude extracts after 48 h in 15°C were equal to 6 h at 34°C, in which cpm reached a maximum and plateaued after 24 h (Table A.2). The reduction in both the uptake of ^{35}S-a a into ovules and incorporation into protein was expected, because 15°C, as a low, non-lethal temperature stress, would suppress overall metabolism and thus fiber growth. Other researchers have shown increased incorporation of ^{35}S- a.a. into protein during cold acclimation after 4 d exposure to low temperature (43). However, the duration of our experiment was only 24 h.

Ovule and fiber growth were severely hindered under constant 15°C, but they could immediately resume rapid elongation after released from the stress (Chapter 2). The quick recovery of elongation after stress at 15°C and the active synthesis of virtually all 34°C-proteins in 15°C suggest that basic cellular machinery was established and was maintained

even at 15°C. Unlike the heat shock response (1), moderate low temperatures have less effects on and generally do not shut off the synthesis of constitutive proteins in favor of CA proteins (42). The quick, full recovery of respiration and the reduced uptake of glucose and synthesis of cellulose (the major cell wall component) in the cycled ovules/fibers (90) indicate that other metabolic adjustments may be made also for elongation to adapt to the cycling condition.

Table 3.3 Time course of total ^{35}S-methionine incorporation into protein under constant 34°C and 15°C (cpm x10^5/µl phenol extract)

	Culture and labeling time in hour						
T°C	1	3	6	9	12	24	36**
34°C	3.56	15.93	33.74	57.49	117.97	218.62	379.02
15°C	1.37	4.07	9.81	11.48	19.30	39.04	47.20
%*	38.61	25.54	29.07	19.96	16.36	17.86	12.45

** Labeled in last 12 h after 24 h culture.
* The percent cpm in 15°C compared to 34°C.
1. Average % cpm in 15°C is 24.57%, (36 h point excluded).

3.3.5 *In vitro* Labeling to Examine Protein Stability

3.3.5.1 Pulse labeling under Constant Temperatures

Pulse labeling experiments were designed to determine if proteins were specifically degraded with each temperature shift in the cycling condition. When ovules of -1 dpa were initially labeled without previous culture for 12 h in 15°C (starting condition as in Fig. 3.7b), then further incubated in unlabeled medium for 12 h in 15°C, the 12 kD protein (marked by square) remained intense (Fig. 3.8a). However, when the ovules were incubated for a further 12 h in 34°C, it disappeared (Fig. 3.8b). Verifying its specificity for synthesis at 15°C and its quick turnover at 34°C, the 12 kD spot also was present if ovules were labeled for 12 h at 15°C after first culturing 12 h at 15°C (Fig. 3.8c) or labeled for 24 h at 15°C (Fig. 3.8e and Fig. 3.7d). However, it did not appear under labeling for 12 h in 34°C after first culturing 12 h in 15°C (Fig. 3.8d). The 12 kD spot reappeared upon re-labeling in 15°C after 12 h culture in unlabeled medium at 34°C (Fig. 3.8f). These results indicate that, in addition to inhibiting synthesis of particular proteins, 15°C could induce synthesis of new proteins like the 12 kD species that disappeared upon shifting to the 34°C

side of the cycle. Such proteins may contribute to the acclimation of fiber elongation to 15°C in the cycling regime.

Besides the inhibition of the 12 kD protein, 12 h culture at 34°C in labeled or unlabeled medium after labeling in 15°C particularly increased some 34°C-specific proteins (diamonds & circles in Fig. 3.8 b & d). The temperature-modulated synthesis of these proteins is shown further by their presence under 24 h labeling at 34/15°C or 15/34°C (Fig. 3.9 a & b). There is no difference in the protein profiles between these two cycling conditions, which differ only in the temperature to which ovules were first exposed after culture. This is consistent with very similar elongation rates of fibers grown under these cycling conditions. The induction at low temperature and termination at warm temperature of the CA protein synthesis was highly correlated with the induction and loss of freezing tolerance in spinach seedlings in which CA proteins were rapidly degraded in early deacclimation (42).

To determine how proteins synthesized at 34°C were affected upon shifting to 15°C, -1 dpa ovules were labeled for 12 h at 34°C (starting condition as in Fig. 3.7a), then further incubated in culture medium for 12 h at 15°C. Under this condition, several proteins of high MW decreased or disappeared (circle in Fig. 3.10a). However, the putative 15°C specific 12 kD protein did not appear. The same proteins remained if the final 12 h of culture was at 34°C with the exception of one (triangle Fig. 3.10b) and reappeared upon relabeling at 34°C after 12 h unlabeled incubation in 15°C (Fig. 3.10c). Also these proteins were present if labeling was done during 24 h in 34°C (Fig. 3.7c) or during 12 h in 34°C after an initial 12 h unlabeled incubation in 34°C (Fig. 3.10d). In addition, labeling for 12 h after culture for 12 h under the same temperature (34°C) showed several proteins were reduced compared to the first 12 h labeling (squares in Fig. 3.10 b & d), supporting the developmental regulation in protein synthesis. Most importantly, 34°C up-regulated low molecular weight proteins (shown in Fig. 3.7), and overall protein profiles are not affected by subsequent incubation in unlabeled medium at 15°C.

Comparing these two pulse labeling experiments (Fig. 3.8 and 3.10) revealed a unique 70 kD protein synthesized during exposure to 34°C (diamond) that was not degraded by the shift to 15°C (Fig. 3.10a), but this protein was not synthesized under 15°C (Fig. 3.7 b & d). This protein may be important for normal fiber elongation. Because only a small fraction of the label taken up by the cells was actively incorporated into protein, a reservoir of unused ^{35}S-a a would have been present and was possibly used later during incubation at the other temperature. The lack of substantial change in protein profiles upon

shifting temperatures as described above may reflect continuous synthesis of protein from an internal labeled amino acid pool, lack of degradation of previously synthesized proteins, or a combination of the two. Nevertheless, these results suggest that loss of particular protein species due to shifting to 15°C is not a major cause of 15°C inhibition of fiber initiation and elongation. This speculation is further substantiated by the results from the 24 h labeling under both cycling conditions (Fig. 3.9). Therefore, the protein population synthesized in 34°C could supply the base for normal fiber elongation under 34/15°C cycling. However, the results cannot exclude the possibility that undetected proteins are lost that could cause the growth inhibition.

3.3.5.2 Pulse labeling under Cycling Temperatures

Ovules at -1 dpa, after culture for 2 days in 34°C or 15/34°C, were labeled with 100 uCi ^{35}S-a.a for 12 h under their initial culture temperatures, and then either harvested or incubated in unlabeled medium for an additional 12 h at the opposite temperature to the one initially used for the labeling. The additional 12 h in 15°C or 34°C altered specific proteins compared with those initially labeled (Fig. 3.11). A shift to 15°C after 12 h labeling in 34°C caused very minor reduction in some high MW proteins and enhanced some low MW proteins (circles in Fig. 3.11 a & b). However, similar to the results of the above section, the overall protein profiles remained very similar. Likewise, incubation in unlabeled medium at 34°C after 12 h labeling in 15°C both increased (circles) or reduced (squares) some high and low MW species (Fig. 3.11 c & d). The specific proteins that increased in intensity upon further incubation in unlabeled medium at 34°C were those normally up-regulated by 34°C as previously described. Comparison of these proteins suggests that some low molecular weight proteins were quantitatively modulated by temperature. Not all of these proteins were the same as those observed in pulse labeling experiments under constant temperatures, indicating specific effects of cycling conditions.

Comparison between the two initially labeled gels (Fig. 3.11 a & c) revealed numerous unique proteins in 34°C, although these could be due to the older physiological age of the 34°C ovules. Consistent with the previous labeling in the 34°C part of the cycle, numerous unique species were not detected under cycling even when the ovules were labeled at 15°C. One protein of about 12 kD (indicated by small square) was faintly visible, and is probably equivalent to the one previously described under constant 15°C. Other proteins were intensified by 15°C (square). These results partially confirmed the results from labeling at the high side of the cycle; few new protein are induced by the cycling

regime. The proteins inhibited by labeling at the 15°C side are consistent with the constant labeling results. Since the ovules were at different developmental (not chronological) ages (1 d lag) at the start of the labeling due to 2 d cycling culture in the experiment and the ovules were 2 d older compared to the constant labeling experiments, the shift in particular protein profiles may be attributed to the effects of the developmental stage. In this experiment only one labeling date was used; other new proteins might be detected if the labeling in 15°C had been started earlier or extended to more cycles.

3.3.6 *In vitro* Translation

Several unique proteins were observed on 2D gels of *in vitro* translation products of mRNA isolated from -1 dpa ovules after 24 h culture at 34°C or 15°C (Fig. 3.12 a & b; proteins unique to 34°C are indicated by the arrow and the circle; those unique to 15°C are marked by a square). Because changes in gene expression is expected to occur with advancing development, the difference in the polypeptide profiles may reflect a causal factor in the temperature-induced delay in development. On the other hand, Weiser (108) proposed that CA, leading to greater freezing tolerance, involved altered gene expression. Low temperature induced changes of mRNA content were correlated with the degree of cold tolerance in spinach leaf (53), tomato (94), rice (14, 44), and alfalfa (72). However, not all the unique protein species labeled in cultured ovules were shown in *in vitro* translation products. For example, the 15°C induced 12 kD spot was not detectable here. This may point to differences in transcriptional versus translational regulation, although more replications and variations of the *in vitro* translation studies would be necessary to be sure. The shift in the polypeptide pattern under 15°C might partially result from the expression of genes involved in adjustment of ovule metabolism to low temperatures, and these polypeptides might play a direct role in leading to growth adaptation.

3.4 Summary

When cotton ovules and fibers were exposed to a 15°C/34°C (12 h/12h) cycling temperature, the elongation rate of fibers reached that of controls after an initial delay, suggesting cold acclimation in cotton fiber elongation. Proteins labeled during the 34°C side of a 34/15°C cycle demonstrate a developmental delay in the synthesis and flux of a specific set of proteins, which parallels the initial delay in the elongation under the cycling temperature. This corresponds well to the adaptation of elongation and suggests a crucial role of these late-appearing proteins in fiber development. These results provide direct

evidence of a molecular basis underlying the adaptation of elongation to low temperature. Presumably, at least some of the proteins at 10, 12, 20 and 92 kD that appear later under 15/34°C are required for rapid fiber elongation. However, other substantial changes in protein profiles were not observed, except those that could be attributed to the developmental delay. Therefore, this elongation adaptation may be related to a metabolic adjustment of preexisting proteins/enzymes to cold conditions, to more time being required for the same essential set of proteins to be produced, and /or to undetected changes in protein population.

Plants often synthesize a set of novel proteins during cold acclimation (41, 105), for review). A positive correlation between the increase in ability to synthesize protein at 4°C and an increase in freezing resistance was reported in alfalfa (70). In contrast, no new proteins were detected by our method when labeling was done during the 34°C side of the cycle across day 1 through 13 compared to constant 34°C. Several limitations in this study may have caused new proteins to remain undetected. For example, the elongation of single fiber cells is driven by turgor pressure (23) and depends on the extensibility of the primary cell wall, which is determined by the cross-linking within and between carbohydrate and protein components. Most cell wall proteins remained unextracted by our methods. Similarly, membranes, being a major target of chilling injury, have a significant effect on the ability of plants to withstand low, non-freezing temperature (41). The induction of chilling resistance was accompanied by increased polyunsaturated fatty acids in cotton seedlings (88) and by changes in membrane protein in alfalfa (71). However, our techniques are not likely to have extracted all membrane proteins. Additionally, the phenol extraction may cause loss of proteins if they are co-precipitated with phenolics at the interface of the aqueous and phenol phases (39). Since the proteins were labeled in the 34°C side of the cycle, any possible new proteins transiently induced after shifting from 15°C to 34°C may be rapidly turned over and not detected after 12 labeling in 34°C as observed in the case of shifts from 28°C to below 22°C and to 40°C. However, data from the 15°C side of the cycle and constant 15°C revealed the synthesis of at least one new protein of 12 kD that appeared in 15°C at the earliest labeling interval possible, 3 h, and remained throughout the 24 h labeling period. This protein was turned over upon shifting into 34°C. Likewise, some minor 34°C specific proteins disappeared during subsequent incubation at 15°C. The re-synthesis of these proteins upon shifting back to 34°C indicated that they were more temperature sensitive than developmentally responsive, and that these proteins may contribute to the elongation adaptation and normal fiber growth.

Constant 15°C, or 15°C as part of a cycling regime, subjected ovules to stress as evidenced by delay in the fiber initiation and primary wall extension (see Chapter 2) and the inhibition of the synthesis of a specific set of proteins by 15°C, which may contribute to normal fiber elongation. Cold acclimation generally does not shut down housekeeping gene expression, and it simultaneously induces or up-regulates the synthesis of selected proteins (42). The overall similarity in the profiles between the cycling, constant 15°C, and 34°C control conditions may reflect the molecular basis for the slow but continuous growth at constant 15°C and a similar rate of later elongation under cycling temperature. The similarity also supports the speculation that alternating 12 h 34°C could partially compensate the inhibitory effects of previous exposure to 15°C. Evidence supporting this possibility include: (1) as in other plant species (19), the proteins present at 34°C were still actively synthesized at 15°C except for a group of low MW proteins; (2) proteins synthesized in previous regimes especially in 34°C were rarely turned over or degraded upon shifting to the opposite temperatures; (3) total protein synthesis (indicated by cpm counts at 34°C side labeling) was not affected by previous 12 h exposure to 15°C. The degradation of minor proteins synthesized at 34°C upon a shift to 15°C, the degradation of a 15°C-induced protein by a shift to 34°C, and inhibition of synthesis of 34°C proteins by the previous 15°C cycling, altogether may be responsible for the initial delay in initiation, initiation of fewer fibers, and ultimately, low dry weight accumulated under 34/15°C cycling (45).

The adaptation of elongation to low cycling temperature may depend on an adjustment in cellular metabolism (such as changes in turnover, synthesis or inhibition of particular protein(s), glucose uptake and cellulose synthesis) that is reflected in different responses at different phases of fiber development (e.g., delayed initiation, equal final fiber length but reduced dry weight). Since induction of cold acclimation often is simultaneously accompanied by a reduction in growth rate (63) and thus developmental changes, the changes in protein synthesis might be expected to partially reflect the growth rates and later adaptation of fiber elongation to the cycling temperature. The latter is favored by the parallel between delay in protein profiles with the developmental delay under the cycling temperature. The temporal difference in both protein profiles and fiber elongation between the cycling and control conditions strongly suggests developmental regulation in the protein changes. Though general protein profiles have been characterized with cotton ovule and fiber, no specific protein marker has been assigned to a given stage (39). Direct comparison can not be made with the previous results in order to separate proteins related

to development and adaptation. However, the dynamic changes in the protein population from ovules *in vivo* and *in vitro* indicate the developmental regulation of protein synthesis under ambient and low temperatures. Sensitivity to low temperature is modulated with developmental stage as reflected at both plant growth (63) and COR-gene expression in barley leaf (19).

Analysis of individual protein spots and total protein patterns from overall 2D gels indicated that proteins could be grouped according to their differential response to temperature: (1) unaffected constitutive proteins that are present across the time course under all regimes; (2) quantitatively reduced proteins, as reflected by a reduced uptake and incorporation of ^{35}S-a.a. into protein, and the reduced intensity of some protein spots by 15°C ; and (3) qualitatively changed proteins that are induced or inhibited in 15°C or 34°C, including both the developmental regulated and temperature-specific protein species. Preliminary results suggests that protein synthesis is regulated at both the transcriptional and translational levels. These differences in temperature response can possibly be explained by the particular function of each group of proteins in ovule/fiber growth. The roles of these low temperature-responsive proteins cannot be determined by our data and deserve further study. Presumably, some of these proteins have important functions in fiber elongation and its adaptation to low temperatures. Microsequencing of these proteins and cloning of the genes would be a first step toward determining their roles in normal fiber development and/or cold adaptation.

Fig. 3.1. Optimal temperature for protein synthesis. 1D fluorograph of -1 dpa ovule protein labeled for 3 or 12 h at different temperature regimes as indicated on the top of the gel. Protein was solubilized in 1D sample buffer (see Materials and Methods). Each lane was loaded with aliquots of the crude protein extract 50 kcpm and subjected to electrophoresis on a 12% acrylamide gel. Letters a and b indicate labeling for 0.5 h at 28°C then 2.5 h at 5°C and 15°C, respectively. The positions of the protein standards are indicated on the left.

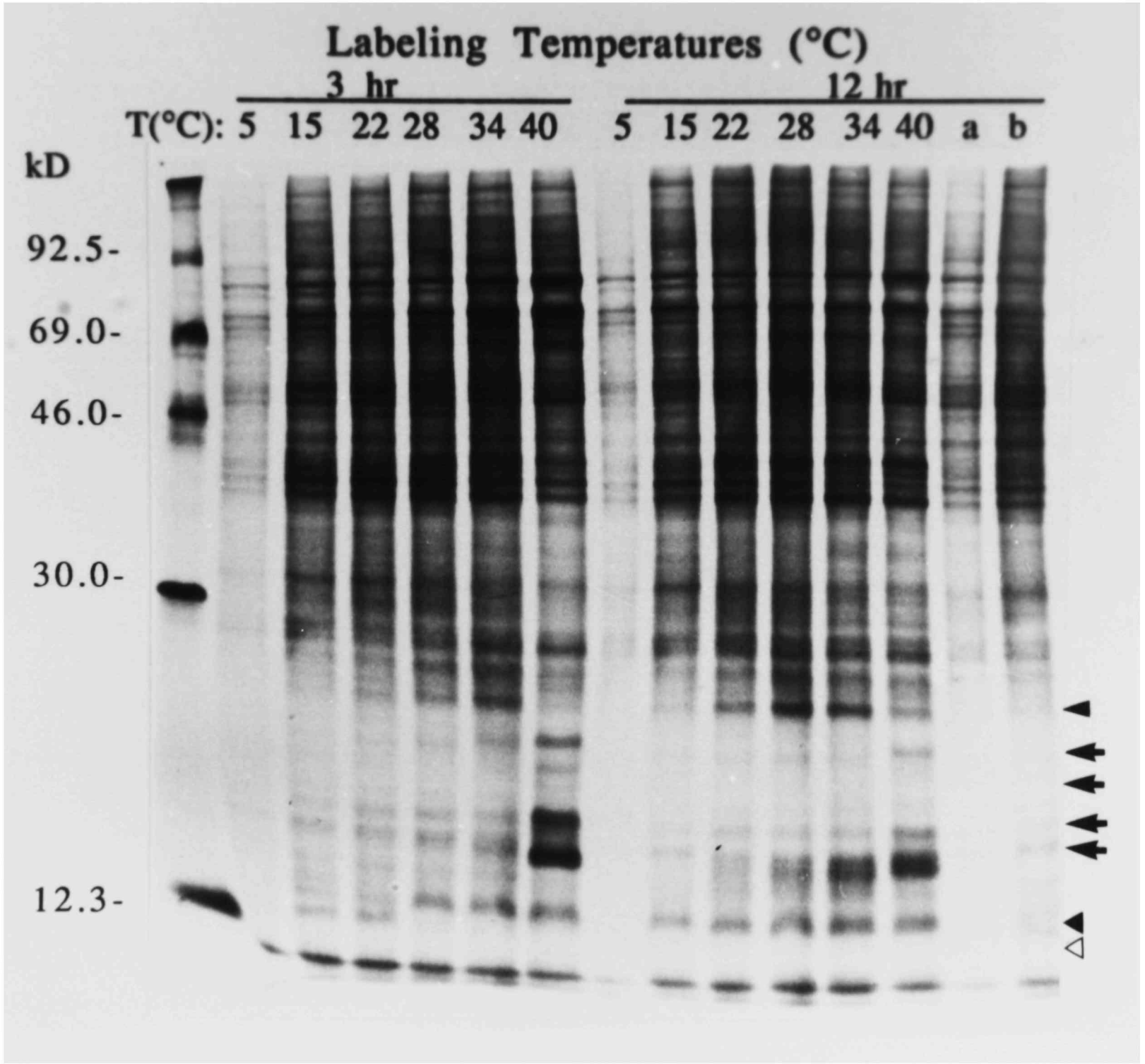

Labeling Temperatures (°C)
3 hr
12 hr
T(°C): 5 15 22 28 34 40 5 15 22 28 34 40 a b
kD
92.5-
69.0-
46.0-
30.0-
12.3-

Fig. 3.2. Silver-stained 2D protein profiles around fiber initiation. Aliquots of the protein extracts (120 ug) from greenhouse ovules were subjected to electrophoresis on a IEF gel then a 12% acrylamide gel. Numbers at the top right corner of each gel indicate the ovule age at -3, 0 and +3 dpa. Circles, diamonds, and arrows indicate proteins that were reduced or enhanced on 3 dpa compared to previous days.

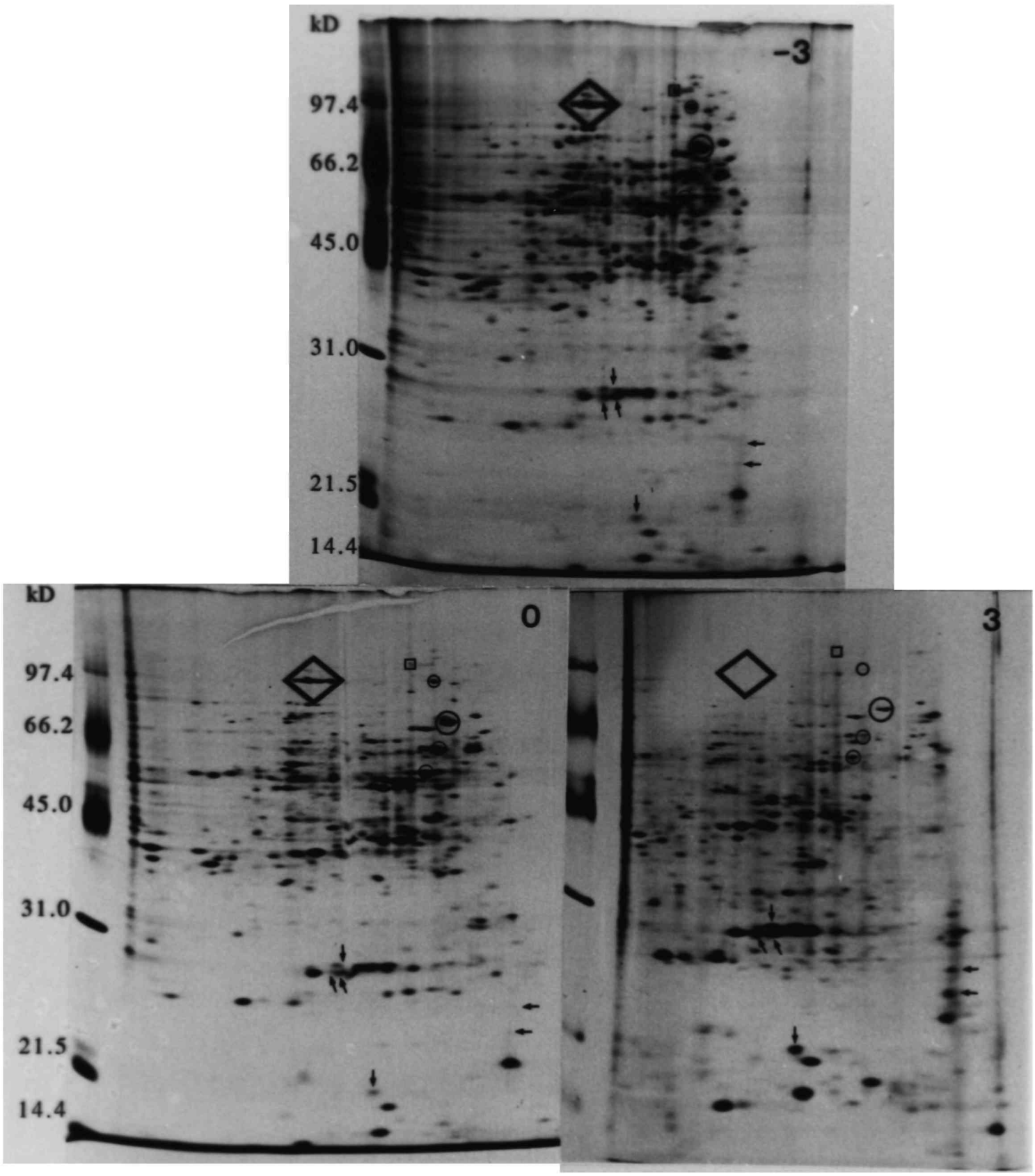

kD
-3
97.4
66.2
45.0
31.0
21.5
14.4
kD
0
97.4
66.2
45.0
31.0
21.5
14.4
3

Fig. 3.3. 2D fluorographs of protein profiles around fiber initiation. Ovules were harvested from greenhouse plants and labeled for 12 h in 28°C prior to protein extraction. The numbers on the gels indicate ovule age in dpa. All gels were loaded with aliquots equal to 1000 kcpm of TCA-insoluble radioactivity except the +3 dpa gel in which 500 kcpm was loaded and exposure time was doubled. Diamonds and arrows indicate proteins that were suppressed and unique/enhanced, respectively, at 3 dpa compared to previous days.

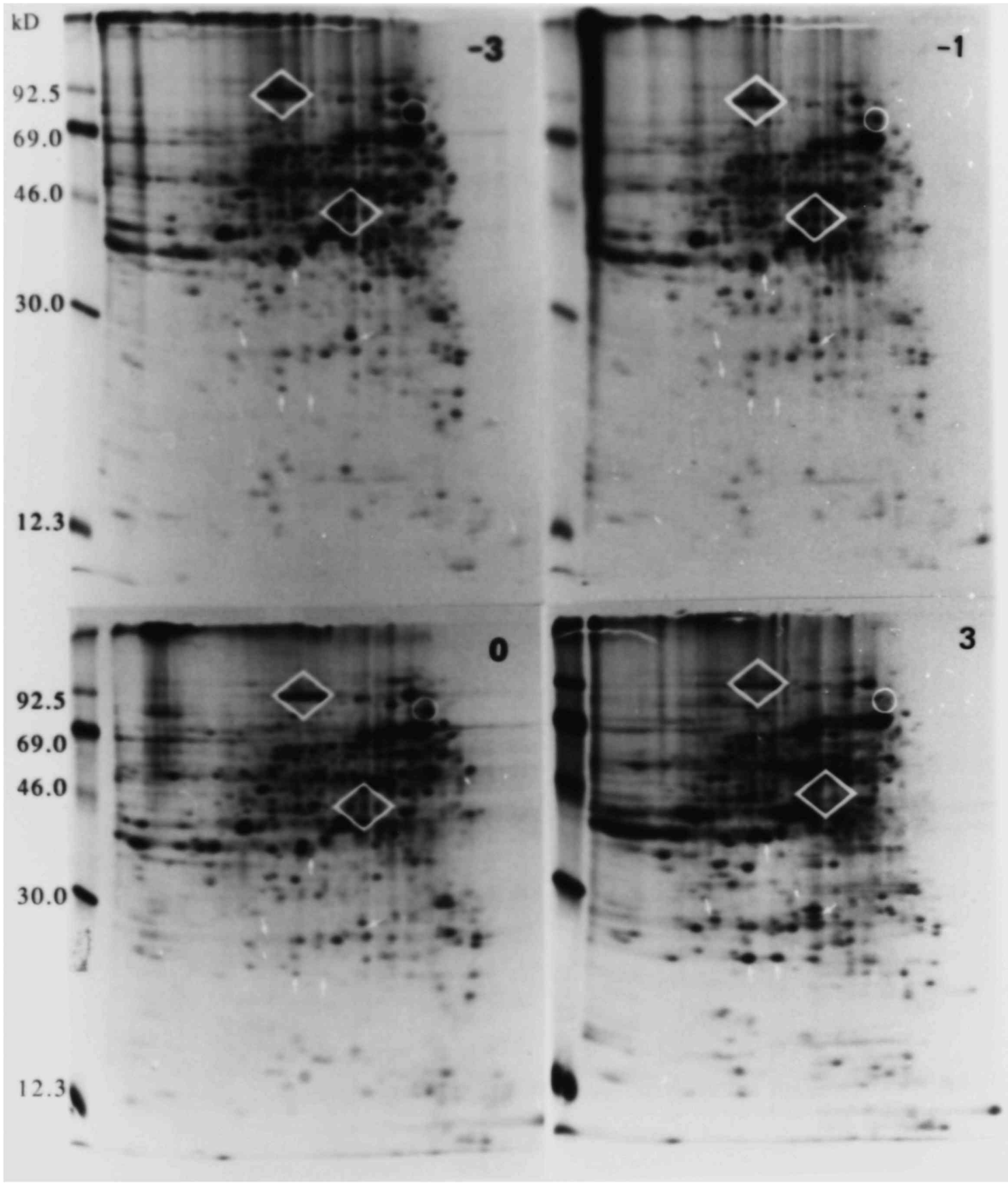

kD
-3
-1
92.5
69.0
46.0
30.0
12.3
0
3
92.5
69.0
46.0
30.0
12.3

Fig. 3.4. 1D gel fluorograph of protein profiles under low cycling temperature. Protein extracts from ovules of -2 or -1 dpa after culture for 1-10 days under 34°C and 15/34°C followed by labeling for 12 h in 34°C were separated by 12% SDS-PAGE. Only the -1 dpa lane corresponds to the -2 dpa starting condition. The number indicates the ovule age in dpa. Arrowheads and arrows point out protein bands suppressed and delayed in disappearance by the cycling, respectively. All lanes were loaded with aliquots equal to 50 kcpm of TCA-insoluble radioactivity. 0*: ovules of 0 dpa harvested and labeled directly without previous culture.

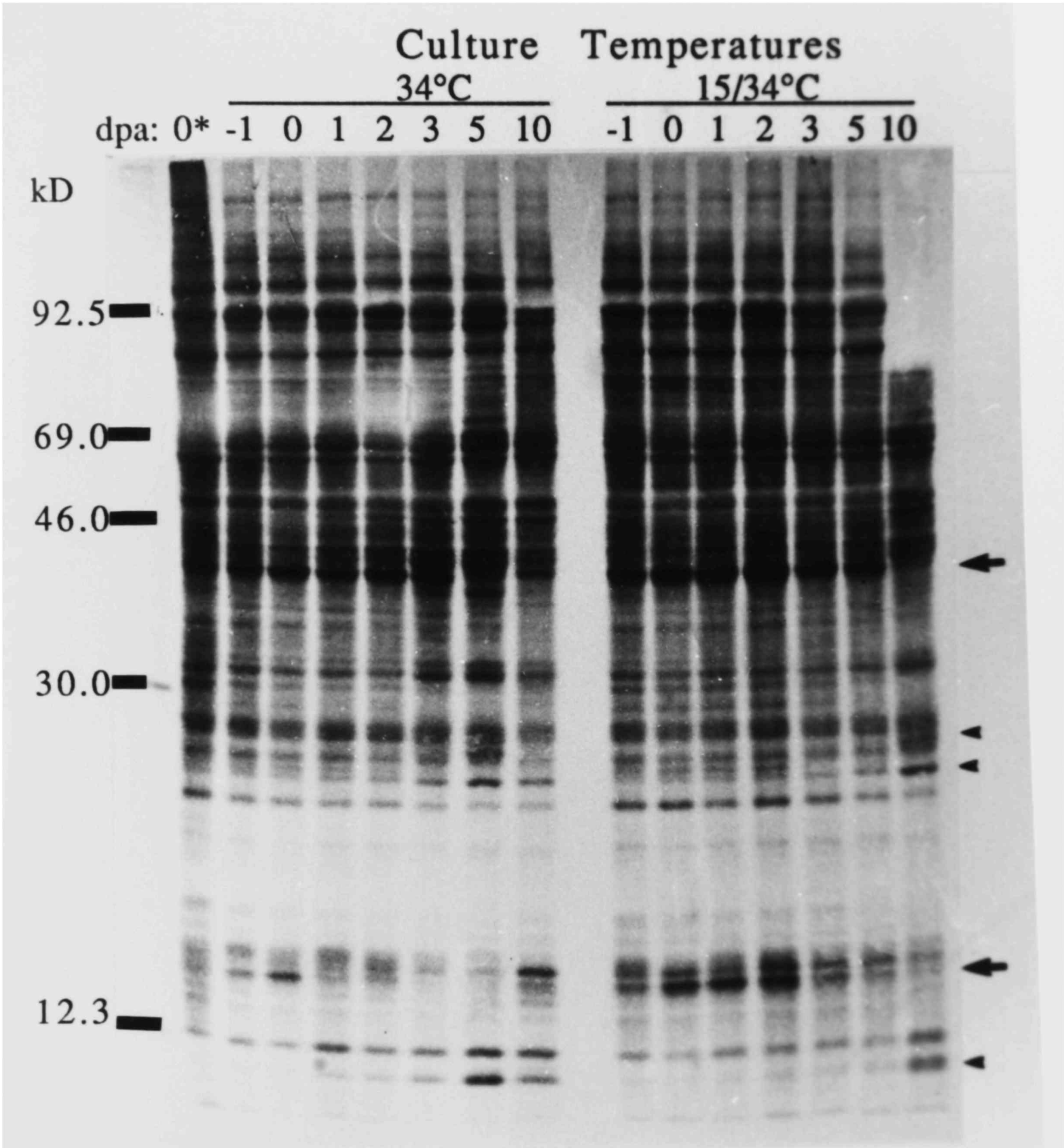
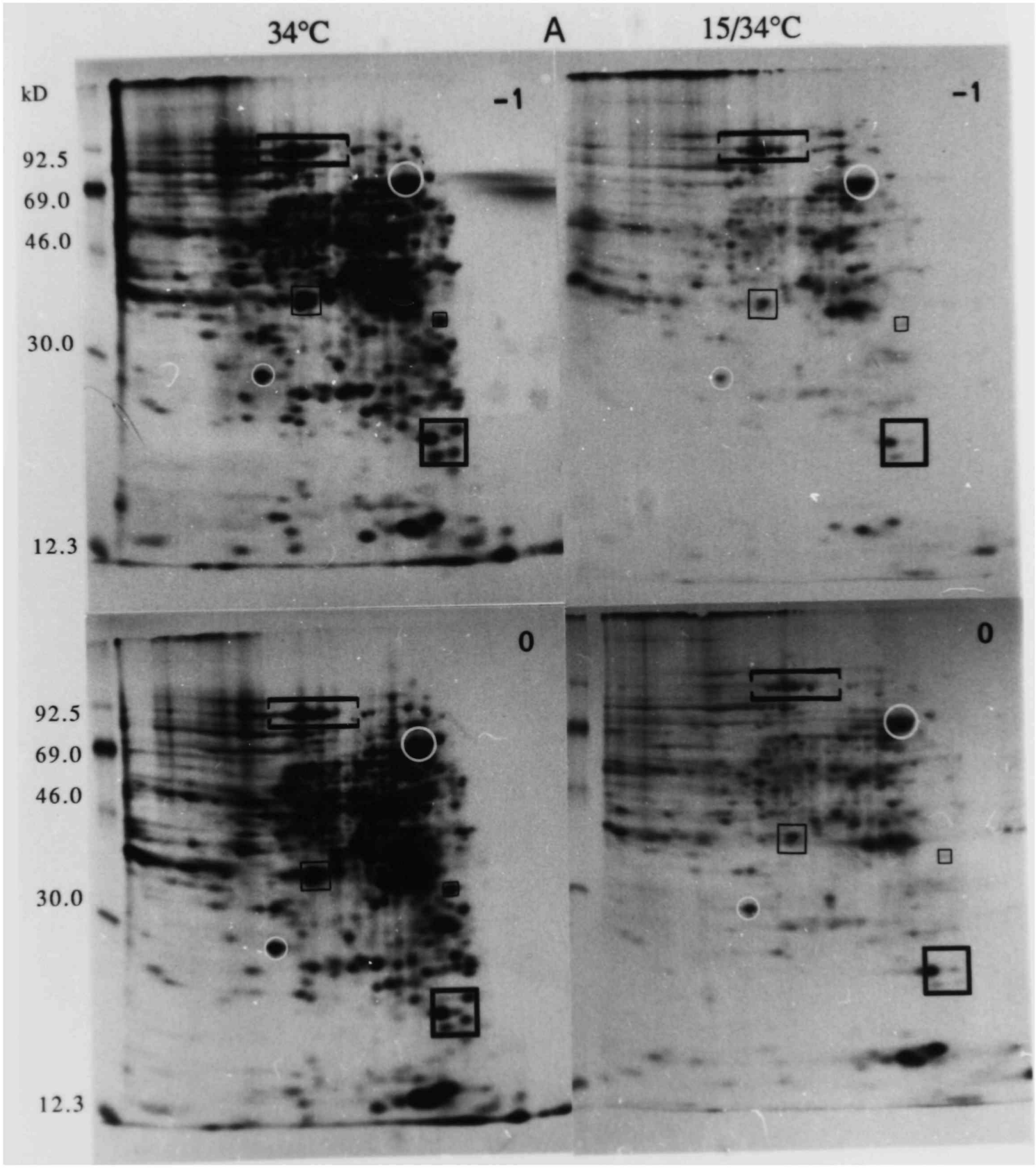
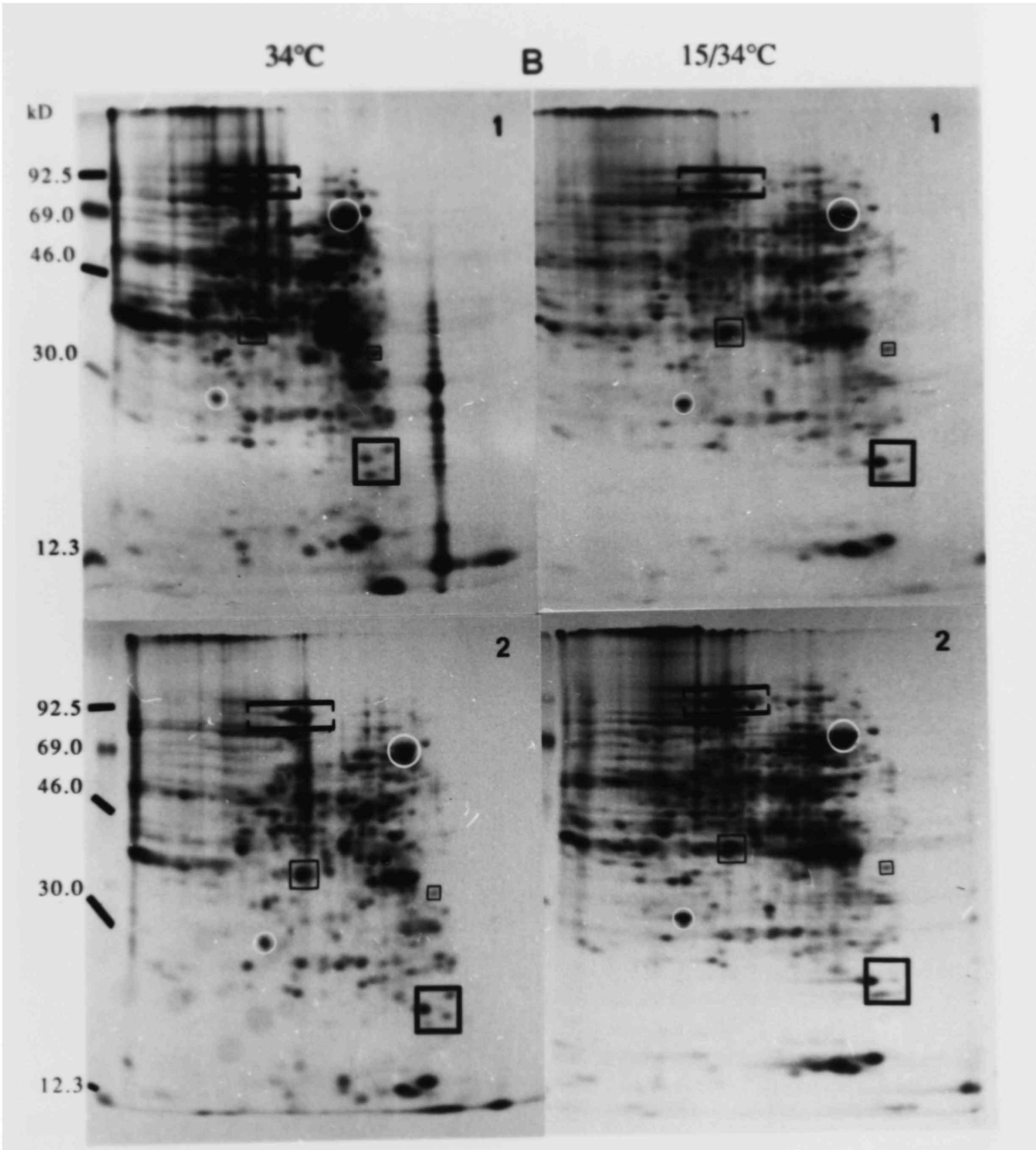
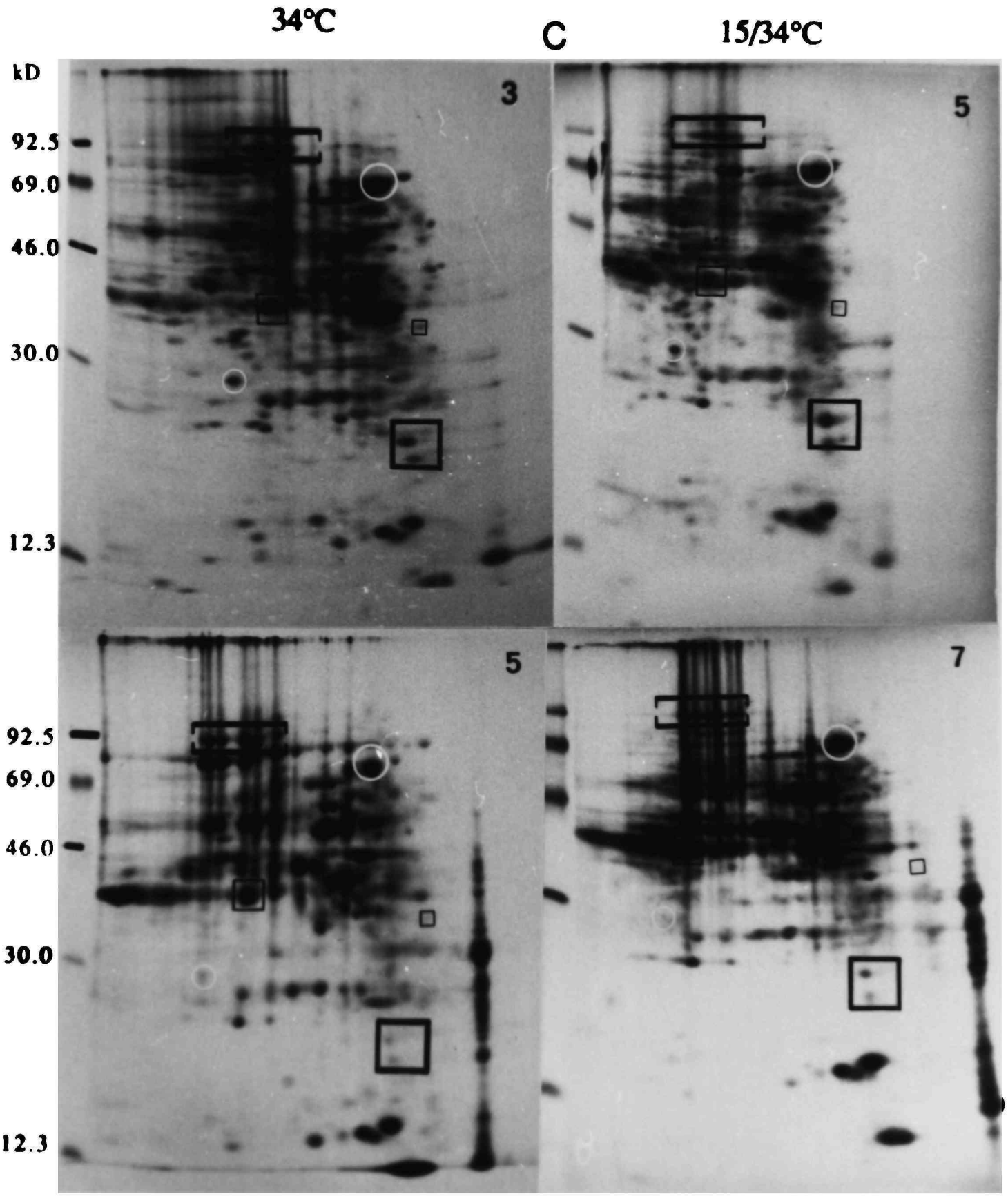

Culture Temperatures
34°C
15/34°C
dpa: 0* -1 0 1 2 3 5 10 -1 0 1 2 3 5 10
kD
92.5
69.0
46.0
30.0
12.3

Fig. 3.5. 2D fluorographs of protein profiles under low cycling temperature. Protein extracts from ovules of -2 or -1 dpa after culture for 1-13 days under 34°C and 15/34°C followed by labeling for 12 h in 34°C were separated by IEF-SDS-PAGE. Only the -1 dpa gels correspond to the -2 dpa starting condition. The numbers on the top right corner of each gel indicate ovule age in dpa. A), -1 and 0 dpa (Page 54); B) 1 and 2 dpa (Page 55); C), 3, 5 and 7 dpa (Page 56); and D), 7, 10 and 13 dpa (Page 57). Black squares and circles point out the proteins repressed by the cycling, white circles point out representative proteins that were less affected by the cycling, and diamonds indicate proteins that were increased later by the cycling. Gels in plate (A) and (B) were loaded with aliquots equal to 1 x 10^6 cpm and exposed for 5 d at -80°C; gels in plate (C) and (D) were loaded with aliquots equal to 500 kcpm of TCA-insoluble radioactivity and exposed for 10 d. The experiments were repeated twice.


34°C
A
15/34°C
kD
-1
-1
92.5
69.0
46.0
30.0
12.3
0
0
92.5
69.0
46.0
30.0
12.3


34℃
B
15/34℃
kD
92.5
69.0
46.0
30.0
12.3
1
1
2
2
92.5
69.0
46.0
30.0
12.3


34°C
C
15/34°C
kD
92.5
69.0
46.0
30.0
12.3
3
5
5
7

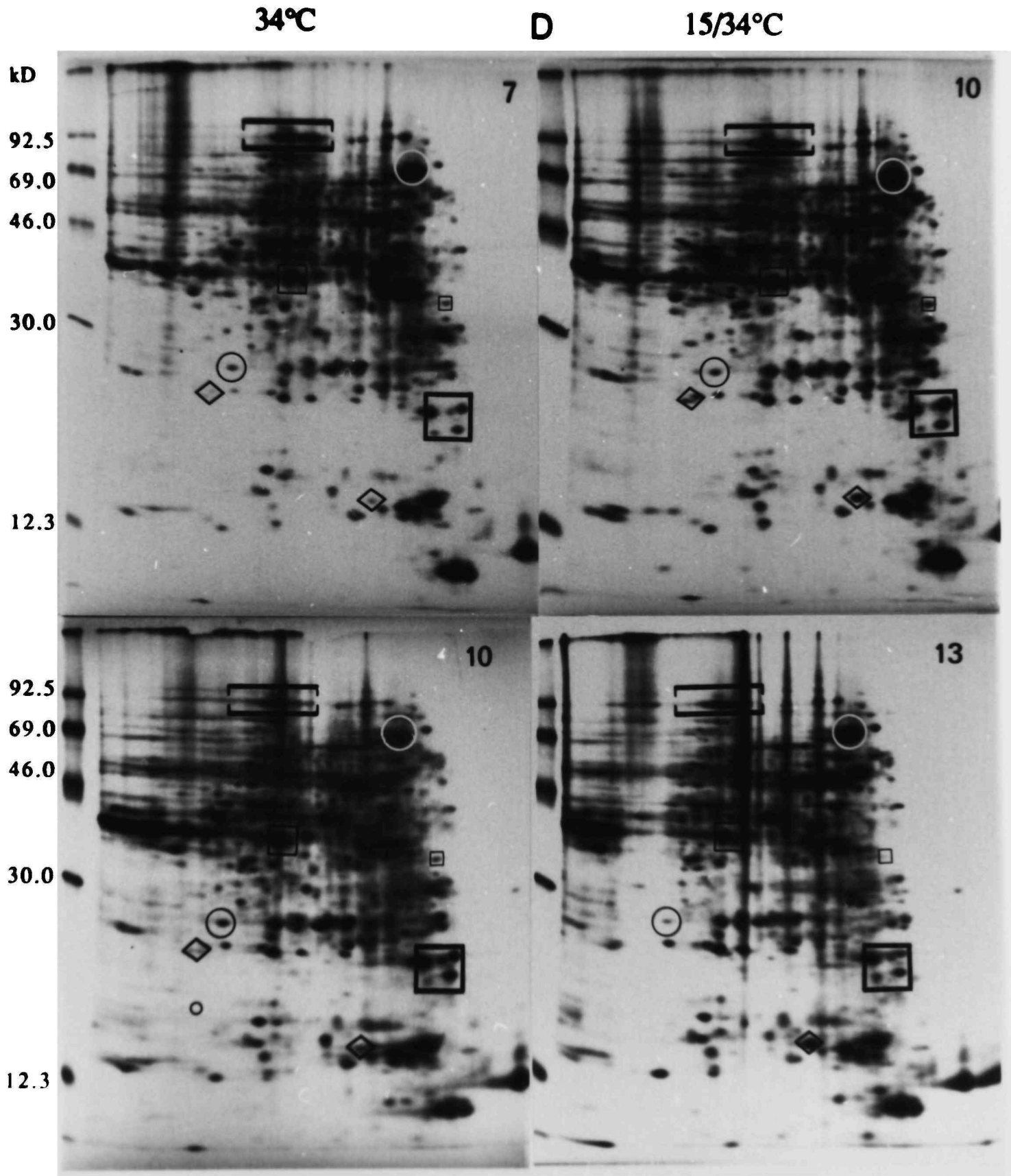
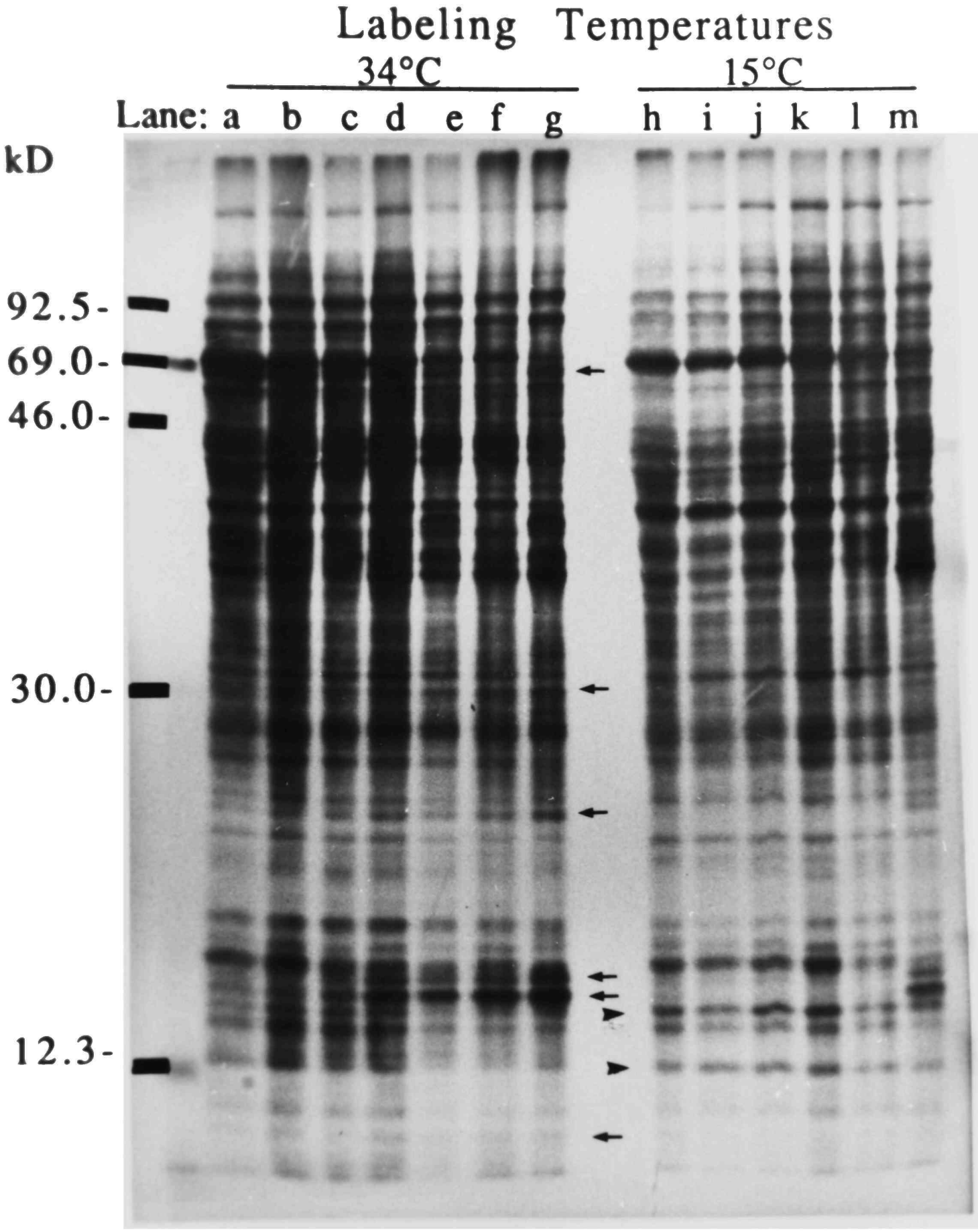

34℃
D
15/34℃
kD
92.5
69.0
46.0
30.0
12.3
7
10
10
13

Fig. 3.6. 1D gel fluorograph of protein profiles under constant temperatures. Protein extracts from -1 dpa ovules after labeling for 1-24 h under constant 34°C, 15°C, and the cycling regime were separated by 12% SDS-PAGE. Lane a-e were labeled at 34°C for 3, 6, 9, 12, and 24 h respectively; lane f and g were labeled for 12 h at 34°C after 24 h culture in 34°C constant and 34/15°C. Lane h-k were labeled at 15°C for 6, 9, 12 and 24 h, and lane l and m were labeled at 15°C for 12 h after 24 h culture in 15°C constant and 15/34°C. Arrows and arrowheads point out proteins suppressed and enhanced, respectively, by 15°C. All lanes were loaded with aliquots equal to 50 kcpm of TCA-insoluble radioactivity. The experiment was repeated 3 times.


Labeling Temperatures
34°C
15°C
Lane: a b c d e f g h i j k l m
kD
92.5-
69.0-
46.0-
30.0-
12.3-

09

Fig. 3.7. 2D fluorographs of protein profiles under constant temperatures. Protein extracts from -1 dpa ovules after labeling under constant 34°C and 15°C were separated by IEF-SDS-PAGE. The labeling times and temperatures are: A) and B), for 12 h in 34°C and 15°C; and C) and D), for 24 h in 34°C and 15°C. Proteins unique or enhanced by 34°C are indicated by circles, diamonds and underlining. The squares indicate 15°C-induced proteins.

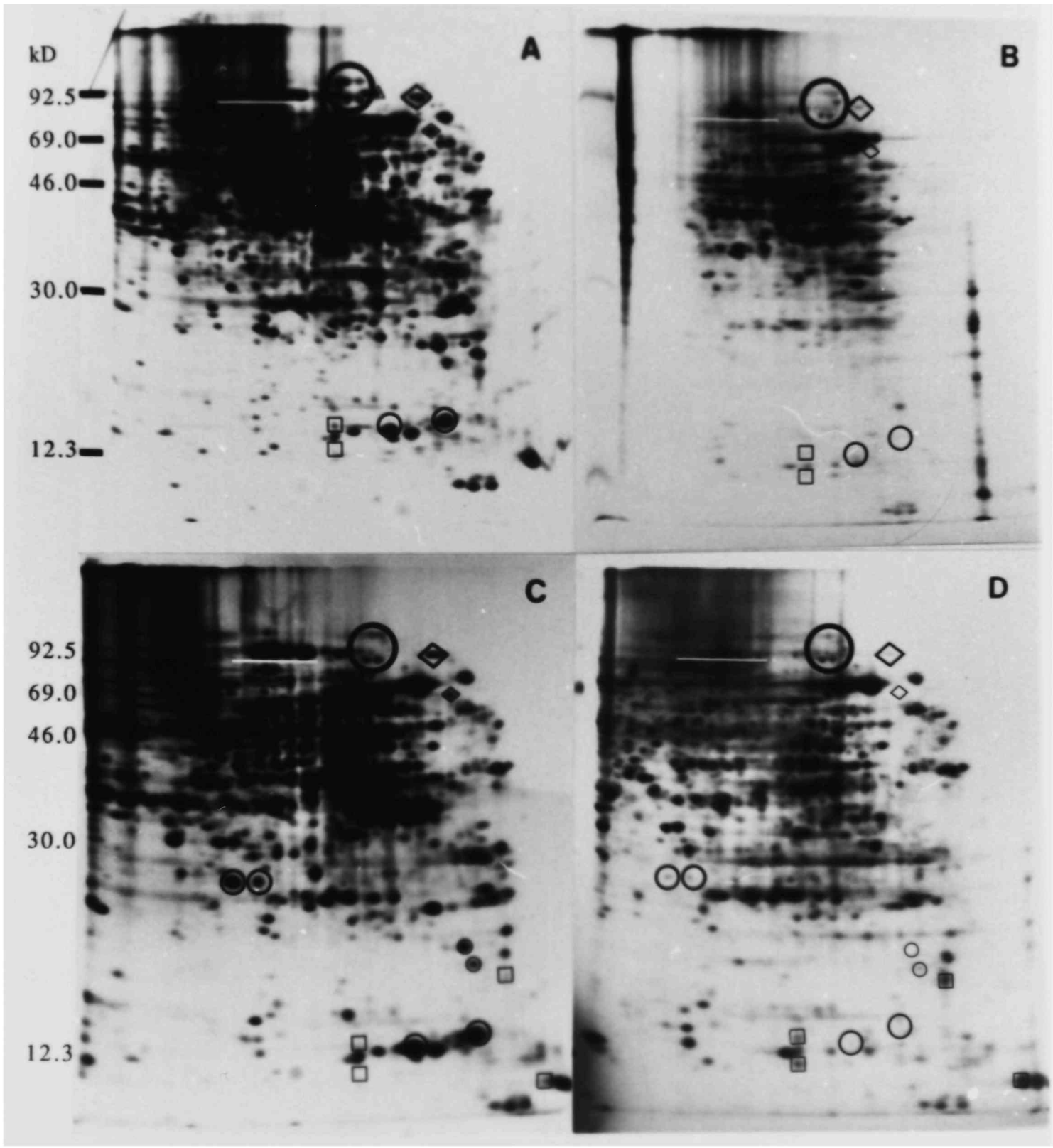

34°C Constant
15°C Constant
kD
92.5
69.0
46.0
30.0
12.3
A
B
C
D

Fig. 3.8. Test for degradation of proteins synthesized at 15°C. 2D gel fluorographs of protein extracts from -1 dpa ovules after labeling 12 h in constant 15°C (Fig. 3.7b). Then: A). 12 h incubation in 15°C; B). 12 h incubation in 34°C; C). First 12 h culture then later 12 h labeling both in 15°C; D). Later 12 h labeling in 34°C after first 12 h culture in 15°C; E). 24 h labeling in 15°C; and F). re-labeling in 15°C after treatment as in (B). Squares indicate the proteins unique to or enhanced in 15°C, and circles and diamonds indicate proteins enhanced in 34°C.

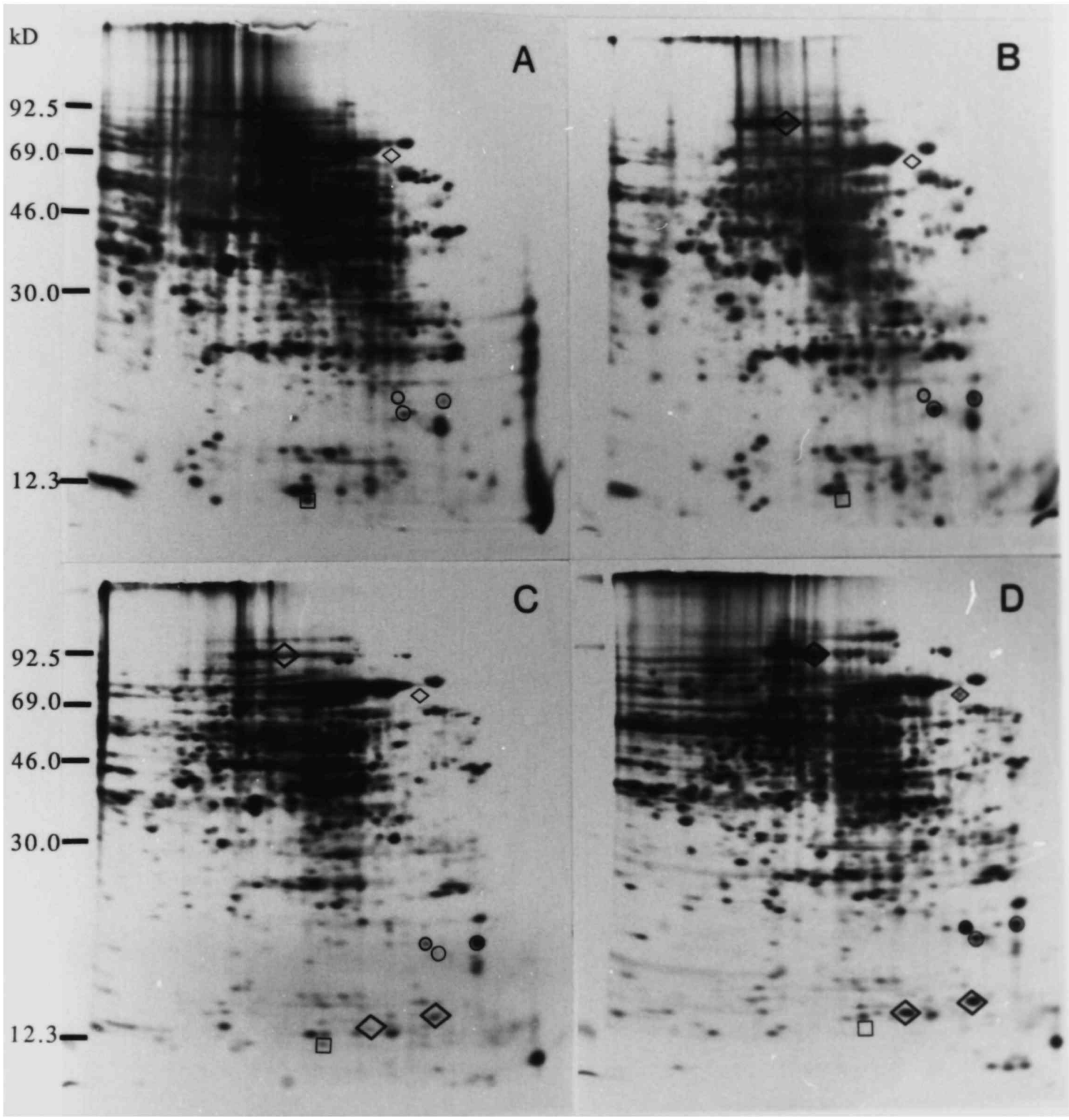

kD
A
B
92.5
69.0
46.0
30.0
12.3
C
D
92.5
69.0
46.0
30.0
12.3

Fig. 3.8. Continued (Top) Test for degradation of proteins synthesized at 15°C. 2D gel fluorographs of protein extracts from -1 dpa ovules after labeling 12 h in constant 15°C (Fig. 3.7b). Then: E). 24 h labeling in 15°C; and F). re-labeling in 15°C after treatment as in (B). Squares indicate the proteins unique to or enhanced in 15°C, and circles and diamonds indicate proteins enhanced in 34°C.

Fig. 3.9. (Bottom) Comparison of protein synthesis under opposite cycling temperatures. Fluorographs of protein extracted from -1 dpa ovules after cultured and labeled simultaneously for 24 h in 12 h/12 h cycling 34/15°C (A) and 15/34°C (B). Circles and diamonds indicate proteins that were up-regulated by 34°C compared to 15°C. Triangles indicate proteins that differed between the two regimes. The experiment was repeated 3 times.

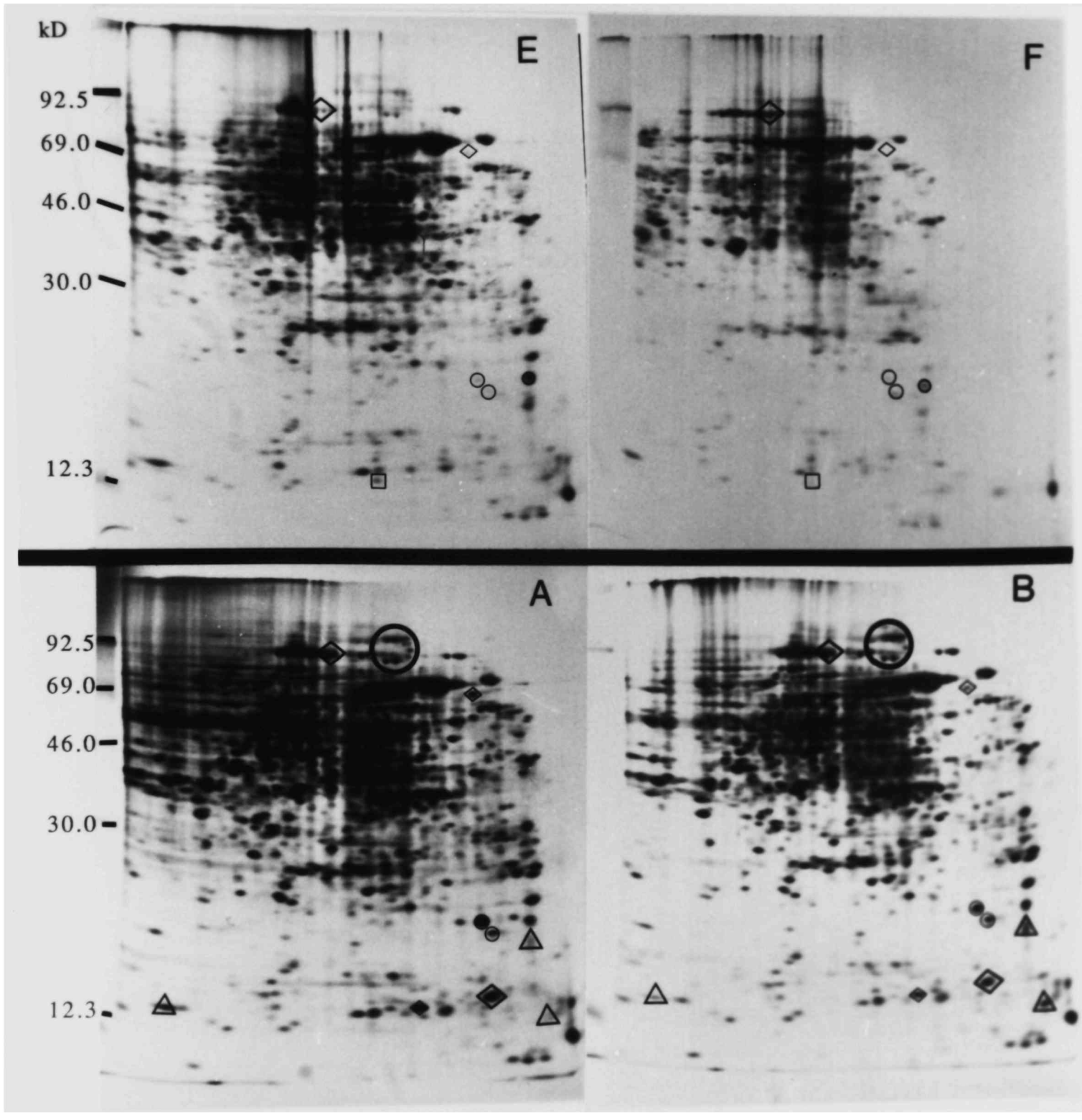

kD
92.5
69.0
46.0
30.0
12.3
E
F
A
B
92.5
69.0
46.0
30.0
12.3

Fig. 3.10. Test for 15°C degradation of proteins synthesized at 34°C. 2D gel fluorographs of protein extracts from -1 dpa ovules after 12 h labeling in constant 34°C (Fig. 3.7a). Then: A). 12 h incubation in 15°C; B). 12 h incubation in 34°C; and C). relabeling for 12 h in 34°C after treatment as in (A); D) condition indicates first 12 h culture then 12 h labeling both in 34°C. Circles indicated the proteins that were enhanced in 34°C or degraded in 15°C, and diamonds indicate proteins unique in 34°C compared with the results from 15°C labeling condition (Fig. 3.8). For other details see text.

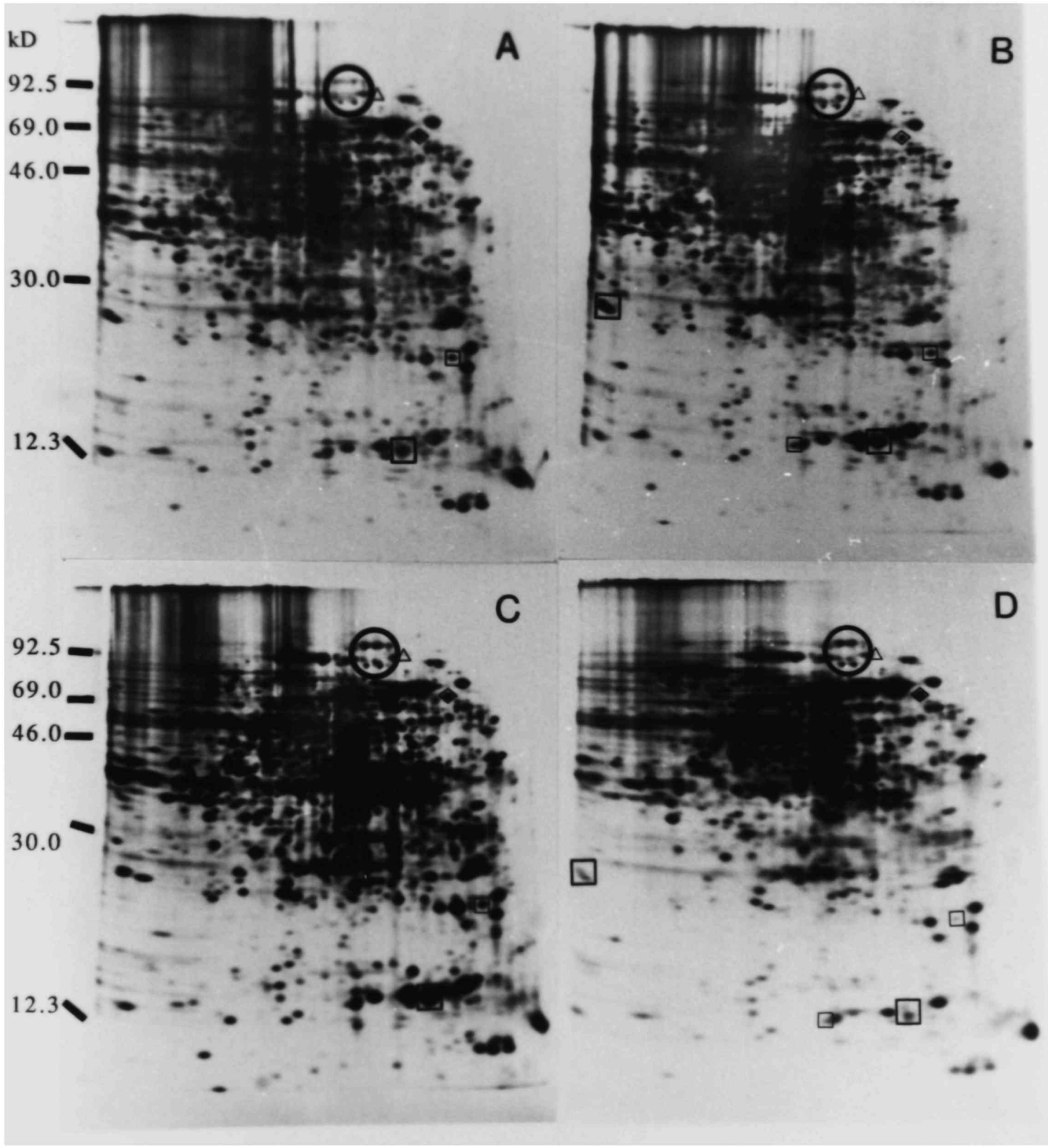

kD
92.5
69.0
46.0
30.0
12.3
A
B
C
D

Fig. 3.11. Protein synthesis and degradation under the low side of the cycling regime. 2D fluorographs of protein extracts from -1 dpa ovules after culture for 2 days, respectively, in constant 34°C or 15/34°C. Then: A). 34°C cultured ovules were labeled 12 h in constant 34°C; B). Further 12 h incubation in 15°C after the initial labeling as in (A); C). 15/34°C cultured ovules were labeled for 12 h in 15°C; D). Further 12 h incubation in 34°C after the initial labeling as in (C). Circles indicate proteins that were enhanced during labeling or subsequent incubation in 34°C. Squares indicate proteins that were induced in 15°C or inhibited by labeling or subsequent incubation in 34°C

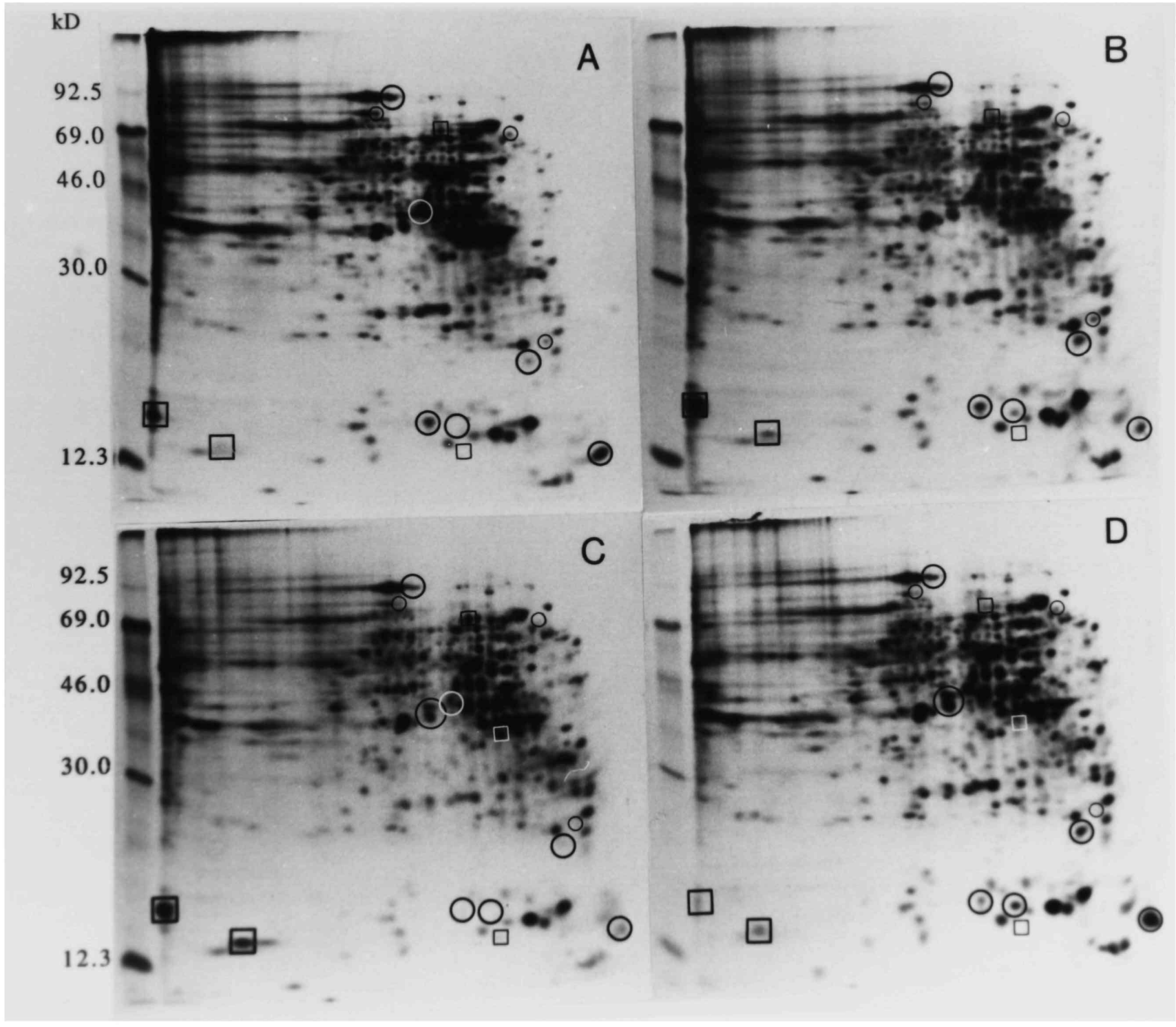
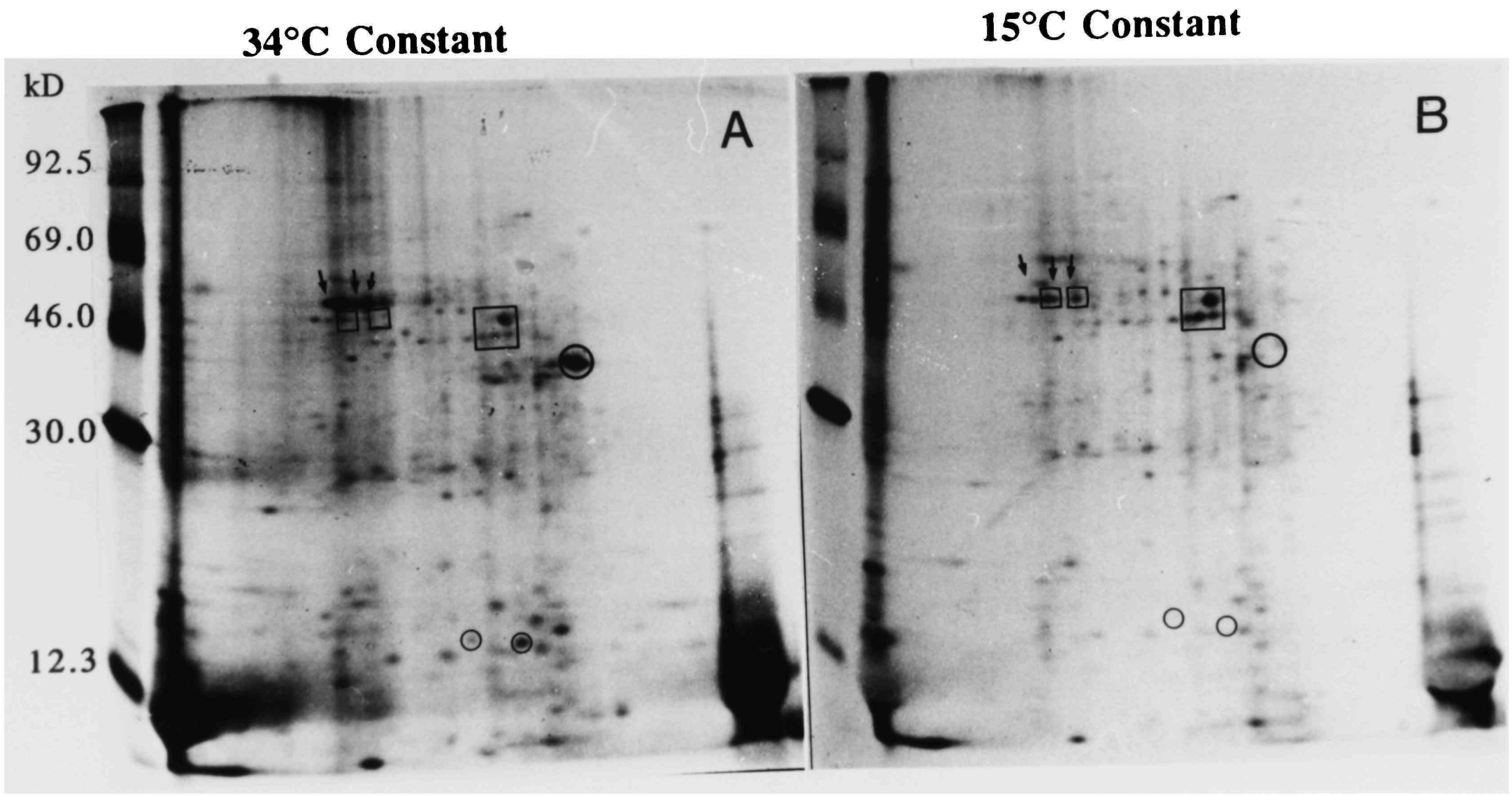

kD
92.5
69.0
46.0
30.0
12.3
A
B
C
D

Fig. 3.12. Fluorographs of *in vitro* translation products of Poly (A^+) RNA extracted from -1 dpa ovules cultured for 24 h in constant 34°C (A) or 15°C (B). Arrows and circles indicate polypeptide species unique to 34°C, and squares indicate polypeptides unique to 15°C.

15°C Constant Can Alter Particular mRNA Levels



CHAPTER 4
PEROXIDASE ACTIVITY OF COTTON OVULES CULTURED *IN VITRO* UNDER LOW TEMPERATURES

4.1 Overview

As an integrated part of changes in protein population, changes in enzyme isoforms and kinetic activities have been reported during cold acclimation (41). Cell wall peroxidases have been used as markers to evaluate thermal stress responses in cotton cell suspensions, and particular isoforms are associated with different culture temperatures and genotypes (49). For cotton plants it is known that oxidizing enzymes (peroxidase and IAA oxidase), together with their substrates (plant growth substances and phenolic compounds), change throughout the process of fiber development (3, 81, 83). Both peroxidase and IAA oxidase activities were greatly reduced around the day of anthesis and sharply increased during secondary wall thickening (86, 103). Phenolic compounds are released at -1 dpa into the cytosol from the vacuole of prefiber cells prior to the initiation event (81) and may be related to auxin metabolism in regulation of initiation (75). These compounds are co-extracted and co-precipatated with proteins, causing an acidic smearing on 2D gels. The decline of the smear coincides with fiber initiation and early elongation (39). It was suggested that oxidative enzymes are required to shift fiber initial cells to an oxidative state for fiber initiation (86). Furthermore, high temperature imitates the effect of GA in promoting fiber production, possibly via increased uptake of IAA (6). These studies suggest that multiple factors affect fiber development and that peroxidase may play a role in control of fiber initiation. Therefore, it is reasonable to hypothesize that low cycling temperature may regulate the activities of peroxidase among other crucial enzymes during fiber initiation and early elongation.

To obtain information concerning changes during fiber development, especially in connection with low temperature stress, the activities of peroxidase enzymes were determined during the period of fiber initiation and elongation with *in vitro* cultures grown under control and low cycling temperatures. Different peroxidase isoforms are present in the ovules and fibers, and contrasting results have been reported with different substrates and the same experimental system (84, 86, 103). In this study guaiacol peroxidase activity was monitored, partially because of availability of suitable color assay method, and because our main goal was to compare the activity of any relevant enzyme between the temperature treatments.

4.2 Materials and Methods

4.2.1 Plant Materials and Ovule Culture

Cotton ovules at -1 dpa were cultured as previously described in Chapter 2. Fibers were hand-separated with forceps from ovules setting on ice, and the naked ovules and fibers were frozen immediately in liquid nitrogen until use.

4.2.2 Preparation of Enzyme Extracts

Cotton ovules were harvested at sequential ages (as specified below for each experiment) after *in vitro* culture under different temperature regimes or from greenhouse plants. They were immediately frozen in liquid nitrogen, stored at -80°C until use or ground into fine powder in liquid nitrogen, and homogenized further in 5-10 ml borate buffer (0.2 M, pH 7.6). The homogenates were centrifuged (4°C, 10,000 rpm, 10 min.) and two volumes of acetone (chilled at -20°C) were added to the supernatants to precipitate the proteins. After incubation on ice for 1 h, precipitated proteins were collected by centrifugation as above. The pellet was dissolved in 2 ml 0.02 M sodium phosphate buffer, pH 6.4, followed by centrifugation to remove undissolved materials. The supernatant was collected as the source of enzyme extract for peroxidase assay.

4.2.3 Protein Content Assay

The protein content in 20 µl ovule extracts was determined at 595 nm using the Bradford Bio-rad microassay procedure (Richmond, CA). The extract was diluted appropriately with phosphate buffer to attain an optical density (OD) reading of 0.5 -1.0, which falls in the linear range of our protein (BSA) standard curve.

4.2.4 Peroxidase Assays

Guaiacol (20 mM) was used as the hydrogen donor in the assay for peroxidase activity with 0.02 M phosphate buffer (pH 6.4) and 9 mM or 0.3% (v/v) commercial 30% H_2O_2 stock (Sigma Co., St Louis, Missouri) as substrate. Kinetic measurements at OD_{470} nm were made using a Gilford Response UV-VIS spectrophotometer (Oberlin, Ohio), which was equipped with an automatic sample changer and recorder that measured 5 samples at 1 min intervals. Assays were continued up to the 5th min from the start of the reaction at room temperature (20-22°C). The change in OD_{470} was determined by using buffer, guaiacol, and active enzyme extract in both the reference and sample curvettes; the reaction was started by adding H_2O_2 only in the sample curvettes. Total volume of the

reaction mix was 1 ml containing 630-650 µl 20 mM phosphate buffer (pH 6.4), 2-20 µl of enzyme extract, 0.25 ml 20 mM guaiacol, and 0.1 ml 0.3% H_2O_2. The range of buffer and enzyme extract volume reflected variation to maintain the color reaction in the readable range (or first order). For the reference, 0.1 ml phosphate buffer replaced the H_2O_2.

The peroxidase activity was then expressed as the rate of change in (ΔA_{470} min^{-1} mg^{-1} protein), using the calculation of the linear regressions of OD over time. Total peroxidase activity as measured reflected contributions of multiple isozymes that use guaiacol as substrate. Each sample was measured with 3-6 replicates and each experiment was repeated at least twice. In one analysis, data were integrated from 3 subsets of samples analyzed over 9 months. The variability was sometimes high for certain replicated data points between subsets, but the trends were highly reproducible. One representative data set is graphed with error bars representing standard deviation from 3-6 samples, and other relevant data are presented in the Appendix B.

4.3 Results

4.3.1 Peroxidase Activity under Constant 34°C or 15°C

Ovules of -1 dpa were collected and cultured at constant 34°C or 15°C. Samples were harvested at 3-12 hour intervals up to 48 h after culture and assayed for peroxidase activity (Fig. 4.1). Peroxidase activity decreased during the initial 3 h culture under both conditions. At 34°C it took another 3 h for activity to recover to the level of freshly isolated ovules. Thereafter, the activity increased rapidly until 12 h, and another increase occurred between 24 and 48 h. In contrast, at 15°C, 18 h was required for the activity to recover to the initial level, and it showed little change thereafter. These results indicate that the culture temperatures greatly affect soluble guaiacol peroxidase activity.

4.3.2 Recovery in Peroxidase Activity at the End of Each Side of the Cycling Regimes

Ovules cultured at -1 dpa under constant 34°C, 15°C, and 12 h/12 h cycling 34/15°C or 15/34°C were harvested at the end of each 12 h interval up to 72 h (3 days). Peroxidase activities assayed at room temperature (22°C) are expressed as percentage of control 34°C (Fig. 4.2). Both 34/15°C and 15/34°C imply opposite starting temperatures for the cycling regimes, which allowed extracts to be prepared at the same time of day from cultures at the end of the 34°C and the 15°C side of the cycle. It is important to emphasize that alternate points in the 34/15°C and 15/34°C curves in Fig. 4.2 reflect extracts made alternatively at 34°C and 15°C. Peroxidase activity was constantly depressed at constant 15°C, whereas

activity under the two cycling regimes was intermediate between constant 34°C and 15°C. Even during the high side of the cycle, extractable peroxidase activity did not recover to the 34°C control level, although it did increase compared to constant 15°C. It is also interesting that exposure to 15°C after 12 h at 34°C causes activity to decrease and remain near the level typical of either cycling regime. Comparison of the two cycling curves would suggest that initial exposure to 15°C was more inhibitory up until 2 d of culture, after which the differences were diminished. This agrees well with SEM data and ruler measurements that show no significant difference in fiber growth over a prolonged time course between the two cycling regimes. These data also suggest that exposure to 15°C as part of a cycling regime causes peroxidase activity to equilibrate at a level typical of that condition.

4.3.3 Peroxidase Activity is Developmentally Regulated

Based on the results above and from previous studies, further experiments were conducted to determine if stages of fiber development were correlated with the amount of guaiacol peroxidase activity present in the tissue. Of particular interest was to determine whether cycled ovules would ever attain the control levels of peroxidase activity, since previous results would suggest that they become physiologically equivalent in elongation rate after an initial developmental delay. Ovules were cultured for 1-12 days under constant 34°C, 15°C, and 15/34°C (12 h/12 h cycling). The 15/34°C cultures were extracted throughout the time course at both 34°C and 15°C, 6 h after shifting from the opposite side of the cycle. Therefore, ovules were harvested 12 h later from 15°C constant and the 15°C side of the cycle as compared to these harvested from 34°C and the 34°C side of the cycle. The peroxidase activity data were normalized for the graph by using day (not hour) of culture on the X-axis (Fig. 4.3). All assays were run at room temperature. As in our previous experiments, when the ovule age advanced, the peroxidase activity increased at 34°C, was depressed completely at 15°C, and had intermediate values in the cycling regimes. Extracts from the 34°C and 15°C side of the cycle did not vary in activity on day 10-12,which demonstrates that isozyme activity did not increase with each period of high temperature and decrease with each period of cool temperature even at later stages of elongation.

Since by day 12, the 15/34°C cultures had not evidenced the very large increase in activity that was observed between day 6 and 12 in control 34°C (Fig. 4.3 and Table B.1), the analysis was extended up to day 18 using 34°C and 15/34°C to test for full recovery of the peroxidase activity in later developmental stages under cycling conditions. The cycling

ovules were harvested after 6 h at 15°C at the same hour as the 34°C constant ovules. Fig. 4.4. includes a new set of data points for all days and shows that peroxidase activity at 15/34°C remains suppressed through day 18. It can be noted that the development of high levels of peroxidase activity at 34°C is delayed about 3 days compared to the previous experiment (Table B.1). Another replicate of day 12, 14, 16 and 18 not showing this delay in attaining the higher peroxidase activity levels also showed only low levels at 15/34°C (these data points in Fig. 4.6A). We did observe, however, that the activity at constant 15°C on 30 d and 50 d (data not shown) reached a level similar to that of the control 34°C on 12 d.

4.3.4 Peroxidase Activity versus Ovule and Fiber Growth

While the majority of our assays for peroxidase activity were done with the ovules plus fibers due to the difficulty in separating young fibers from ovules, assays with pure fibers or naked ovules only at day 12-18 also were made. Cycling temperature reduced the activity in both tissues compared to the 34°C control (Fig. 4.5 a & b), though the ovular tissue constituted the major part of the total activity and the sum of activity from the two tissues is much lower than that of unseparated fibers and ovules (Ref. Fig. 4.4). The lower activity may reflect loss of fiber contents when they were removed from the ovules.

The relatedness of the peroxidase activity and fiber elongation under both temperature regimes becomes evident when both are plotted against ovule age (Fig. 4.6 a & b, note the different scales for peroxidase activity between the two regimes). The diagrams show two important points. First, the overall shape of the peroxidase activity curves for later days of development were similar under both regimes, but the sharp increase in activity occurred 2 days later under the cycling regime (day 12 versus day 10). Note that the rapid increase occurs in each case when fibers are about 2 mm long, which seems to mark the transition to the later elongation phase (Chapter 2). Second, by analogy with previous work, the highest levels of peroxidase activity in the 34°C control on day 15-18 would correlate with the time of secondary wall deposition and this event is likely delayed under 15/34°C cycling (45). Therefore, in the *in vitro* culture system, the guaiacol peroxidase activity appeared more highly associated with the secondary wall deposition phase than the elongation phase. We cannot yet explain the generally suppressed activity under 15/34°C. However, if the peroxidases assayed were involved in wall polymer crosslinking, the suppressed activity might result in a more extensible cell wall that could account for the recovery of elongation rate under 15/34°C.

4.3.5 Peroxidase Activity *in vivo*

To confirm the accuracy of our assay method by comparison with previous studies, ovules were harvested from greenhouse plants (day/night approx. 30/22°C) from day 0-11 dpa (equivalent to 1-12 dac under *in vitro* system) and assayed for soluble peroxidase activity (Fig. 4.7). The activity increased, peaked at 5 dpa, and dropped to the initial 0 dpa level by 11 dpa. The absolute values for activity was similar to previous studies (103). Previous studies suggest that the enzyme activity will rise again on day 18 and 30 to 50 (92, 103), which correlates with our observation of an increase at the secondary wall stage. However, comparison of the *in vitro* cultured ovules showed that the absolute measurements of the activity *in vivo* were much lower (10 and 100-200 fold less than at 15/34°C and 34°C, respectively). This difference in activity between *in vivo* and *in vitro* suggests that the culture manipulation and temperatures may place ovules under stress, a possibility also supported by the fact that fibers grown *in vitro* are shorter than those *in vivo*, although the physiological events are similar (67). Also, it is possible that the *in vivo* condition, endogenous inhibitors for the enzymes may be present in the ovary wall. It needs to be pointed out that the low cycling regime resembles more the greenhouse condition, and that the activity levels at 15/34°C are close to the greenhouse condition.

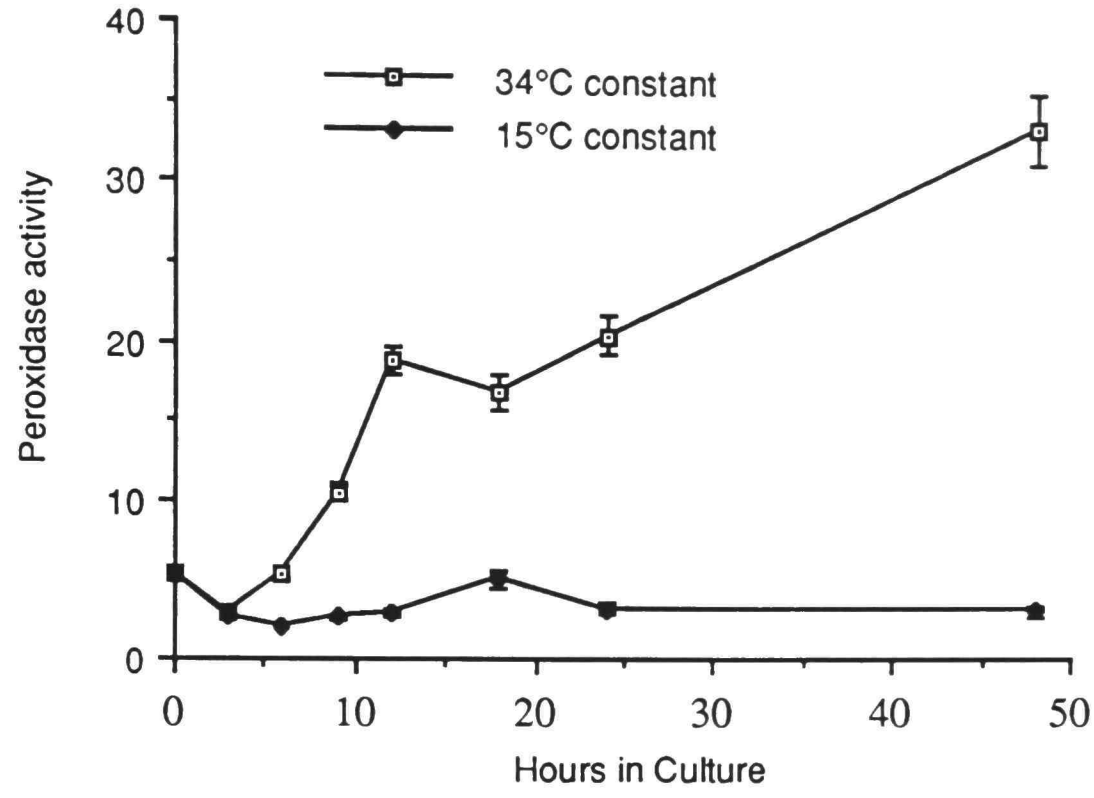


Fig. 4.1. Soluble peroxidase activity in cotton ovules cultured at -1 dpa under 34°C or 15°C constant. Enzyme activity (ΔA_{470} min^{-1} mg^{-1} protein) was determined with guaiacol as substrate. Vertical bars represent standard deviation from 3-5 replicates at each data point. The experiment was repeated twice.

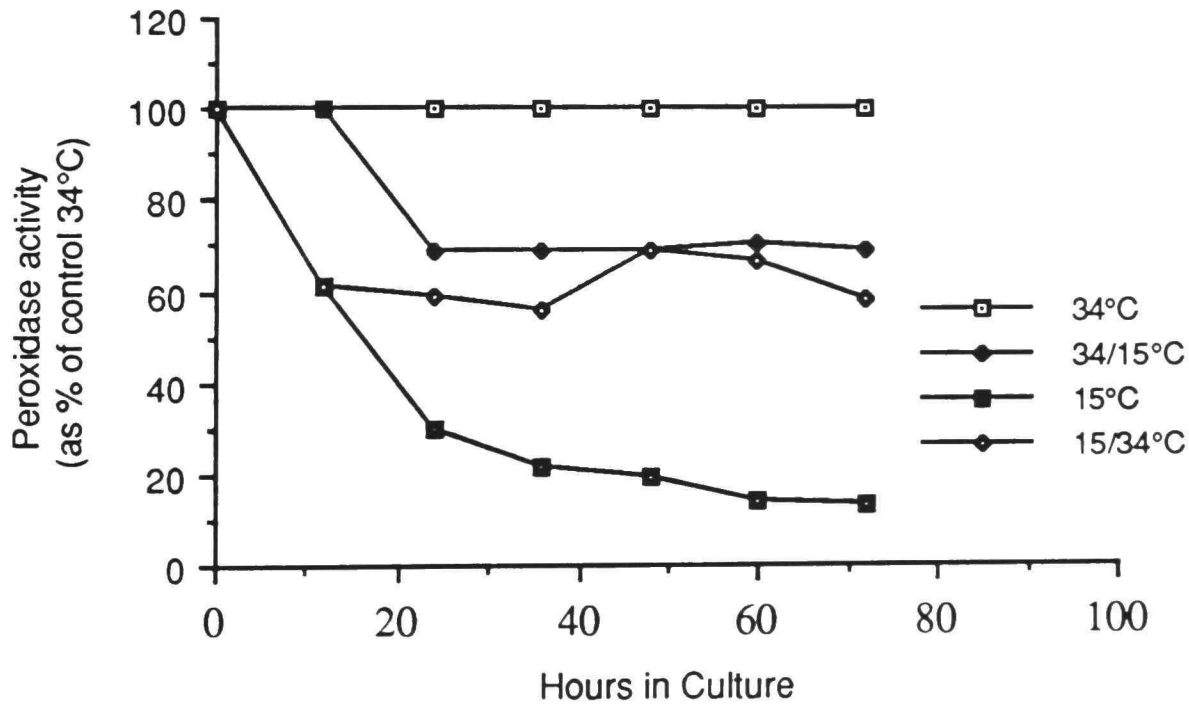


Fig. 4.2. Soluble peroxidase activity in cotton ovules cultured at -1 dpa under 34°C or 15°C constant, 12 h/12 h cycling 15/34°C or 34/15°C. The ovules were harvested at 12 h interval at the end of each cycling side. Enzyme activity (ΔA_{470} min^{-1} mg^{-1} protein) was determined with guaiacol as substrate and expressed as percentage of the 34°C control. The experiment was repeated twice.

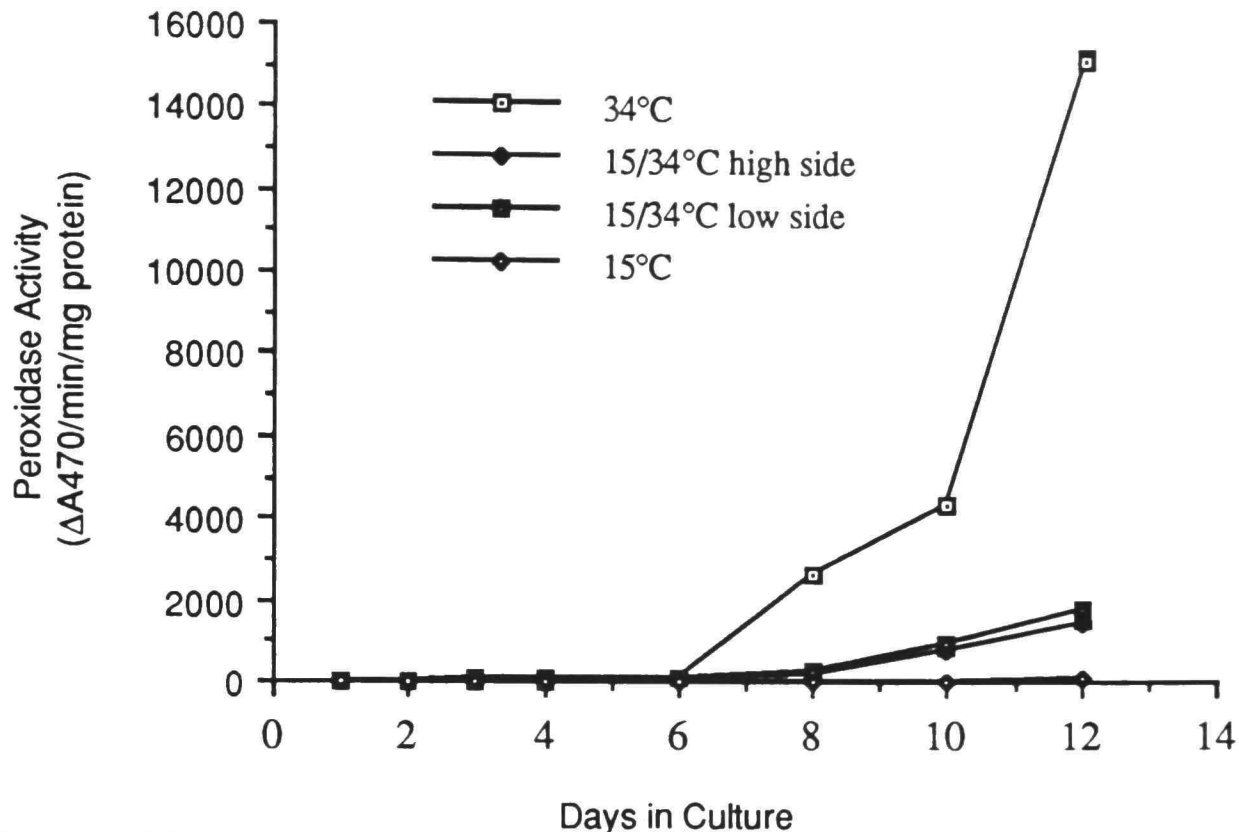


Fig. 4.3. Peroxidase activity in -1 dpa ovules cultured for 1-12 days under 34°C or 15°C constant and 12 h/12 h 15/34°C cycling. Samples from cycled cultures were harvested after 6 h into each side of the cycling. Enzyme activity and bars (which are mostly so small that they are invisible) as in Fig. 4.1. The experiment was repeated 3 times.

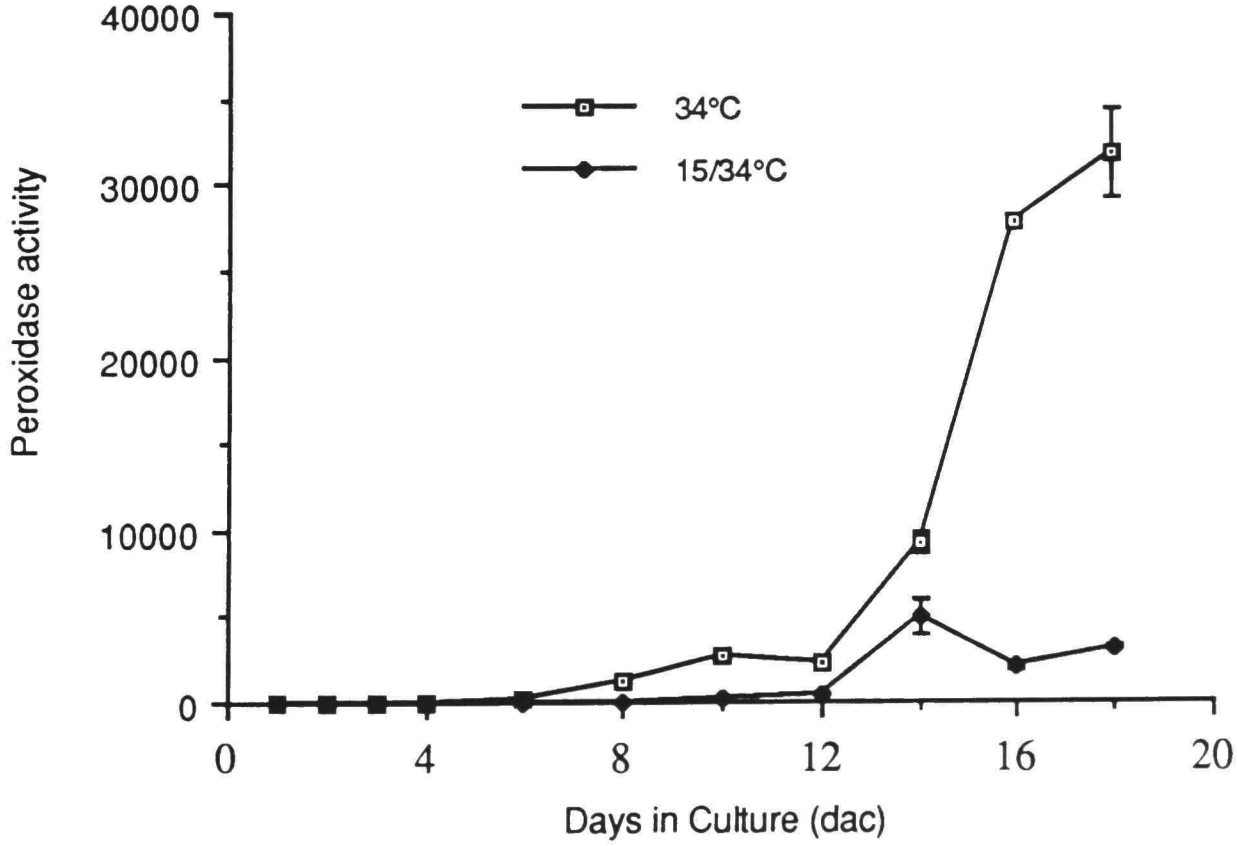
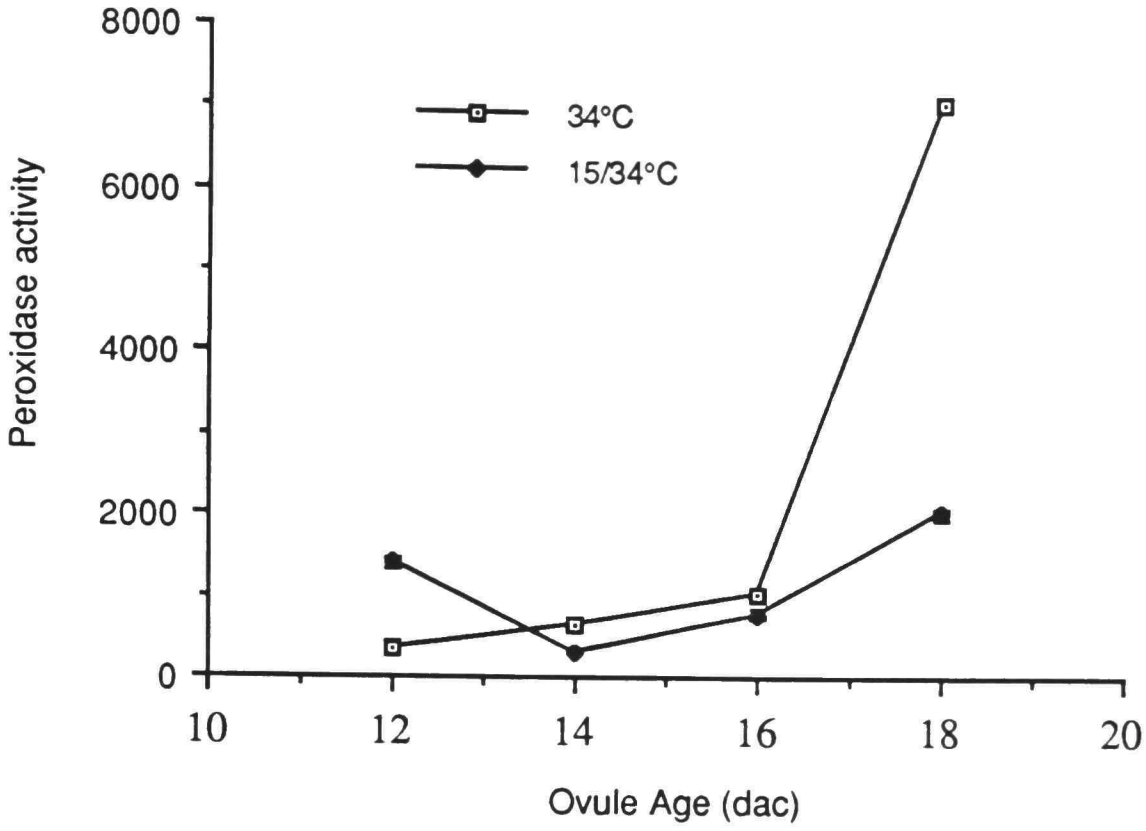


Fig. 4.4. Soluble peroxidase activity in -1 dpa ovules cultured for 1-18 days under 34°C constant and 12 h/12 h 15/34°C cycling. Samples from cycled cultures were harvested after 6 h into the low side of the cycle. Enzyme activity (ΔA_{470} min^{-1} mg^{-1} protein) and bars as in Fig. 4.1. The experiment was repeated 2-4 times for each day.



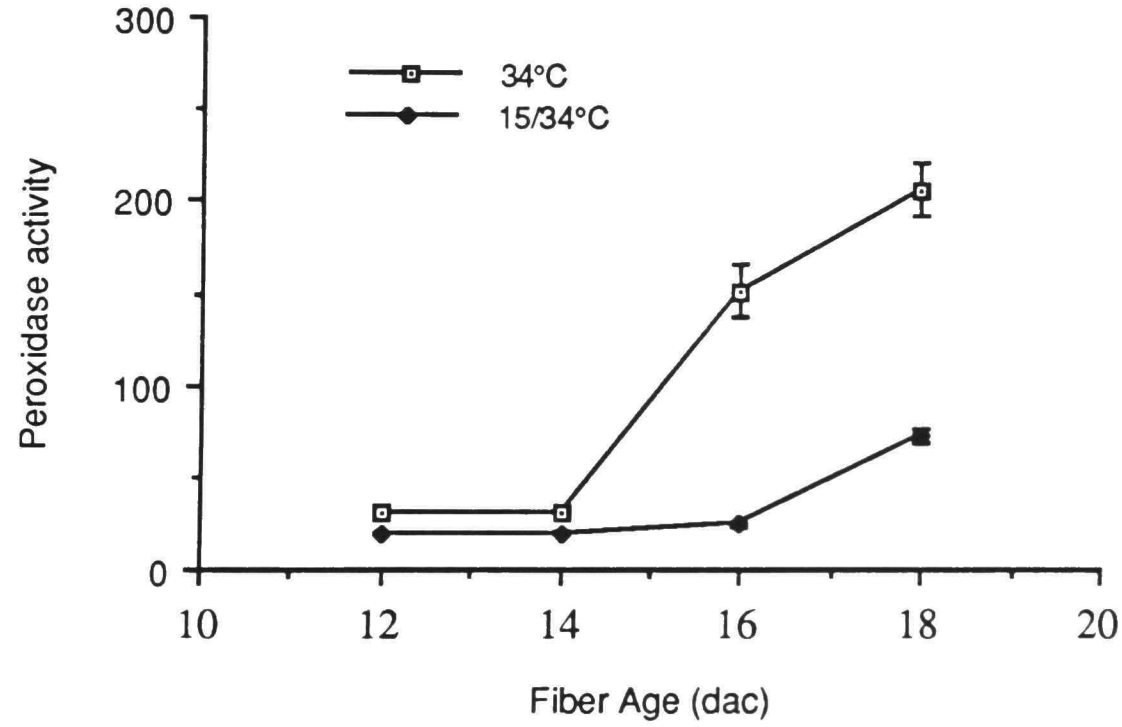


Fig. 4.5 Soluble peroxidase activity in naked (A) ovules and fibers (B) grown *in vitro* under 34°C constant and 12 h/12 h 15/34°C cycling temperature at 12-18 dac. Enzyme activity (ΔA_{470} min^{-1} mg^{-1} protein) was determined with guaiacol as substrate. Bars as in Fig. 4.1.

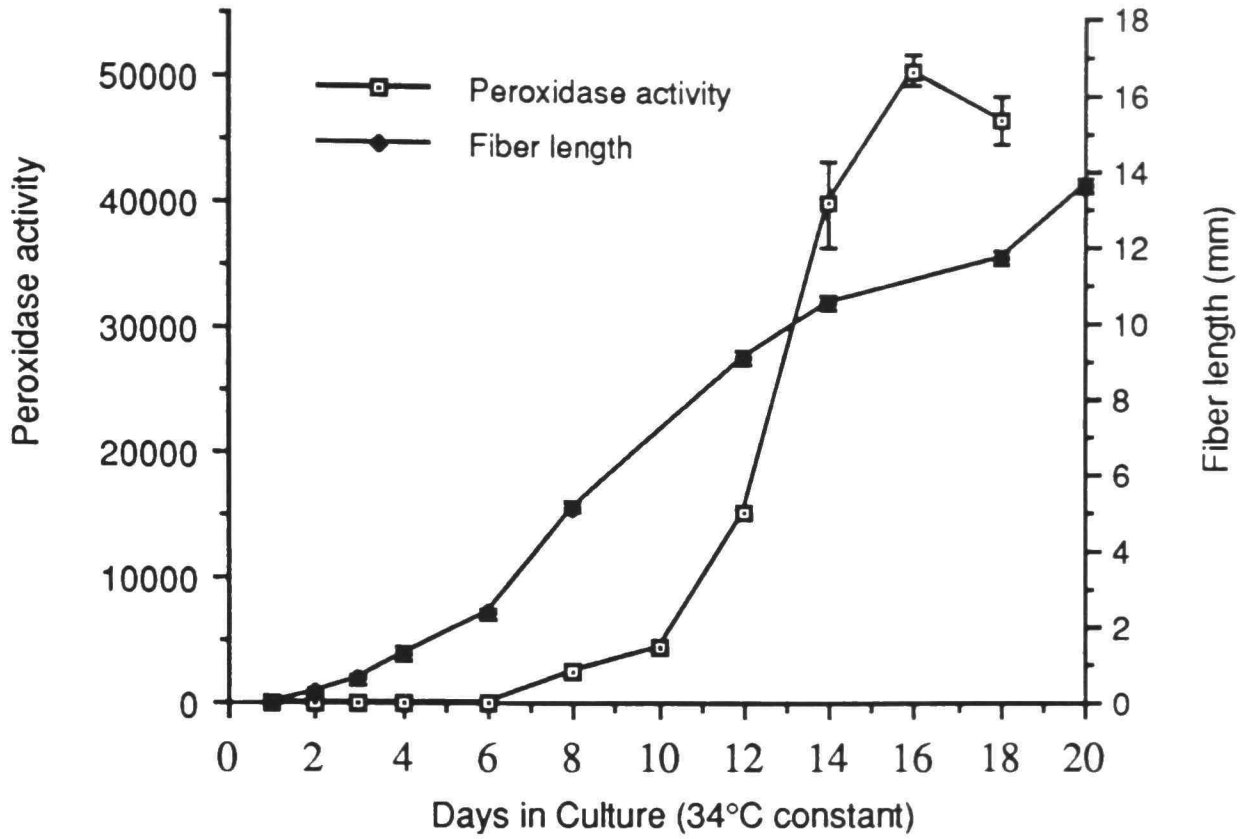


Fig. 4.6. Soluble peroxidase activity (ΔA_{470} min^{-1} mg^{-1} protein) versus fiber length (mm) under constant 34°C (A) and 12 h/12 h 15/34°C cycling (B) regimes. Notice the similarity in the curve pattern and the difference in the scale used under the two temperature regimes.

Fig. 4.7. Soluble peroxidase activity on cotton ovules harvested directly from greenhouse-grown plants at 0-11 dpa. Enzyme activity (ΔA_{470} min^{-1} mg^{-1} protein) was determined with guaiacol as substrate. Bars as in Fig. 4.1

4.4 Discussion

After initial culture, a reduction in guaiacol peroxidase activity occurred under both 34°C and 15°C constant temperatures (Fig 4.1). This observation, together with the discrepancy in the activity between *in vivo* and *in vitro* ovules (Fig. 4.3 & 4.7), suggests a metabolic shock or a thermal stress for the ovules. The peroxidase activity was consistently depressed and remained at the initial level under 15°C constant at day 12, but it increased dramatically under 34°C, the 15/34°C cycling regime, and under the *in vivo* condition (approx. 32/22°C cycling) (Fig. 4.7). Therefore, peroxidase is probably a valid marker for low temperature stress in this system. The low activity may indicate reduction in general metabolic activity under low temperature causing a slower growth rate. Correspondingly, higher activity under 34°C may be a reflection of a high metabolic activity that is the cause of faster fiber and ovule growth at 34°C compared to low cycling or constant conditions (Fig. 4.4 & 4.5). In cotton, the optimal temperature for enzyme

activity is 22.5-32°C (17). Since peroxidases secreted from ovule culture media are highly thermostable in the assay temperature of 10-55°C (68), the incubation temperatures apparently caused the differential activities observed. Because an increase in peroxidase activity under cycling condition was not observed, it is unlikely that this enzyme is up-regulated to protect the cell or tissue from oxidative damage of cool temperature stress as occurs with cold-stress (e.g., 5°C) in other systems (77). It is possible that other scavenging enzymes such as catalase or other peroxidase isoforms perform this function in cotton or that 15°C is not low enough to cause oxidative damage. Our data can not exclude the possibility of a shift in isoform profiles which may contribute to the differential activity because more than 350 isozymes of peroxidase have been reported with many different, even contrasting roles (103). Peroxidase was highly temperature-dependent and more isoforms were present at 34°C and 18°C than at 28°C in cotton suspension cultures (49). A 10-fold increase in the peroxidase activity in transgenic tobacco plants induced wilting at the time of flowering (60).

The peroxidase activity at the two sides of the cycle showed little differences after 3 d as did fiber growth rate (Chapter 2, data not shown). Compared to 34°C controls, 15/34°C cultures followed the same pattern of increase in activity throughout the culture period (Fig. 4.3 & 4.4). The increasing activities under both conditions corresponded well with stages of fiber elongation, although the pattern is delayed in time and reduced in scale for 15/34°C (Fig. 4.6). More importantly, the activity in the fiber resembles the patterns for ovules alone and both tissues combined (Fig. 4.5). These results indicated a developmental delay in the peroxidase activity. They are consistent with the results from SEM and protein studies (Chapters 2 and 3). The analyses indicate that the primary effect of exposure to cool temperature is to induce a developmental delay, not to modulate enzyme activity through each fluctuation in temperature. However, the activity under cycling condition never reached the control 34°C level even after the growth rates became similar. It is possible that the suppressed activity under 15/34°C leads to a more extensible cell wall, and is, therefore, part of 15/34°C adaptation. Based on previous studies (92, 103), it is possible that the 15/34°C activity would eventually catch-up. Our observation that ovules, after being cultured in constant 15°C for 30 or 50 days, achieved a similar scale of peroxidase activity as the 34°C at day 12-18 (data not shown) supports this possibility. Because the initial developmental delay is perpetuated throughout the elongation period, fibers grown under the cycling and the 34°C regimes are always in different physiological stages based on the fiber length. The parallel fluctuations in peroxidase activity with stages

of fiber elongation under constant and cycling regimes strongly suggest that peroxidase levels are important in fiber development, but they are not in a direct relationship with growth rate. In fact, they may be in a precise correlation with other unidentified cellular factors as a way of regulating growth rate. In agreement with these studies, peroxidase was observed only in the distal part of the primary wall of fiber at 5 dpa (Berlin, 1977, unpublished data), presumably participating in the wall extension.

In addition to its parallel with fiber elongation, peroxidase activity increased abruptly and apparently coincided with the onset of the secondary wall thickening around 12 dpa at 34°C or later at 15/34°C (Fig. 4.6). Fibers on ovules cultured at 2 dpa began secondary wall synthesis at 14 and 20 dpa under 34°C and the 34/15°C cycling, respectively (45). Onset of secondary wall deposition was not determined directly in this study. However, -1 dpa ovules cultured at 15/34°C required 3-4 day less to reach the same length as those on ovules cultured at 2 dpa. Therefore, we can hypothesize that, in our cultures, the secondary wall thickening may begin around 12 dpa at 34°C and 16-18 dpa under the cycling. Continuous increase up to day 35 (the last assay date) in guaiacol peroxidase activity has been also observed for *in vitro* cotton ovule culture medium (68). Wall-bound guaiacol peroxidase paralleled dry matter accumulation in secondary wall thickening (84). Furthermore, studies with plant grown ovules and fibers, assayed with different phenolic substrates (chlorogenic acid and diaminobenzidine) showed that peroxidase in crude homogenates increased throughout fiber development (92, 103), but wall-bound activity increased only up to the end of primary wall formation and remained constant afterwards (92). Cytochemical test showed that peroxidase reaction product was localized in primary walls (Berlin, unpublished data; 92), suggesting binding of peroxidase to primary walls only. Our results, together with these previous finding indicate that both soluble and wall-bound peroxidase are developmentally regulated and participate in the whole process of fiber development, probably becoming more important in later stages.

The biological significance of peroxidase activity remains unclear. Peroxidase, as part of IAA oxidizing system, has been implicated in oxidation of phenolics (102), lignification processes, degradation of IAA (107), and induction of IAA oxidase (73). Exogenously applied IAA may induce increased peroxidase activity in roots and in other parts of cotton plants (27). An enzyme having such spectrum of roles may have several different metabolic functions. The current results apparently contradict the role of peroxidase in degrading IAA that is essential for fiber initiation and elongation (12) because both fibers grew faster under 34°C which led to a higher peroxidase activity. Other

evidence negates the possibility of such a destructive role by the high temperature up-regulated peroxidase because inclusion of NH_4^+ and additional IAA in the medium were required for fiber production on ovules cultured under sub-permissive temperature, and high temperature promoted fiber elongation, all probably by stimulating the uptake of IAA (6). Since cotton fiber and young ovules contain no lignin, the higher activity of peroxidase at 34°C may preferentially oxidize some of the phenolic compounds that accelerate degradation of IAA (69) and, thereby, protect IAA. There is, however, no direct evidence to support this. Alternatively, high activity may be required for destroying phenylpyruvate to prevent the accumulation of excessive quantities of such phenolic compounds. In either case, this speculation is consistent with the observation that enzyme extracts from 34°C cultures appeared to be much less brown than those from the cycling and constant 15°C and the color of extracts diminished with increased age. Brown color often signifies the accumulation of phenolic compounds, which causes acidic smearing on 2D gels (39), and may interfere the peroxidase activity. High activity of peroxidase under 34°C may be well suited for a major role in the *in vitro* regulation of IAA levels through its versatile effects on IAA oxidase activity, and thus may lead to faster initiation and growth of fiber than in low and cycling regimes. Therefore, how IAA levels and the IAA oxidase responded to different temperatures in relation to fiber development is an intriguing question. We found that IAA oxidase activity was lower and basically not detectable after 5 dpa in the *in vitro* culture (data not shown); this might facilitate accumulation of IAA for fast elongation. A number of workers have demonstrated that the activities of guaiacol peroxidase and IAA oxidase may be dual catalytic functions of a single enzyme in *Betula* leaf (48, 98). If this is true in the culture system, the high peroxidase activity may also account for the lower IAA oxidase activity observed. An internal link seems to exist among physiological factors such as phenolic substances, growth regulators, IAA oxidase and peroxidase, and culture temperatures. Continuing study with peroxidase would be necessary to determine its possible relation with these factors and its roles during fiber development.

CHAPTER 5
SUMMARY AND CONCLUSIONS

This research was undertaken to examine changes in growth, protein synthesis and peroxidase activity of developing cotton ovules and fibers, especially during the very early stages of fiber elongation under cycling 15/34°C and constant 34°C temperatures. The investigation was intended to determine general mechanisms governing the inhibition of fiber initiation and elongation *in vivo* by cool temperatures. Several approaches were used to accomplish the goals with *in vitro* ovule cultures as a model system. The main discoveries from this work were the following:

1. Fiber initiation on cultured ovules followed exactly the pattern observed *in vivo* in terms of growth polarity toward the micropylar region, providing further evidence that ovule cultures are a valid model for studying fiber development. Most interestingly, the timing of initiation on the ovules cultured at -1 dpa did not begin until the actual day of anthesis *in vivo*, though all the external physiological factors required for the growth are accessible. This indicates that an internal clock has been set during the pre-fiber differentiation for the time of initiation. Mechanisms controlling the timing of fiber initiation demand further study as a possible means to manipulate fiber development. Results on the difference in peroxidase activity between *in vivo* and *in vitro* cultures do, however, indicate that caution is appropriate in considering *in vitro* cultures as an appropriate model for all aspects of ovule and fiber metabolism.

2. Based on SEM observation and growth measurements of fibers on the control and cycled ovules, fiber elongation can be divided into 3 substages: initiation, early elongation to achieve a length of 0.5-2 mm, and later elongation to reach a final genetically determined potential length. The time required for early elongation to be completed depends upon the duration of exposure to low temperature, and the elongation period will be prolonged to compensate for the early inhibition. This study demonstrates that the first two stages are extremely sensitive to low temperature. In contrast, the later elongation rate is similar under both cycling and control 34°C, perhaps reflecting a metabolic priority for elongation under stress as compared to secondary wall thickening. Other studies in this lab suggest that the rate of cellulose synthesis for secondary wall thickening is constantly depressed by 15/34°C cycling. These observations suggest that a primary effect of low cycling temperatures is to induce a developmental delay in fiber elongation.

3. Radioactive labeling experiments demonstrated a developmental delay in the changes of a specific set of protein profiles caused by low cycling temperatures. The timing of this delay correlated well with the physical delay in fiber initiation and early elongation observed by SEM. The later recovery in the synthesis of these proteins (10, 12, 20 and 92 kD) coincided with the adaptation in elongation rate, suggesting that they may be candidates for proteins that are essential for fiber elongation.

4. Either applied constantly or as the low side of a cycle, 15°C greatly reduced total protein synthesis and particularly inhibited synthesis of a group of proteins (e.g., 10, 14, 16, 20, 25, 33 and 70 kD) that presumably are important for fiber elongation and low temperature response. However, other proteins that were present in 34°C also were actively synthesized in 15°C. This provides a molecular basis for fibers to resume growth quickly after release from constant 15°C stress.

5. Either applied constantly or as the low side of a cycle, 15°C induced a 12 kD protein that was degraded under 34°C. This protein may represent a class that plays a role in adaptation to the low temperature.

6. Other labeling experiments demonstrated that few proteins synthesized at 15°C or 34°C were permanently degraded upon shifting to opposite sides of the cycling regime. Only minor changes in protein quantity were observed. Therefore, protein degradation at 15°C is not a likely cause of the 15/34°C inhibition of elongation. Furthermore, the lack of degradation at 15°C of proteins synthesized at 34°C could be part of the mechanism by which adaptation to 15°C side of the cycle occurs. This implies that proteins required for elongation might function at 15°C once they are present in sufficient quantities. Alternatively, 12 h or less at 34°C may be sufficient to allow total elongation for that day.

7. Guaiacol peroxidase activity correlated well with fiber elongation, both of them are highly mediated by growth temperatures. The low cycling temperature delayed the development of high peroxidase activity typical of 34°C control. Our data appeared to support the use of peroxidase as a marker for both low temperature stress and fiber developmental stages.

Information from this research should stimulate further work on cellular and molecular mechanism related to fiber development under low temperatures. It provides a sound basis upon which strategies can be formulated to obtain genes for adaptive proteins. Some specific questions that need to be answered include:

1. Identification of the mechanism of temporal regulation of fiber initiation;

2. Determination of function of temperature-mediated proteins (such as the 12 kD and 70 kD) in fiber elongation and its adaptation to cool temperatures. Making a subtractive library to obtain genes for these and other similarly regulated proteins will be a natural extension of this work. Once genes are available, the function of the related proteins can be tested by plant transformation experiments with the hope that the resulting transgenic plants could avoid the early delay in fiber elongation under cool temperature conditions.

3. Zymogram analysis of peroxidases would allow identification of particular isozymes that are responsive to low temperatures and help elucidate their possible functions during fiber growth.

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APPENDIX

Table A.1 Ovule ages versus ^{35}S-methionine uptake and incorporation into protein of cotton ovule from plants (cpm count/µl phenol-extracts)

Ovule age (dpa)	-3	-1	0	+3	+3*
cpm 1x10^5/µl	2.23	1.96	3.33	0.542	1.08

* the value after being adjusted based on equal numbers of ovules.

Table A.2. Time course of ^{35}S-a.a. uptake and incorporation into protein of ovules cultured under constant 34°C and 15°C (cpm/µl crude extract)

	Culture and labeling time in hour					
T°C	1	6	12	18	24	48
34°C	18,431	95,903	143,281	145,962	176,029	178,162
15°C	3,796	25,050	36,251	53,277	65,589	105,426
%*	20.60	26.12	25.30	36.50	37.26	59.17

NOTE: *The percentage of cpm in the 15°C extract compared to 34°C.

The absolute value for ovules labeled for 24 h in 15°C is equal to that for 6 h in 34°C; the uptake in 15°C is only 22-60% of control 34°C, which plateaued at 24 h.

Table A.3 Time course of ^{35}S-a.a. uptake and incorporation into protein of ovules cultured under constant 34°C and 15°C (cpm/µl crude extract)

	Labeling time in hour				
Tempt °C	3	6	9	12	24
34°C	21,243	-	-	85,116	85,116
15°C	6,705	9,701	17,602	19,396	36,249
%*	31.56	-	-	22.79	42.59

The cpm from 15/34°C culture is 81,947/µl at 24 h, and 15°C is 44.24% of the cycling.
*. The percentage of cpm in the 15°C extract compared to 34°C.

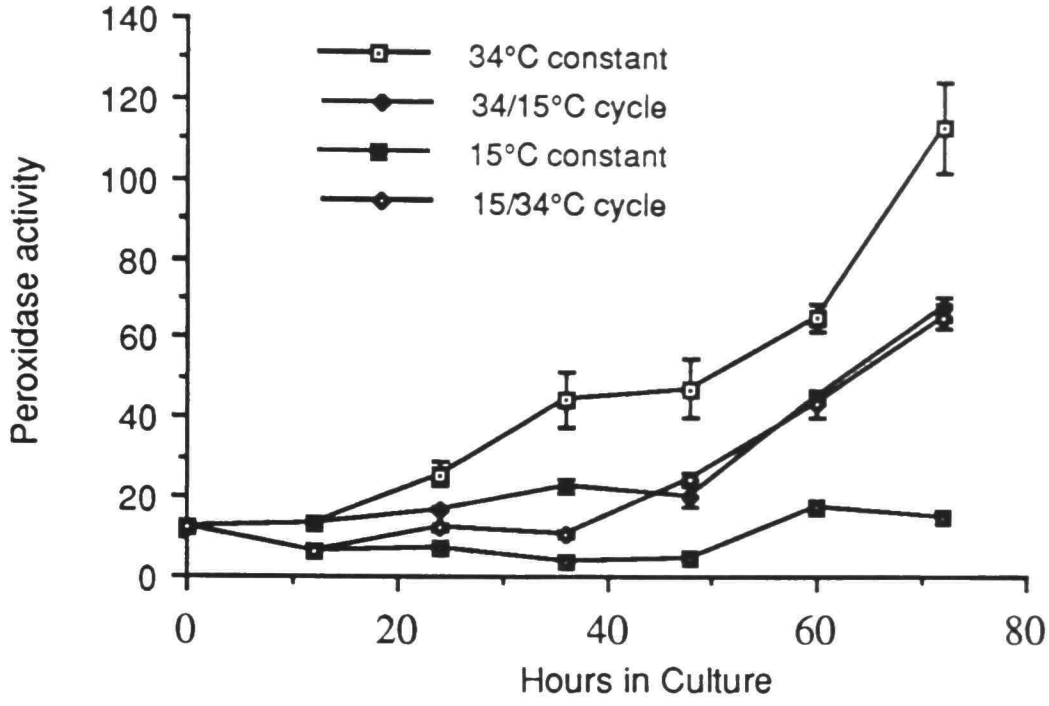

Figure B.1. Peroxidase activity in cotton ovules cultured at -1 dpa under 34°C or 15°C constant, 12 h/12 h cycle 15/34°C or 34/15°C. The ovules were harvested at 12 h interval at the end of each cycle side. Enzyme activity (ΔA_{470} min^{-1} mg^{-1} protein) and bars as in Fig. 4.1. The experiment was repeated twice (Ref. Fig. 4.2 in text).

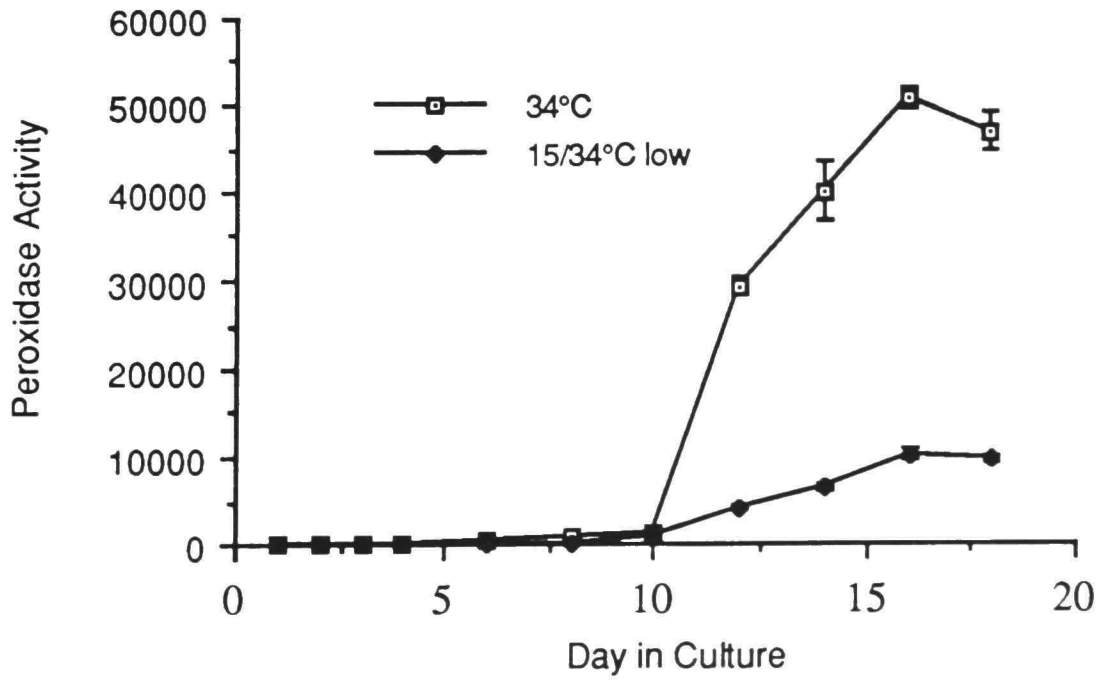

Figure B.2. Peroxidase activity in cotton ovules cultured at -1 dpa under 34°C and 12 h/12 h 15/34°C after d 1-18. Samples from cycled cultures were harvested after 6 h into the 15°C. The activity (ΔA_{470} min^{-1} mg^{-1} protein) and bars are same as in Fig. 4.1.

Table B.1. Guaiacol peroxidase activity (ΔA_{470} min^{-1} mg^{-1} protein) in the extracts from ovules cultured under different temperatures for 1-18 days

Days in culture	34°C	SD	15/34°C H	SD	15/34°C L	SD	15°C	SD
	15.43	0.83	6.85	0.25	12.79	1.01	5.75	0.15
1	16.33	1.48	8.26	0.37	13.45	0.29	6.38	0.69
	22.5	1.95			9.66	0.80		
2	26.79	2.78	13.50	1.760	20.33	0.99	7.12	0.20
	34.90	1.51	15.59	0.58	17.99	0.69	7.53	0.42
	31.96	1.98			12.78	0.48		
3	42.09	3.05	17.92	4.11	30.19	1.45		0.47
	48.18	4.99	25.70	4.69	28.25	3.40	9.25	0.47
	46.43	5.25			19.34	2.47		
4	60.15	7.01	22.52	2.10	42.90	2.84	8.23	0.72
	79.72	9.12	29.56	4.43	29.98	2.77	12.64	0.48
	59.11	6.70			22.24	0.39		
6	523.31	1.95	63.68	1.52	45.21	1.17	28.52	0.46
	100.32	12.02	53.30	1.20	46.25	1.23	20.14	1.00
	120.69	2.36			34.90	2.76		
8	836.31	5.93	105.38	6.03	133.00	2.15	48.15	0.97
	2618.05	46.45	172.28	2.53	270.86	10.12	26.13	1.36
	1165.10	5.00			69.93	1.03		
10	1235.6	40.38	453.12	60.66	763.41	17.27	68.20	3.42
	4388.27	30.62	770.29	43.21	982.69	30.35	38.05	1.71
	2649.70	32.65			176.28	4.31		
12	29311.25	1063.78	2556.25	71.76	4110.61	180.05	83.19	3.25
	15252.31	200.91	1469.94	61.91	1775.19	41.77	55.99	15.44
	2298.70	134.35			456.42	4.81		
14	40151.68	3466.69	6383.1	505.36	6383.1	505.36		
	9134.583	567.18	4913.0	958.88	4913.0	958.88		
16	50587.97	1251.25	10088.55	682.74	10088.55	682.74		
	27781.26	300.12	2020.23	179.26	2020.23	179.26		
18	46995.0	2013.65	9686.14	388.06	9686.14	388.06		
	31924.33	2694.91	3124.50	88.63	3124.50	88.63		