J. Derek Bewley · Kent J. Bradford
Henk W.M. Hilhorst · Hiro Nonogaki

Seeds

Physiology of Development, Germination and Dormancy

Third Edition

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Physiology of Development, Germination and Dormancy, 3rd Edition

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With thanks to our spouses Barbara Zadra, Ingrid Hilhorst, and Mariko Nonogaki for their invaluable support and patience during the writing of this book.
To the memory of Christine Bewley.

Preface

In this third edition of the *Seeds* book, several changes have occurred compared to the previous one of nearly two decades ago. First, of course, is that Michael Black is no longer a coauthor, for he chose to apply his time and talents to other scientific and educational endeavors. We are grateful for his encouragement to undertake the task of completing this edition and for the use of material that he wrote for the second edition. We also express our appreciation to our new publishers for their support and their agreement to produce the book.

Plant research has moved forward with remarkable speed in many areas since the publication of the last edition, especially with the development of new molecular-, cellular-, and computer-based technologies. These have provided opportunities for numerous advances to be made in our understanding of the physiology of development and germination of seeds. In addition, burgeoning knowledge of dormancy and of how seeds respond to their environment led to the expansion of these topics in the book and to the addition of "Dormancy" to the title of this third edition. We also consider events that occur in seeds during dispersal or storage as part of their "development." In covering all of these topics it was appropriate to restructure the contents of the book to generate a logical flow of ideas and information, thus putting them in perspective to the all-important "big picture" of seed biology, additionally encompassing ecology and agriculture. Unsurprisingly, the vast area of seed biology can only be introduced at this level of text, but we trust that the reader will garner an understanding of how seeds function, and that more in-depth information can be gained by pursuing the relevant references. Given the huge number of publications on seed biology, only a few pertinent articles are cited, and many of equal importance and impact had to be omitted. Not all of the information herein is new, for some studies conducted even as long as 50 or more years ago have withstood the test of time and remain fundamental in our understanding of seeds. On the other hand, many substantial sections of the book contain information that was completely unknown at the time of the previous edition. We have attempted to balance the coverage of basic information, which someone new to seed biology can appreciate,

with more detailed examples illustrating the state of the art in 2012 that even experts should find useful.

We are grateful to the many authors who have given us permission to use their published and unpublished research, and to several of our colleagues who have helped us by generously spending time reading sections of the book and providing information and clarification on specific topics.

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Contents

Chapter 1
Structure and Composition

Abstract Seeds are very diverse in their shape and size. In the mature state they contain an embryo, the next generation of plant, surrounded by a protective structure (the seed and/or fruit coat) and, in species in which the nutritive reserves are not stored within the cotyledons, by an alternative storage tissue (endosperm, perisperm, or megagametophyte). Most seeds contain large and characteristic quantities of polymeric reserves. The major ones are carbohydrates, oils, and proteins, with minor amounts of phosphate-rich phytin. Starch, a polymer of glucose, is the most common form of stored carbohydrate, contained within cytoplasmic starch granules; less common are the hemicelluloses, stored in secondary cell walls, usually as mannan polymers. Oils are triacylglycerols, each composed of glycerol and three fatty acids that are specific to the oil; these are present within oil bodies. Storage proteins, of which there are three types, albumins, globulins, and prolamins, are sequestered in protein storage vacuoles. These reserves are vital components of human and animal diets, and their production in crops is a basis of agriculture.

Keywords Seed structure • Embryo • Endosperm • Seed coat • Storage reserves

1.1 Introduction

An obvious first question is: why should we be interested in seeds? The answer is clearly because they are important to the plant as a major means of regeneration, and to humans for without their constant production and propagation we would ultimately have no food, fiber, or shelter! Seeds are the staple of the human diet and of our domesticated animals, and their value lies in the storage reserves of protein, starch and oil synthesized during development and maturation. Initially, seeds were picked by our hunter–gatherer ancestors as they followed herds of wild game, and later they became the foundation of settled agriculture as they were sown in fertile areas in the Middle East, Asia, and in South and Central America. Over the millennia farmers have selected seeds from plants that showed improved yields and following

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our quite recent understanding of breeding and genetics, seed food quality and production have accelerated to what it is today. The major food seeds are those of the cereals and legumes; annual world production is now approaching 3 billion tons. Almost three-quarters of the food we eat come directly from seeds, and many important dietary components are manufactured from them.

Seeds come in many shapes and sizes, from the smallest orchid seed that weighs about a millionth of a gram, to the huge 25 kg seed of the double coconut palm (coco de mer). One or more of a seed's features may be used in taxonomic identification. Nevertheless, each seed contains an embryo, which is the next generation of plant in miniature; it is usually formed by sexual reproduction and arises from the ovule during development. The seed is the means by which the new individual is dispersed, although frequently the ovary wall or even extrafloral organs remain in close association to form a more complex dispersal unit as in grasses and cereals. Thus the seed occupies a critical position in the life history of the higher plant. The success with which the new individual is established—the time, the place, and the vigor of the young seedling—is largely determined by the physiological and biochemical features of the seed. Of key importance to this success are the responses of a seed to its environment, and the food reserves it contains; these are available to sustain the young plant in the early stages of growth before it becomes an independent, autotrophic organism, able to use light energy. People also depend on these activities for almost all of their utilization of plants. Cultivation of most crop species depends on seed germination; there are exceptions when propagation is carried out vegetatively.

The biological and economic importance of seeds is evident, therefore. In this book we will give an account of processes involved in their development, in germination and dormancy and their control, and in the utilization of seed reserves during the early stages of seedling growth. Important topics such as the responses of seeds to their environment and their improvement, storage, and utilization are also covered. While considerable advances have been made due to research on seeds of economically important species, our understanding of many cellular and molecular processes has also been enhanced by the use of seeds of "model" species, most notably those of Arabidopsis (thale cress), whose genome sequence and plethora of mutants have been invaluable research assets. And of course, the study of seeds of nondomesticated species has allowed for a greater understanding of their behavior in the natural environment.

1.2 Seed Structure

In angiosperms and gymnosperms, the so-called seed plants or Spermatophytes, the seed develops from the fertilized ovule; this is discussed in more detail in Sects. 2.1, 2.2. At some stage during its development the angiosperm seed is usually composed of: (1) the embryo, the result of the fertilization of the egg cell nucleus in the embryo sac by one of the male pollen tube nuclei; (2) the endosperm, which arises from the

Table 1.1 Is the dispersal unit a seed or a fruit?—some examples

Seed	Fruit (and type)
Brassica species (e.g., rapeseed, mustard, cabbage)	Ash, maple, elm (samara)
Brazil nut	Buckwheat, anemone, avens (achene)
Castor bean	Cereals (caryopsis)
Coffee bean	Hazel, walnut, oak (nut)
Cotton	Lettuce, sunflower and other Compositae (cypsela)
Legumes (e.g., peas, beans)	
Squashes (e.g., cucumber, marrow)	
Tomato	

fusion of two polar nuclei of the central cell in the embryo sac with the other pollen tube nucleus; (3) the perisperm, a development of the nucellus; and (4) the testa (seed coat), formed from one or both of the inner or outer integuments around the ovule. Although all mature seeds contain an embryo (sometimes poorly developed), and many are surrounded by a distinguishable seed coat, the extent to which the endosperm or perisperm persists varies among species. Sometimes the testa exists in a rudimentary form only, the prominent outermost structure being the pericarp or fruit coat derived from the ovary wall; in these cases, the dispersal unit is not a true seed in the strict botanical sense, but a fruit (Table 1.1). In gymnosperm seeds, there is no fusion of the male and polar nuclei leading to the formation of a triploid endosperm—in these the storage tissue in the mature seed (which is functionally similar to the true endosperm) is haploid and is the modified megagametophyte (Fig. 1.1).

More rarely, the embryos within seeds are produced by nonsexual processes, such as apomixis (Sect. 2.3.5), and thus contain only the diploid maternal complement of genes. Such seeds are often indistinguishable from those of the same species resulting from sexual reproduction. The dandelion and some other composites have come to rely exclusively, or almost exclusively, on apomictic reproduction.

Each of the seed components will now be considered briefly.

1.2.1 Embryo

The embryo is composed of the embryonic axis and one or more cotyledons. The axis is composed of the embryonic root (radicle), the hypocotyl to which the cotyledons are attached, and the shoot apex with the first true leaf primordia (plumule). These parts are usually easy to discern in the embryo of dicotyledonous (dicot) (Figs. 1.1, 2.2), but in the embryos of monocotyledonous species (monocots), particularly the Gramineae, identifying them is considerably more difficult. Here, the single cotyledon is much reduced and modified to form the scutellum (Figs. 1.1, 2.3); the basal sheath of the cotyledon is elongated to form a coleoptile covering the first leaves, and in some species (e.g., maize) the hypocotyl is modified to form a mesocotyl. The coleorhiza is regarded as the base of the hypocotyl sheathing the radicle.

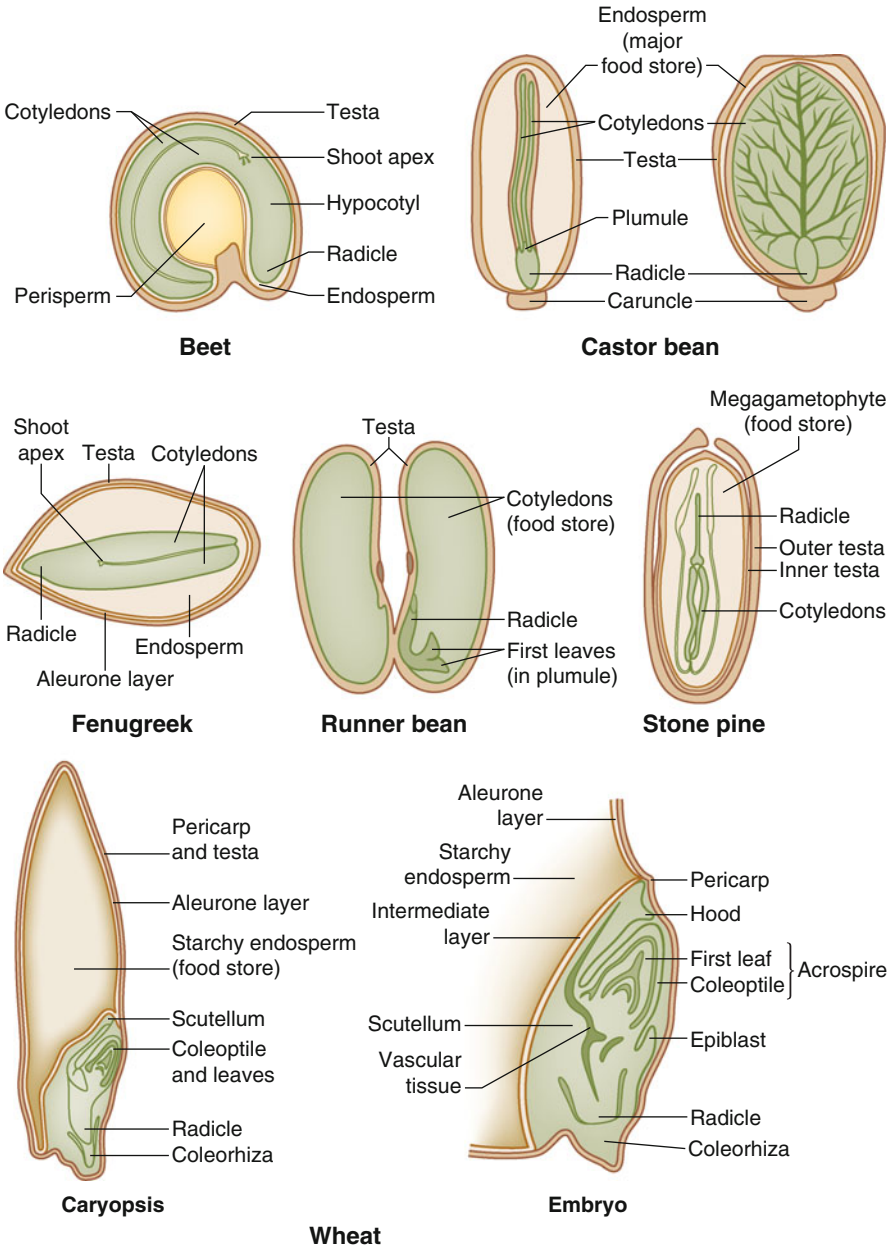


Fig. 1.1 The structure of a monocot grain (caryopsis) and seeds of a gymnosperm and some dicot species showing the location of their tissues and organs. Not drawn to scale

The shapes of embryos and their sizes in relation to other structures within the seed are variable. In monocot and dicot species with a well-developed endosperm in the mature seed, the embryo occupies less of the seed than in non-endospermic

seeds (Fig. 1.1). Cotyledons of endospermic seeds are often thin and flattened since they do not store much in the way of reserves (e.g., castor bean); in non-endospermic seeds, such as those of many of the legumes, the cotyledons are the site of reserve storage (embryonic storage tissue) and account for almost all of the seed mass (Fig. 1.1). Cotyledons of non-endospermic, epigeal (Fig. 5.1) species (such as some members of the squash and legume bean family) that are borne above the ground after germination and become photosynthetic are relatively not as large, nor do they contain as much stored reserves as the subterranean, hypogeal type (Fig. 5.2). The cotyledons are absent from seeds of many parasitic species; in contrast, the embryos of many coniferous species contain several cotyledons (polycotyledonous) (Fig. 1.1).

In the Brazil nut, much of the edible kernel (embryo) is the hypocotyl, which is unusually large and is the site where the stored reserves are deposited.

Polyembryony, i.e., more than one embryo in a seed, occurs in some species, e.g., *Poa alpina*, *Citrus,* and *Opuntia* spp. This can arise because of division of the fertilized egg cell to form several zygote initials, development of one or more synergids (accessory cells in the embryo sac), the existence of several embryo sacs per nucellus, and the various forms of apomixis. In flax and other species some of the embryos formed by polyembryony are haploid.

Not all seeds contain mature embryos when liberated from the parent plant. The final developmental stages of the embryo occur after the seed has been dispersed, e.g., ash spp., carrot (Fig. 6.2b), and hogweed. Orchid seeds contain minute and poorly formed embryos, with no storage reserves, and no endosperm (Fig. 6.2a). Their further development and germination requires that they first form a symbiotic association with soil fungi or other microorganisms.

1.2.2 Non-embryonic Storage Tissues

In most species the diploid perisperm, derived entirely from the maternal nucellar tissue of the ovule, fails to develop, or develops for only a short time following fertilization, and is quickly absorbed as the embryo (and endosperm where present) becomes established. In the seeds of several species, including beet, *Yucca*, and some cacti, the perisperm is the major store of the food reserves. The endosperm is virtually absent from these seeds, although in others it may be developed to a greater (*Acorus* spp.) or lesser (*Piper* spp.) extent than the perisperm. In beet seeds (Fig. 1.1) both the perisperm and cotyledons of the embryo contain substantial reserves: there is only a single layer of endosperm cells covering the radicle tip.

Seeds can be categorized as endospermic or non-endospermic in relation to the presence or absence in the mature seed of a well-formed triploid endosperm. Even though an endosperm is present, some seeds are generally regarded as being non-endospermic because this tissue is mostly broken down and is only a flimsy remnant of that formed during development (e.g., soybean and peanut), or it may be robust but only one to a few cell layers thick (e.g., lettuce, Arabidopsis). In these cases, other structures, usually the cotyledons, are the principal storage organs. Some endosperms are relatively massive and are the major source of stored reserves within

the mature seed, e.g., in cereals, castor bean, date palm, and endospermic legumes such as fenugreek, carob, and honey locust. In the cereals and some endospermic legumes (e.g., fenugreek) the majority of cells in the endosperm are nonliving at maturity, the cytoplasmic contents having been occluded by the accumulation of reserves during development, accompanied by the occurrence of programmed cell death (Sect. 5.5.4). But on the outside of the endosperm remains a living tissue, the aleurone layer, which does not store any of the major reserves, but rather is responsible ultimately for the production and release of enzymes for their mobilization (Figs. 1.1, 5.5). Endosperms with a high water retention capacity may have a dual role: to regulate the water balance of the embryo during germination, and to provide reserves that are utilized for early seedling growth (e.g., fenugreek). An unusual endosperm is that of the coconut palm seed, in that part of it remains acellular and liquid.

In gymnosperms the haploid megagametophyte, a maternal tissue that persists after fertilization of the egg, surrounds the embryo and is the major nutrient tissue.

1.2.3 Testa (Seed Coat)

Variability in the anatomy of the testa is considerable, and it has been used taxonomically to distinguish between different genera and species. Hence, a discussion of the range of seed coat structures is well beyond the scope of all but specialist monographs. The testa is of considerable importance to the seed because it is often the only protective barrier between the embryo and the external environment (in some species the fruit coat, and even the endosperm, support or provide a substitute for this role). The protective nature of the seed coat can be ascribed to the presence of an outer and inner cuticle, often impregnated with fatty and waxy substances (e.g., suberin), and one or more layers of thick-walled, protective cells (Fig. 6.5). Mechanical reinforcement through the synthesis of secondary cell walls, impregnated with impermeable fats/waxes or lignin can occur, as can the production of polyphenolics as protectants from insects. Layers of crystal-containing cells (calcium oxalate or carbonate, silica) occur in the seed coats of many species (e.g., sesame); these may play a protective role, dissuading insect predation also, for example. Coats may contain pectin-rich cell walls that erupt upon contact with water, releasing the pectin as mucilage, such as in linseed (flax), providing a water-retaining barrier around the seeds; the hydrophilic mucilage also aids passage of the seed though the digestive system of dispersing birds and animals. Such barriers may also restrict oxygen uptake, as will the presence of phenolics, and there are other structural features in some coats that restrict exchange of gases between the internal structures (embryo and endosperm) and the environment. Some coats, e.g., of many legumes, are largely impermeable to water and consequently can restrict the metabolism and growth of inner tissues.

The coloring and texture of seed coats are distinguishing features of many seeds but sometimes cannot be used taxonomically because they may change due to environmental and genetic influences during development. Upon detachment from the

parent plant, the seed coat bears a scar, called the hilum, marking the point at which it was joined to the funiculus. At one end of the hilum of many seed coats can be seen a small depression, the micropyle. Hairs or wings may develop on the testa to aid in seed dispersal (e.g., in willow, lily, *Epilobium* spp.); more usually the dispersal structures are a modification of the enclosing fruit coat. As an attraction to birds and animals, to aid in their dispersal (Sect. 7.1), the outer layer of the testa in some seeds may be fleshy and colored, and is then termed the sarcotesta, while the inner layer is rigid and protective (e.g., *Zamia* and *Taxus* spp., Ginkgo).

Outgrowths of the hilum region occur: the strophiole, which restricts water movement into and out of some seeds, and the aril. These often contain chemicals, e.g., oils that attract ants, which carry the seeds away for later consumption, thus dispersing them (Sect. 7.1). In seeds of several species, e.g., castor bean and other members of the Euphorbiaceae, the aril is associated with the micropyle and is called the caruncle; this may also contain oils and be important in dispersal. Arils are variable in shape, forming knobs, bands, ridges, or cupules, and are often brightly colored. Some are of commercial value; that of the nutmeg is used as a source of the spice mace, while the seed coat contains different chemicals and is ground up for the spice named after the seed itself.

1.3 Seed Storage Reserves

About 70% of all food for human consumption comes directly from seeds (mostly those of cereals and legumes), and a large proportion of the remainder is derived from animals that are fed on seeds. It is not surprising, therefore, that there is a wealth of literature concerned with the chemical, structural, and nutritional composition of seeds of cultivated species since they make up such a large share of our food source and also provide many raw materials for industry. Information on seeds of wild species and wild progenitors of our cultivated crops is relatively scarce. But with increasing interest in novel sources for food, and in improved genetic diversity within domesticated lines, the seeds of wild plants are receiving more attention.

In addition to the chemical constituents common to plant tissues, seeds contain extra amounts of substances stored as a source of food reserves to support early seedling growth. These are principally carbohydrates, oils, and proteins. Present also are more minor, but nevertheless important compounds; of these, several are recognized as being nutritionally undesirable or even toxic (e.g., alkaloids, lectins, proteinase inhibitors, phytin, and raffinose family oligosaccharides [RFOs]).

The chemical composition of seeds is determined ultimately by genetic factors and hence varies widely among species and their varieties and cultivars. Some modifications of composition may result from agronomic practices (e.g., nitrogen fertilizer application, planting dates) or may be imposed by environmental conditions prevalent during seed development and maturation; but such changes are usually relatively minor. Through crossing and selection, plant breeders have been able to manipulate the composition of many seed crops to improve their usefulness and

Table 1.2 The food reserves of seeds of some important crops

	Average percent composition			
	Protein	Oil	Carbohydrate[a]	Major storage region
Cereals				
Barley	12	3[b]	76	Endosperm
Dent corn (maize)	10	5	80	Endosperm
Oats	13	8[b]	66	Endosperm
Rice	7	3	75	Endosperm
Rye	12	2	76	Endosperm
Wheat	12	2	75	Endosperm
Legumes				
Broad bean	23	1	56	Cotyledons
Garden pea	25	6	52	Cotyledons
Peanut	31	48	12	Cotyledons
Soybean	37	17	26	Cotyledons
Other				
Castor bean	18	64	Negligible	Endosperm
Oil palm	9	49	28	Endosperm
Pine	35	48	6	Megagametophyte
Rape	21	48	19	Cotyledons

The percentages are approximate and vary between cultivars and with field conditions during seed development. For example, there are cultivars of soybean that produce over 40% protein, and 20% oil, and under conditions of high nitrogen fertilization and favorable weather conditions wheat may produce up to 16% protein

[a]Mainly starch

[b]In most cereals oils are stored within the scutellum, an embryonic tissue, with less in the aleurone layer, but in oats it is present in the starchy endosperm also, and in some cultivars up to 11–18% of grain weight

yield; more recently, with the advent of genetic engineering, there is considerable investment in modifying and improving the quality and quantity of the stored reserves (Chap. 3).

Modern cultivars of many cereals and legumes store substantially higher quantities of reserve material than either earlier cultivars or their wild progenitors. Even so, some nutritional deficiencies remain to be rectified; e.g., the composition of the storage proteins of legumes and cereals is such that they do not provide all of the amino acids required by simple-stomached (monogastric) animals such as humans, pigs, and poultry (Sect. 1.3.3). Some indication of the differences in the constituent stored reserves of seeds is to be found in Table 1.2; the important storage tissue within each seed is noted.

In many seeds the stored reserves may occur within both embryonic and extra-embryonic tissues, but in different proportions, e.g., in cereals the major starch and protein content is in the endosperm, but the oil is present in the embryo (scutellum). Different reserves may even be located within different storage tissues; in fenugreek seeds, for example, the endosperm is the exclusive source of carbohydrate (as cell wall galactomannan), but the cotyledons contain the protein and oils. The reserves

may be distributed unevenly within any one storage tissue; in the maize kernel, there are protein-rich regions (horny endosperm) and starch-rich regions (floury endosperm) within the endosperm. Chemical differences can also exist within a species in relation to the distribution of any particular reserve: in rapeseed, the oil in the cotyledons and hypocotyl contains different proportions of erucic and palmitic acids.

Each of the major reserves will now be considered in more detail, along with a brief description of some of the minor components present in seeds. Chap. 3 deals with the deposition of the main stored reserves, and therein will be found further discussion of their chemical composition and localization.

1.3.1 Carbohydrates

Carbohydrates are the major storage reserves of most seeds cultivated as a food source (Table 1.2). Starch is the carbohydrate most commonly present in seeds, although cell wall-associated hemicelluloses may be present, and sometimes are the major carbohydrate reserve. Frequently present also are the raffinose family (or series) oligosaccharides (RFOs, RSOs), the proportions of which vary among species, often as much as 2–6% of seed dry weight. Other carbohydrates that occur in nonstorage forms are cellulose, pectins, and mucilages.

(1) *Starch* is stored in seeds in two related forms, amylose and amylopectin; both are polymers of glucose. Amylose is a straight-chain polymer some 100–10,000 glucose units in length: adjoining glucose molecules are linked by $\alpha(1 \rightarrow 4)$ glucosidic bonds (Fig. 1.2a). Amylopectin is much larger (10^2–10^3 times); it consists of many amylose chains linked via $\alpha(1 \rightarrow 6)$ bonds to produce a multiple-branched molecule, each chain of the branch being 6–80 glucose residues in length (Fig. 1.2a). Starch is synthesized in nonphotosynthetic plastids called amyloplasts, which contain one to many starch granules. Granules typically are composed of dense concentric rings (a few hundred nm in thickness) interspersed with less-dense amorphous zones (each combination being a "growth ring"). The amylopectin, present in a highly ordered fashion in the dense regions, contains clusters of aligned double helices of amylopectin, which alternate with less-compact lamellar regions (together these constitute the "crystalline region") (Figs. 1.2b, 3.9). The amylose chains are thought to be interspersed in the amylopectin as well as reside in the amorphous zones in which there are less-well-organized forms of the latter. Most granules are composed of about 70–75% amylopectin and 20–25% amylose. Certain mutants of cereals have a higher- or lower-than-average content of amylose, and starch granules of *waxy* mutants of maize are devoid of this polymer; this means that the structure of granular starch is not dependent upon the presence of amylose. In wrinkled peas, amylose accounts for two-thirds or more of the starch compared with about one-third in smooth peas and other legumes.

Starch granules may have an appearance characteristic of an individual species, and they may be predominantly spherical (barley), angular (maize), or elliptical

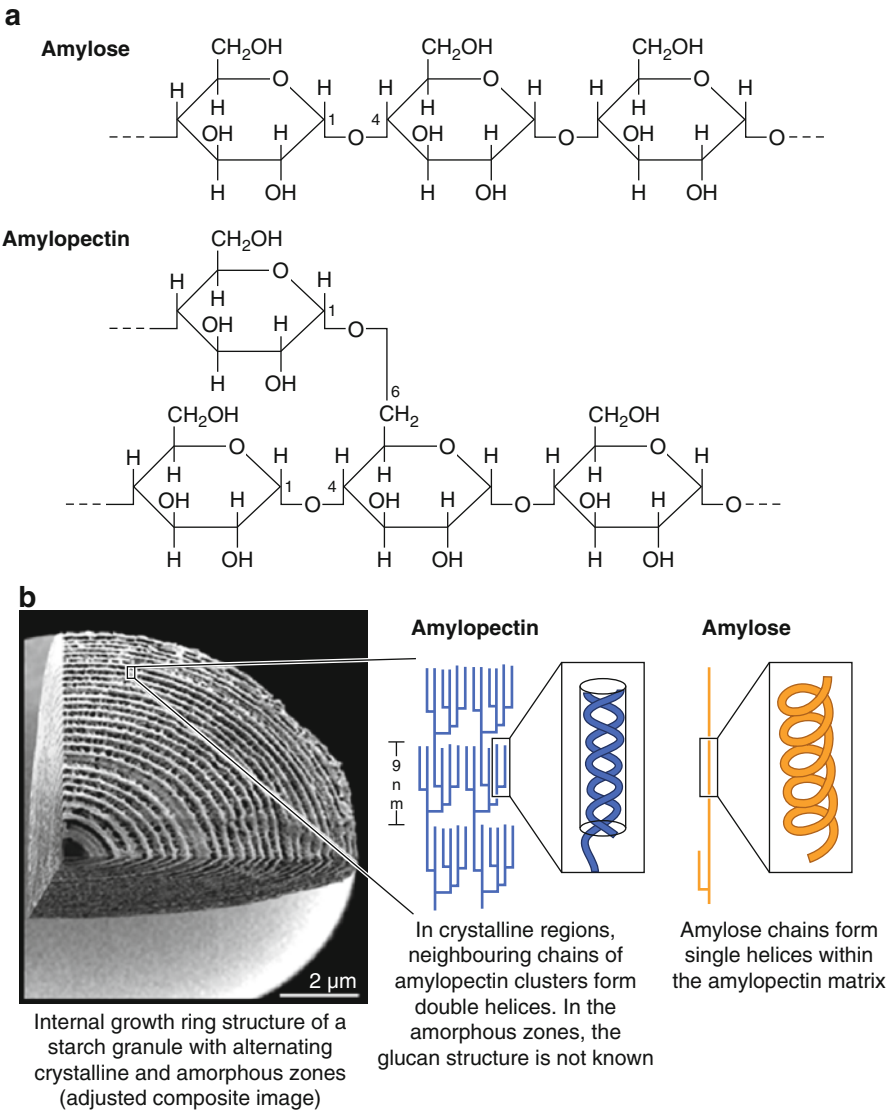


Fig. 1.2 (**a**) The chemical composition of amylose and amylopectin. (**b**) Scanning electron micrograph showing the regular periodicity (several hundred nm) of rings in a starch granule. The double and single helical structures of amylopectin and amylose are shown. For more details see Zeeman et al. (2010). Courtesy of S.C. Zeeman, Zurich Institute of Plant Sciences, Switzerland

(runner bean). The diversity of granule size, morphology, and composition reflects the different functional properties, and hence end-uses, of starch. Granule size is very variable, from 2 to 100 µm in diameter, even in the same seed. In the rye endosperm, for example, there are large oval starch grains that are up to 40 µm in

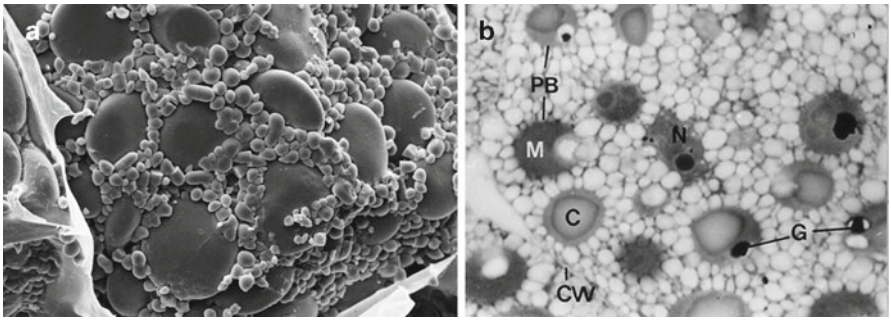


Fig. 1.3 (**a**) Scanning electron micrograph of large and small starch granules in the barley grain endosperm. To the *left* and lower *right* of the micrograph the lighter colored cell wall is visible. Courtesy of K. Brismar, Swedish Univ. of Agricultural Sciences, Alnarp. (**b**) Light micrograph of an endosperm cell of castor bean showing extensive packing of oil bodies around larger protein storage vacuoles (protein bodies, PB) C, crystalloid (protein); G, globoid (phytin); M, matrix (protein); N, nucleus; CW, thin cell wall. Courtesy of J. S. Greenwood, Univ. Guelph

diameter; there are also numerous smaller grains less than 10 μm in diameter embedded in a fine network of cytoplasmic protein. In barley, the spherical starch grains are separable into two groups, large ones and small ones (Fig. 1.3a). Although the latter account for about 90% of the total number of grains, they comprise only 10% of the total starch by weight.

Starch is the most important carbohydrate in the human diet; it has a multitude of uses both in the food and nonfood industry, but its functionality varies with its composition related to amylose and amylopectin content, for this affects its ability to gelatinize at specific temperatures. Gelatinized starches with a high amylose content form firm gels (a property useful in puddings, soups, pie fillings, and salad dressings, for example) and strong tough films (for paper coatings and adhesives). High amylose starches are also known nutritionally as "resistant starches" because they escape digestion in the intestine of healthy humans thus desirably increasing the fiber content of the diet. Low amylose starches, the most prevalent starch in foods, do not retrograde when frozen and thus can be used in puddings, pies, and crusts in frozen foods. Maize starch is now modified industrially to make high fructose corn syrup, and is a "green" source for conversion to ethanol.

(2) *Hemicelluloses* are the major form of stored carbohydrates in some seeds, particularly in certain endospermic legumes. Usually starch is absent from tissues where hemicelluloses are present in appreciable quantities in cell walls; there are no hemicellulose storage bodies. The endosperm of the ivory nut palm, date palm, and coffee are extremely hard because of hemicelluloses laid down as very thick cell walls. In several "hard" seeds, the endosperm around most of the embryo (lateral endosperm) is composed of cells with very thick and rigid walls, whereas in the micropylar endosperm close to the radicle (which will eventually emerge to complete germination) the walls are thin and more easily penetrable (Fig 1.4a–c). Many of the hemicelluloses are mannans, i.e., long-chain polymers of mannose

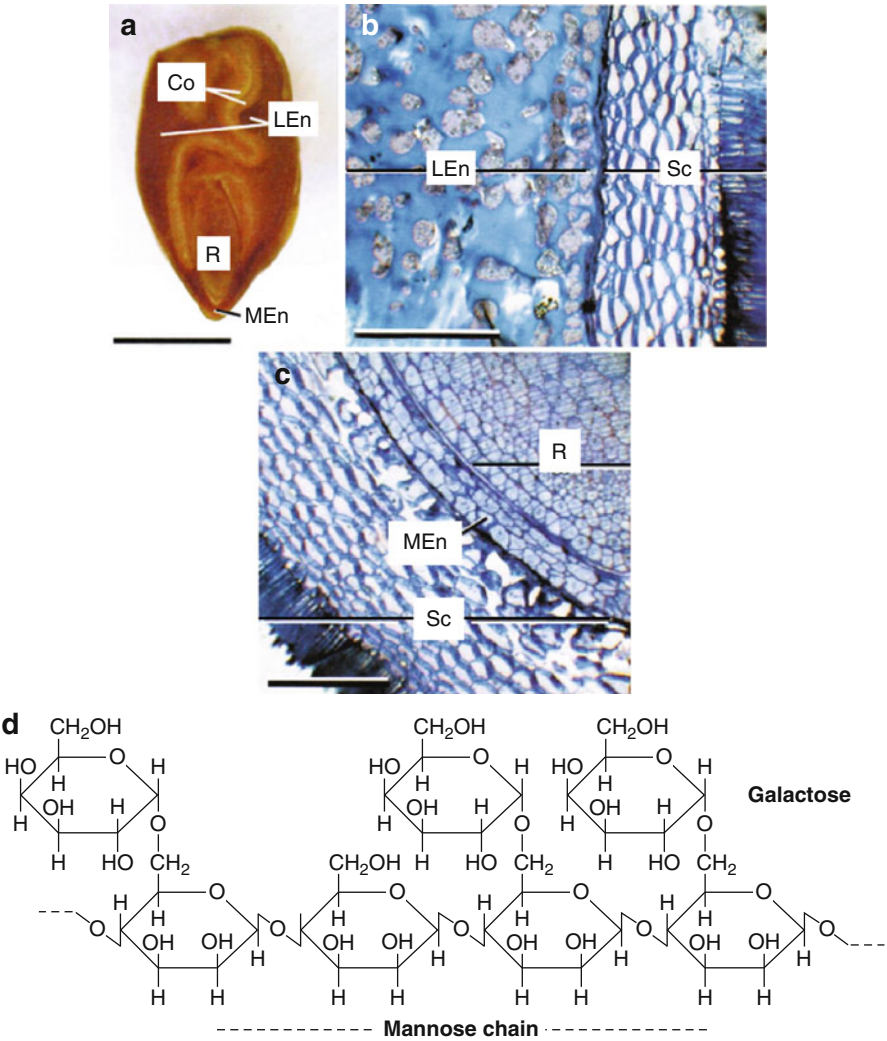


Fig. 1.4 (**a**) Transversely dissected seed of Chinese senna showing the embryo embedded in the hard endosperm. (**b**) Lateral endosperm cells with thick walls, and seed coat to the outside. (**c**) Micropylar endosperm with thin-walled cells adjacent to the radicle. Co: cotyledon; R, radicle; LEn, lateral endosperm; MEn, micropylar endosperm; Sc, seed coat. Scale bars: a, 2 mm; b and c 100 μm. From Gong et al. (2005). With permission of Oxford Univ. Press. (**d**) Structure of galactomannan, showing the β1→4 linkages in the mannose backbone and α1→6 to the galactose side-chains

(Man, linked by β1→4 bonds) with variable but small quantities of sugar present as side chains, e.g., galactose (Gal, linked by α1→6 bonds) in galactomannans (Fig. 1.4d). The number of Gal side chains affects the consistency of the galactomannan; it varies from being very hard in almost pure mannans (coffee mannans

contain 2% Gal) to being mucilaginous at much higher Gal contents. Within the Leguminosae the Man:Gal ratio may have a taxonomic significance as well. Carob and guar seed galactomannans are very important commercially, with many uses including in processed foods as hydrating, thickening, and stabilizing agents; as a thickener in toothpastes, carpet and fabric dyes, and cosmetics; as a component of the hydrophilic matrix of pharmaceutical drugs to aid controlled release of the active agent; as biodegradable mulches for hydroseeding; and even in the mining and explosives industries as waterproofing for stick dynamite.

Glucomannans, in which there are repeats of glucose and Man units in a linear chain joined by $\beta 1 \rightarrow 4$ links, occur only in the endosperm of certain monocots, specifically members of the Liliaceae and Iridaceae. They, too, are components of thickened cell walls. Other hemicelluloses include the xyloglucans, which, strictly, should be called galactoxyloglucans since they are substituted cellulose-type molecules, i.e., a linear $\alpha(1 \rightarrow 4)$-linked glucose backbone with short xylose (some with a Gal attached) side chains. They are present in cell walls of certain dicot embryos (cotyledons) and endosperms, such as in members of the Caesalpinoideae, a subfamily of the Leguminosae, e.g., tamarind. Xyloglucans in the cell walls have also been called "amyloids" because they react positively to a stain for starch.

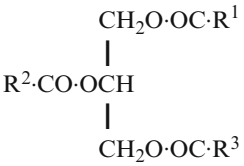
In some wild grasses, such as *Brachypodium distachyon*, the endosperm cell wall has a high mixed glucan content (β-$1 \rightarrow 3$, $1 \rightarrow 4$ glucans), along with lesser amounts of cellulose and xylans, which is several times more than the amount of storage starch present; thus the wall is likely the major source of storage carbohydrate.

(3) *Free sugars* are present in very variable amounts in seeds of different species. They are rarely the main storage carbohydrate but can account for up to 12–16% of the dry mass of the axes of some legumes (e.g., lupin, soybean); however, in other species, especially the cereals, there may be less than 1%. The disaccharide sucrose and the oligosaccharides raffinose, stachyose, and verbascose (RFO members, Sect. 5.3) are the most common free sugars present. They are important to the embryos of many seeds as a source of respirable substrate during germination and early seedling growth, but also play a role in the desiccation tolerance and storability of seeds; those with a higher than 1.0 ratio of sucrose to RFOs having a shorter viability period during storage of the mature dry seed than those with a lower ratio. RFOs are of interest with respect to human and domestic animal nutrition for they are not digested or absorbed in the upper intestinal tract. Instead, they are degraded by the microbial flora of the colon, producing H_2 and CO_2. This causes flatulence, and the expulsion of these gases can have embarrassing and malodorous consequences.

A large number of different cyclitols (e.g., *myo*-inositol, *chiro*-inositol, pinitol, ononitol) and their respective galactosyl derivatives (galactinol, fagopyritol, galactopinitol and ciceritol, galactosyl ononitol) are also present in seeds, especially those of legumes, but with few exceptions (e.g., buckwheat, chickpea, castor bean) they account for only a minor proportion of the total oligosaccharide content. Their role in the seed is unclear, but they may contribute as antioxidants, respirable substrates, and stress protectants in the same manner as RFOs.

1.3.2 Oils (Neutral Lipids)

Chemically, seed storage lipids are triacyglycerols (TAGs, formerly called triglycerides), most of which are oils, i.e., they are liquid above about 20°C; some seeds may contain appreciable quantities of phospholipids, glycolipids, and sterols too. TAGs are insoluble in water but soluble in a variety of organic solvents including ether, chloroform, and benzene. They are esters of glycerol and fatty acids, with the latter attached to the backbone of the former:



The number of carbon atoms in each of the fatty acid chains, denoted as R^1, R^2, and R^3, is usually not the same.

Fatty acids are identified according to the number of carbon atoms and double bonds in their chain, those with ~12–18C being termed long-chain fatty acids (LCFA), and with more than 18C, very long chain fatty acids (VLCFA). Saturated fatty acids contain an even number of carbon atoms and no double bonds; e.g., palmitic acid (16:0; 16 carbon atoms:no double bonds) is the most common saturated fatty acid in seed oils. But the predominant fatty acids in seeds are the unsaturated ones; of these, oleic (18:1Δ9; one double bond in position 9 of the fatty acid chain) and linoleic (18:2Δ9,12, with two double bonds at positions 9 and 12) account for more than 60% by weight of all oils in oil seed crops, with α-linolenic acid (18:3Δ9,12,15) being much less abundant. The position of the double bond (Δ) in the fatty acid chain is specified by counting from its carboxyl (–COOH) end (the one condensed with the glycerol backbone). Less common fatty acids are erucic acid (22:1Δ13), a component of some rapeseed oils (although it has now almost been bred out of most commercial cultivars, e.g., canola, because it has an unpleasant taste and smell) and the oil of *Crambe abyssinica*; ricinoleic acid (12-hydroxy 18:1Δ9), the major component of castor (bean) oil; eicosenoic acid (20:1Δ5), which accounts for 60% of the total fatty acids in meadowfoam seeds; and petroselinic acid (18:1Δ6) which is 85% of the total fatty acids of seeds of the Umbelliferae. Oils containing such fatty acids are important because they are the raw materials for products such as lubricants, pharmaceuticals, biodiesel fuels, cosmetics and soaps, plastics, coatings, and paints. Cacao (cocoa) oil, with its unique arrangement of fatty acids on the glycerol backbone, is used to produce cocoa powder and butter, and therefrom chocolate. The storage lipids of jojoba are unusual in that they are wax esters of long-chain unsaturated fatty acids and alcohols, not TAGs, and they are liquid. They are an important source in the manufacture of cosmetics because of their similarity to sperm whale oil, and of surfactants, pharmaceuticals and high-pressure lubricants.

A different nomenclature for the position of the double bonds in fatty acids is also in use, particularly in the food industry. In this case the position of the double

Table 1.3 The percentages of major fatty acids in oils of various commercially important seeds

Species	Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)
Cotton	27	3	17	52	0
Linseed (Flax)	–	–	–	77	17
Maize	12	2	24	61	<1
Oil palm	44	39	4	10	0
Peanut	12	2	50	31	0
Rapeseed (Canola)	5	2	55	25	12
Soybean	11	3	22	54	8
Sunflower	6	4	26	64	0
Animal fat (lard)	29	13	43	10	0.5

The percentage of fatty acids in an oil is determined genetically, but there are small variations between cultivars and with year of harvest dependent upon growth conditions in the field. The oils of many species also contain other fatty acids in low amounts (0.5–5%), often one or more of C20:0, C22:0, C22:1, and C24:0

bond is denoted as the one closest to the terminal methyl (–CH3, ω) end of the fatty acid, opposite from the carboxyl end. The most common examples are the omega-3 and omega-6 (ω-3, ω-6) fatty acids, linoleic acid being a ω-6 fatty acid, and α-linolenic acid a ω-3. These are also known as essential fatty acids because they cannot be synthesized by humans, and must be obtained in the diet.

The fatty acid compositions of the oils and fats of some important crop species (major oilseeds) are shown in Table 1.3. Both maize and sunflower oils are widely used for cooking, and in margarine. Among their desirable properties for this purpose is their high linoleic acid content. Catalytic hydrogenation of seed oils changes their melting point and lowers their linolenic acid content. This triunsaturated fatty acid oxidizes readily during food storage and produces off-flavors. The degree of unsaturation left after hydrogenation determines whether a fat or oil is a solid at room temperature (required for margarine) or a liquid (cooking oil). Variable hydrogenation of peanut oil yields peanut butter of different consistencies. The advantage of using plant oils over animal fats in the diet is that because of their higher unsaturated fatty acid content (polyunsaturated) after hydrogenation, they are purported to reduce atherosclerosis, a claim not wholly substantiated in medical tests. Fats such as lard (Table 1.3) from animal sources are high in oleic acid, and hydrogenation causes rapid saturation of the one double bond per molecule. Drying oils, such as those used in paints and lacquers (e.g., linseed oil from flax, and rapeseed oil), contain a high linolenic acid content after processing. Upon exposure to air a free-radical polymerization reaction occurs, initiated by oxygen, which cross-links the oils to form a tough film, trapping the colored pigment in place.

There are many "minor" oil seeds, so called because they have little impact on the global oilseed economy (e.g., neem, almond, *Jatropha curcas*, tonka bean); they are grown for the distinctive fatty acid composition of their oils, and are useful as culinary oils and in cosmetics, soap, medicinals, pesticides, surfactants and lubricants. Consideration is being given to expanding the production of some

species, or to transfering genes relevant to the synthesis of their useful oils to major crops.

The TAG reserves in seeds are laid down in discrete subcellular organelles called oil bodies (also called lipid bodies, oleosomes, spherosomes or, in jojoba, wax bodies). They range in size from 0.2 to 6 μm in diameter, according to species; they are bounded by a phospholipid membrane monolayer in which several unique proteins, especially oleosins, are embedded. Oil body size typically correlates with the amount of associated oleosin. These aid in the stabilization of the membrane and are also important during maturation drying in preventing the oil bodies from coalescing. In seeds with high oil content, the oil bodies occupy a substantial volume of the cell, as in the castor bean endosperm (Fig. 1.3b). The ontogeny of these bodies is discussed further toward the end of Sect. 3.2.3.

1.3.3 Proteins

Proteins (polypeptides) are polymers of approximately 20 different amino acids, and may be composed of a single (peptide) chain, or a number of associated chains of similar or different sizes. These may be linked by relatively weak (non-covalent) hydrogen bonds, or strong, covalently linked disulphide (–S–S–) bonds between cysteine residues (interchain links); intrachain links between cysteines in the same polypeptide chain also occur. Protein size is determined largely by its amino acid content and is expressed either as a molecular weight (MW or Mr, e.g., 45,000) or more commonly as a molecular mass, in kiloDaltons (e.g., 45 kDa). The net charge of a protein (positive or negative) depends upon its amino acid composition, resulting in a distinct isoelectric point (pI). Many proteins, especially storage proteins, are encoded by several genes (multigene family), and exhibit small differences in pI and molecular mass (isozymes). The size of some proteins is also affected by posttranslational addition of sugars (glycoproteins), methyl or phosphate groups; proteins encoded by a single gene, but differing in this way are termed isoforms.

Since the late nineteenth century T. B. Osborne's classification has been used to divide seed proteins into four classes in relation to their solubility: (1) *albumins*—soluble in water and dilute buffers at neutral pHs; (2) *globulins*—soluble in salt solutions but insoluble in water; (3) *glutelins*—soluble in dilute acid or alkali solutions; (4) *prolamins* (*prolamines*)—soluble in aqueous alcohols (70–90%). However, not all proteins adhere to these solubility parameters, and glutelins are in effect prolamins with internal disulfide linkages; hence they are no longer regarded as a separate solubility class. Prolamins are now divided into 3 separate subclasses: S-rich, S-poor, and High Molecular Weight (HMW). Solubilization of some proteins requires harsh extraction procedures (e.g., boiling in buffer containing the detergent sodium dodecyl sulfate [SDS], or a chaotropic agent such as urea). Prolamins are a storage protein class in cereals and grasses, but do not form a valid fraction in other species; they are absent from dicot and gymnosperm seeds.

Table 1.4 Storage protein classes present in seeds and grains of some commercially important crops

	2S albumins	7–8S globulins	11–12S globulins	Prolamins
Major component in:	Legumes	Legumes	Legumes	Cereals
	Cotton	Cotton	Cucurbits	
	Composites	Palms	Composites	
	Castor bean		Oats, rice	
	Brassicas		Brassicas	
	Brazil nut			
Minor component in:	Cereals	Cereals	Most cereals	Oats, rice

There are no genes for 7S globulins in the Brassicas (e.g., rapeseed) nor in Composites such as sunflower. Modified from Shewry and Casey (1999)

Table 1.5 The approximate percent storage protein composition of some cereals by class and their common names

Cereal	Albumin	Globulin	Prolamin
Barley	13	12	75 (hordein, hordenin)
Maize	4	2	85 (zein)
Oats	11	70 (avenalin)	20 (avenin)
Rice	5	60[a] (oryzenin)	20 (oryzin)
Sorghum	6	10	80[b]
Wheat	9	5	85 (gliadin, glutenin)

[a]Confusingly, this protein is also called a glutelin because of similarities in the structure and sequence of this globulin with those of prolamins of the glutelin type

[b]This has been called kafirin, but because of the unsavory connotations of part of this word, a more suitable name is required (perhaps sorghin)

There are usually several to many proteins that make up a particular class, varying in size and charge, e.g., in wheat there are at least two types of each of the high and low molecular weight glutenins, and three gliadin types (α, γ, ω, of which the latter contains 6 subtypes). The percentage content of each class and type of storage protein will vary somewhat with the environmental and nutritional status of the parent plant during grain development

For a detailed account of the complexity of seed storage proteins see Shewry and Casey (1999)

Most investigations on these various classes of proteins have been conducted on storage proteins in the edible seeds of crop species, a storage protein being one whose primary role is to store C, N and S to support early growth of the seedling. The major and minor storage proteins in grains or seeds of some commercially important crops are listed in Table 1.4, and the approximate proportions of the main protein classes in cereals are shown in Table 1.5, along with their commonly used names. The major storage protein in the endosperms of most cereals, e.g., maize, barley, rye, wheat and sorghum is of the prolamin type, whereas globulins predominate in oats and rice. Globulins are the storage proteins of cereal embryos, but as such they represent only a very small proportion of the total reserve protein of the whole grain; likewise in the aleurone layer the storage proteins are typically globulins and account for about 25% dry weight of this tissue. The amino acid composition of

Table 1.6 Amino acid composition of the prolamins in maize, barley, and wheat, and of the globulins of oat and soybean (7S and 11S fractions)

				Soybean	
Amino acid	Maize (zein)	Barley (hordein)	Oats (avenalin)	7S (β-conglycinin)	11S (glycinin)
Alanine	11.3	3.0	6.0	3.7	6.2
Arginine	1.7	2.6	6.6	8.8	5.6
Aspartic acid	3.9	1.9	9.2	14.1	11.7
Cysteine[a]	1.9	2.9	1.1	0.3	0.6
Glutamic acid	20.7	31.8	19.1	20.5	21.4
Glycine	3.8	3.2	7.5	2.9	7.5
Histidine	2.1	1.2	2.2	1.7	1.7
Isoleucine[a]	2.9	4.1	4.8	6.4	4.1
Leucine[a]	15.3	7.4	7.4	7.8	7.0
Lysine[a]	0.2	1.0	2.9	7.0	3.9
Methionine[a]	1.9	0.6	0.9	0.3	1.3
Phenylalanine[a]	3.8	5.2	5.2	7.4	4.6
Proline	13.0	20.1	4.9	4.3	6.5
Serine	5.9	4.9	7.0	6.8	6.0
Threonine[a]	3.2	2.5	4.1	2.8	3.8
Tryptophan[a]	0.3	–	1.0	0.3	0.8
Tyrosine[a]	3.9	2.6	3.5	3.6	2.7
Valine	4.5	5.1	6.4	5.1	5.2

Glutamic acid and aspartic acid values include glutamine and asparagine, respectively
Based on data from Shewry and Casey (1999) and Derbyshire et al. (1976)
All values expressed as mole %
[a]Essential amino acids that must be provided in the diet of animals because they are unable to synthesize them

the total protein fractions present in select cereal grains and a legume (Table 1.6) is strongly influenced, not surprisingly, by the nature of the major storage protein. Albumins and globulins are not seriously deficient in specific amino acids, and hence, from a nutritional point of view, oats are a good source of dietary protein, particularly for breeding stock. In normal barley and maize grains, however, where prolamins are high, lysine is seriously limiting; tryptophan is low and threonine content is nutritionally inadequate. Prolamins are high in proline and glutamic acid/glutamine; hence their name. Attempts have been made to modify the amino acid composition of the prolamin-rich cereals such as maize and barley, and in particular to increase their lysine content (Sect. 3.2.4.2).

Characteristically, storage proteins are oligomeric; that is, the complete protein (holoprotein) is made up of two or more subunits that can be separated, after extraction, using mildly dissociating conditions. These subunits in turn may be made up of a number of polypeptide chains, which may vary in amino acid composition between "homologous" subunits. This variation results in considerable heterogeneity of the native protein. For example, the prolamin fraction of maize consists of proteins of mol. mass between 10 and 27 kDa, called the α-, β-, δ-, and γ-zeins, of which the

α-type classes of 19 and 22 kDa account for about 70% of the total. Even within the α-zeins, however, there may be many (40–50) distinct polypeptides with small variations in their amino acid composition, indicative of their being encoded by a multigene family; for the α-zeins it is calculated that there are as many as 100 genes (although some may be untranslatable pseudogenes because there are many more genes than there are different polypeptides). There are probably only single genes (unigenes) for the other zein types. A similar complexity is shown by the wheat prolamin gliadin, which is separable into four major component protein types (S-rich α, β, γ, and S-poor ω), made up of at least 46 discrete polypeptides. This large variation in component polypeptides is also found in wheat glutenins and in the prolamins of other cereals. Hence, most storage proteins should not be thought of as a single protein, but rather as a complex of individual proteins bound together by a combination of intermolecular disulphide groups, hydrogen bonding, ionic bonding, and hydrophobic bonding.

Legume seeds are the second most important plant protein source, on a world basis, after cereals. Nutritionally, they are generally deficient in the S-containing amino acids (cysteine and methionine), but unlike cereal grains, their lysine content is adequate (Table 1.6). The major storage proteins are globulins, which account for up to 70% of the total seed nitrogen. The globulins consist of two major families of proteins that differ in molecular mass and that sediment during ultracentrifugation with sedimentation coefficients (Svedberg or S values) of approximately 7 (average 7–8) and 11 (average 11–13). Hence they are termed the 7S (vicilin group) and 11S (legumin group) proteins; both are holoproteins composed of regularly assembled subunits. Characteristically, the 7S proteins are glycosylated (glycoproteins), but the 11S only rarely.

The 11S storage globulins occur as hexameric complexes of 320–400 kDa composed of 6 nonidentical subunits (52–65 kDa). Each subunit contains an acidic polypeptide (pI ~ 6.5) of 33–42 kDa and a basic polypeptide (pI ~ 9) of 19–23 kDa. The acidic and basic polypeptides are linked by a single disulphide bond (Fig. 1.5). Compared to the 11S, the 7S storage globulins have more varied structures. They are present in seeds as trimeric complexes of 145–190 kDa, composed of 3 nonidentical polypeptides of 48–83 kDa. In some legumes, e.g., pea, these larger polypeptides undergo a series of proteolytic cleavages after assembly as the trimer, to range in size from 12 to 75 kDa (Table 1.7). In other species, including soybean, alfalfa and *Phaseolus vulgaris*, no proteolytic processing occurs. Most, if not all 7S proteins completely lack cysteine and thus the polypeptides are not linked by disulphide bonds. Other examples of the complexities of the subunit polypeptide composition of 7S and 11S legume storage proteins are given in Table 1.7.

Although relatively minor in amounts compared to the globular storage proteins, 2S albumins are invariably present in dicot seeds. Their molecular mass ranges from 10 to 15 kDa; they are not glycosylated, are composed of several polypeptides, and arise from multigene families. Those of some species are rich in the S-containing amino acids (e.g., Brazil nut, and two of the 13 albumins in sunflower), and have been of interest with respect to the genetic engineering of seeds to improve their protein S content.

All storage proteins, in spite of their great diversity in composition and size, are derived from a single ancestral gene encoding about 30 amino acids. Triplication of

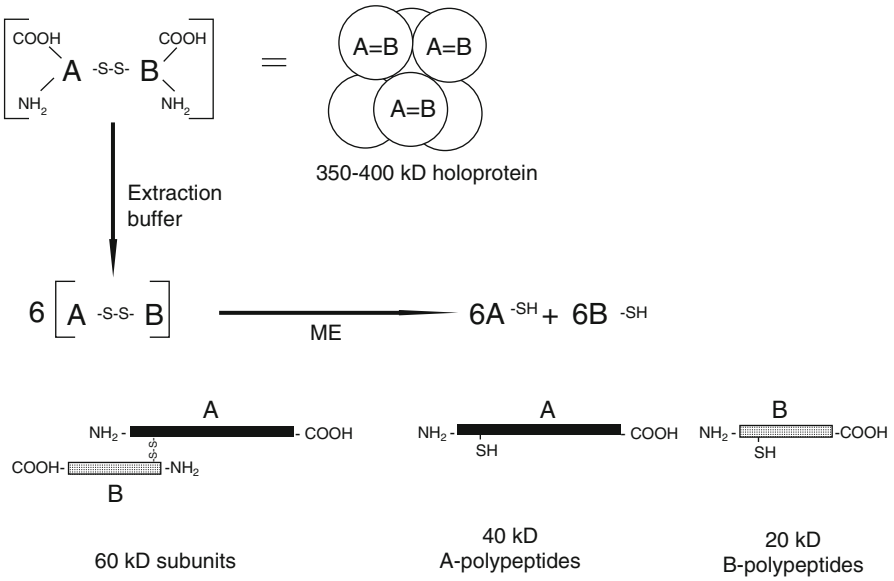


Fig. 1.5 Components of an 11S (legumin) storage protein as affected by a high-salt extraction buffer and the reducing agent β-mercaptoethanol (ME). The holoprotein is made up of 6 subunits, each containing one acidic (A) and one basic (B) polypeptide joined by a disulphide (–S–S–) bond. During extraction in high-salt buffer the protein is dissociated into its individual 60 kDa subunits because of the disruption of the non-covalent, electrostatic bonds holding them together. These subunits further dissociate into their component 40 and 20 kDa polypeptides in the presence of ME, which disrupts the interchain disulphide bonds. After Krochko and Bewley (1988)

Table 1.7 Subunit composition of the globulin storage proteins of legume seeds

Species	Approximate sedimentation coefficients (S)	Average mol mass (kDa)	Name of holoprotein	Subunits mol mass (kDa)
Garden pea	7–8	186	Vicilin	12, 14, 18, 24, 30, 50, 75
	12–13	360	Legumin	18, 20, 25, 27, 37, 40
Broad bean	7	150	Vicilin	31, 33, 46, 56
	11–14	328	Legumin	20, 37
French bean	6.5–7.5	150	Glycoprotein II (G2, β-phaseolin)	43, 47, 53
	11	340	G1	30, 32, 34
Alfalfa, lucerne	7	150	Alfin	14, 16, 20, 32, 38, 50
	11	360	Medicagin	59, 63, 64, 65, 67, 69
Soybean	7–8	160	β-Conglycinin	42, 57
	12	330	Glycinin	19, 37, 42

this gene resulted in three regions in the protein (A, B, C) and, over evolutionary time, deletions, insertions, and various repetitions of base sequences in one or more of these regions has led to the different classes and types of proteins in seeds of present-day species.

Seed storage proteins are usually deposited within a special cellular organelle called a protein storage vacuole (PSV), previously termed a protein body. These range in diameter from 0.1 to 25 μm and are surrounded, at least during development, by a membrane originating from the cellular endomembrane system (Sect. 3.2.4.1). In mature, dry storage tissues of some seeds, e.g., certain cereal grain endosperms, the membrane is incomplete or absent, leaving the protein dispersed in the cytoplasm. This is relatively uncommon, however. Some PSVs are simple in that they consist of a protein matrix surrounded by a limiting membrane. Inclusions frequently occur, however, particularly of crystalloids and globoids and, more rarely, druse (calcium oxalate) crystals. The crystalloids are water- or buffer-insoluble proteinaceous inclusions embedded in the soluble protein matrix; e.g., in castor bean (Fig. 1.3b) the crystalloid is an insoluble 11S protein, and the matrix is made up of 2S and 7S soluble proteins, including lectins. Globoids are the sites of deposition of phytin—the potassium, magnesium, and calcium salts of phytic acid (Sect. 1.3.4). Barley aleurone layer PSVs also contain carbohydrate but in a unit distinct from the globoid, known as the protein–carbohydrate body. Various enzymes may be present within the PSV in mature seeds, and during reserve mobilization other enzymes are added so that it eventually becomes an autolytic vacuole (Sect. 5.8.3).

PSVs may contain only one type of storage protein. In certain legumes, for example, some contain only albumin or vicilin or legumin, although most seem to contain both vicilin and legumin. When there is more than one protein in the same PSV, its distribution can be uneven, e.g., in the maize endosperm the major storage protein, α-zein, is deposited centrally in the PSV, and is surrounded by a thin layer of β- and γ-zein (Fig. 3.16). PSVs in the aleurone layer of this and other cereals contain mostly albumins and globulins, although in maize there a minor amount of prolamin present.

Not all seed storage proteins have nutritionally desirable properties. Enzyme inhibitors (Sect. 5.8.4) may reduce the effectiveness of food-degrading enzymes in the digestive tract of animals. Lectins, which are usually glycoproteins, have the capacity to bind to animal cell surfaces, sometimes causing agglutination (particularly of erythrocytes), e.g., concanavalin A in jack bean seeds: hence lectins are sometimes called phytohemagglutinins. This property is probably superfluous as far as the seed is concerned, and many lectins are innocuous from a nutritional standpoint. Some lectins are highly toxic, however, including ricin D from castor bean and abrin from rosary pea, both of which are mixtures of non-agglutinating toxins and nontoxic agglutinins. Any toxic effect of lectins can usually be eliminated by proper heat treatment, e.g., castor bean meal can be fed as a protein supplement to cattle after extraction of castor oil, and appropriate heating.

Mandelonitrile lyase (MDL) is a glycoprotein sequestered in the PSVs of the endosperm of black cherry seeds, and is an enzyme involved in the degradation of the cyanogenic disaccharide, amygdalin. It may be important in this and other cyanogenic seeds in producing HCN for protection when tissues are damaged by pathogens. Many seeds contain proteins that may be part of their defense mechanisms against pests and predators, e.g., wild species of *Phaseolus vulgaris* contain the glycoprotein arcelin, which confers resistance against some brucid beetles, whereas seeds of domestic species appear to have such resistance conferred by an

α-amylase inhibitor. Chitinase, an enzyme that increases resistance to fungal attack, has been isolated from mature dry seeds of several monocots and dicots. Some of these enzymes may have the dual function of being storage proteins, as well as deterrents to seed predation.

The consumption of some seed storage proteins may result in serious allergenic or intolerance reactions in humans. The most common intolerance response, affecting about 0.5% of the population of Western Europe, is gluten-sensitive enteropathy, also known as coeliac disease, a result of damage to the lining of the small intestine leading to malabsorption of nutrients and liquids, and diarrhea. This occurs in response to the ingestion of wheat gliadins and related proteins in barley and rye. Another intolerance response to these proteins in some humans is a form of dermatitis. The 2S albumins also include a variety of allergens, and are present in seeds of Brazil nut, mustard species (*Brassica juncea, Sinapis alba*), and castor bean, for example. The serious and sometimes fatal allergic hypersensitive reaction to peanut seeds is caused by the 7S globulin *Arah 1*. This protein provokes an inappropriate immune response, resulting in the production of IgE antibodies and other anaphylatoxins which, via induced histamine release, cause anaphylactic shock due to constriction of the airways, and heart failure.

1.3.4 Phytin

Phytin (*myo*-inositol-6-phosphate, $InsP_6$ or IP_6) is the insoluble mixed potassium, magnesium, and calcium salt of *myo*-inositol hexaphosphoric acid (phytic acid), and although present in relatively minor quantities compared to the aforementioned major reserves, it is an important source to the seed of phosphate and mineral elements. Additionally, iron, manganese, copper, and, more rarely, sodium occur in some phytin sources (Table 1.8).

Phytin is located exclusively within the globoid (Fig. 1.3b) in some, but not in all PSVs in a seed, nor is the mineral element composition of phytin the same in all cells, e.g., calcium is in highest amounts in the globoids of the radicle and hypocotyl regions of the dicot embryo, but little, if any, of this element is present in those of cotyledons. Globoids are noncrystalline globular structures and are the most commonly occurring inclusion in PSVs, although they vary in both size and number. In some species, the globoids are present in PSVs of one region of a seed but not of another; e.g., the aleurone-layer PSVs (aleurone grains) and those in the scutellum of cereal grains usually contain globoids, but those in the starchy endosperm never do.

Phytic acid and its conjugates are generally regarded as being nutritionally undesirable since the negatively charged phosphate groups can bind essential dietary minerals (e.g., zinc, calcium, and iron), thus making them wholly or partially unavailable for absorption. This is an identified problem in children in developing countries, where food is often consumed that is less-well processed than in the developed world. Phytin in domestic animal feed of cereal grains and legume seeds reduces mineral availability; also, the undigested phosphate in the animal waste can

Table 1.8 The content of the main inorganic elements of phytin in seeds of various species, and their location

Species (and location)	Mg	Ca	K	P	Fe	Mn	Cu
Barley (A, E)	0.16	0.03	0.56	0.43			
Broad bean (C)	0.11	0.05	1.13	0.51			
Cotton (C)	0.4	0.13	2.18	0.79	0.059	0.003	0.005
Oat (A, E)	0.4	0.19	1.1	0.96	0.035	0.008	0.005
Soybean (C)	0.22	0.13	2.18	0.71			
Sunflower (C)	0.4	0.2	1	1.01			

A aleurone layer. *E* embryo, including scutellum. *C* cotyledon (there may be small amounts in the radicle/hypocotyl axis). Values are expressed as a % of seed dry weight
Variability in mineral ion content occurs between cultivars and lines of the same species, and is also influenced by the mineral content of the soil in which the parent plant is grown
After Weber and Neumann (1980)

be released by microbial action and enter runoff water, causing proliferation of algae in ponds and streams (eutrophication). Phytin in grains fed to animals, especially fowl, causes thinning of bones by binding calcium, to the extent that their legs are not able to withstand the weight of the bird. Attempts are being made to breed low-phytin lines of grains, as well as to reduce phytin content using genetic engineering (Sect. 3.2.5). A novel approach to the problem has been to engineer pigs with phytin-degrading phosphatases (phytases) in their saliva.

1.3.5 Other Constituents

There is a wealth of minor constituents present within seeds that cannot be regarded strictly as storage components since they are not utilized during germination or subsequent growth. Some, nevertheless, are worth mentioning briefly because of their medical applications (Table 1.9), although many also have deleterious effects on humans and domestic animals. Certain alkaloids, which are nonprotein nitrogenous substances, are important commercial sources of stimulants and drugs, e.g., theobromine from the cacao bean, caffeine from coffee and cocoa, strychnine and brucine from *Strychnos nux-vomica* (present as 2.5% of seed dry weight), and morphine from certain types of poppy. Phytostigmatine from the Calabar or ordeal bean was used in Nigeria for trials by ordeal, involving the drinking of this as a poison (if, rarely, the accused survived, this was taken as a verdict of not-guilty). In nature, such compounds can prevent insects and animals from using a seed for food. The alkaloids vicine and convicine from faba (fava) beans cause hemolytic anemia (favism) when in the diet of individuals, mostly men, in eastern Mediterranean and Middle Eastern populations.

Phytosterols such as sitosterols and stigmasterols are present in some seeds, e.g., in soybean. The latter is important pharmaceutically because it can be converted to the animal steroid hormone, progesterone. Certain nonprotein amino acids may be present in considerable quantities in some seeds; e.g., canavanine, which is toxic

Table 1.9 Examples of pharmacologically active compounds in seeds and their uses

Chemical type	Compound	Seed source	Pharmacological use
Purine alkaloid	Caffeine	Coffee, cola nut, cocoa	CNS[a] stimulation
Tropane alkaloid	Hyoscyamine	Thornapple	Relaxant, nausea reduction
Indole alkaloid	Phytostigmine	Calabar bean	Cholinesterase inhibitor
Other alkaloid	Colchicine	Autumn crocus	Anti-mitotic, anti-inflammatory
Amino acids/amine	L-DOPA[b]	*Mucuna* spp., faba bean	Parkinson's disease treatment
Cardiac glycoside	Digoxin	*Digitalis* spp.[c]	Strengthening heartbeat
Saponin glycoside	Aesculin	Horse chestnut	Reducing varicose vein inflammation

Based on an article by P.J. Houghton on Pharmaceuticals and pharmacologically active compounds in *The Encyclopedia of Seeds. Science, Technology and Uses*, Black et al. (2006)
[a]Central nervous system
[b]L-Dihydroxy-L-phenylalanine
[c]Mostly extracted from leaves, but present in the seed

to some herbivores, accounts for 8.3% of the dry weight of seeds of the legume *Dioclea megacarpa*, hydroxytryptophan accounts for 14% of the dry weight of *Griffonia*, and dihydroxyphenylalanine (L-DOPA) makes up 6% of dry *Mucuna* seeds. These amino acids are probably mobilized by the seed as a nitrogen source after germination. Glucosides are bitter-tasting components of some seeds, e.g., amygdalin from almonds, peaches, and plums and aesculin from horse chestnut; some, such as saponin from tung seeds may be deadly to humans and animals. Amygdalin extracted from apricots gained some notoriety when it was touted as Laetrile (or erroneously as "vitamin B17") to be a cure for cancer. This has now been soundly refuted as an example of medical "quackery"; alarmingly, it can cause fatal cyanide poisoning.

Phenolic compounds such as coumarin and chlorogenic acid, and their derivatives, and ferulic, caffeic, and sinapic acids occur in the coats of many seeds. These may inhibit germination of the seed that contains them or, being leached out into the soil, may inhibit neighboring seeds (a form of allelopathy). Another germination inhibitor present in many seeds is abscisic acid (ABA), and germination promoters and growth substances such as gibberellins (GAs), cytokinins (CKs), and auxins also occur.

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Chapter 2
Development and Maturation

Abstract Fertilization, the fusion of pollen and egg nuclei, produces a diploid zygote, which differentiates into the embryo, the vital next generation of the plant. Embryo development, or embryogenesis, is accompanied by the production of storage tissues, such as the endosperm or megagametophyte, and the maternal integument layers, which in the mature seed become the testa (seed coat). Seed development requires close interactions among these three basic seed structures in terms of the regulation of gene expression and nutrient supply, which also determines seed size and quality. Hormones play important roles in seed development and maturation. The fates of embryonic cells and tissue domains depend on the distribution of auxin during their early embryogenesis; the deposition of stored reserves and prevention of precocious germination is controlled by abscisic acid during late stages of seed development. The accumulation of specific proteins and oligosaccharides during maturation is associated with the acquisition of desiccation tolerance, which allows seeds to survive maturation drying, a spontaneous decline in water content at the end of the developmental program.

Keywords Fertilization • Embryogenesis • Endosperm development • Testa development • Hormones • Germinability • Desiccation tolerance

2.1 Fertilization

Male sexual gametes in the angiosperms are produced in the anthers as pollen, and the female gametes in the embryo sac of the ovule as egg cells. Pollen grains (microspores) contain a tube nucleus and a generative nucleus, each with a haploid chromosome complement. Pollen that lands on the stigma as a result of pollination germinates to form a pollen tube that grows through the style towards the ovule; in some species the generative nucleus has already divided to form two sperm nuclei, while in others this occurs as the pollen tube is progressing through the style. After the pollen tube finds its way into the ovary through the micropyle of the ovule,

J.D. Bewley et al., *Seeds: Physiology of Development, Germination and Dormancy, 3rd Edition*, DOI 10.1007/978-1-4614-4693-4_2,

the sperm nuclei are ultimately released into the embryo sac. This is developed from the megaspore mother cells in the ovule through meiosis and division to result in eight haploid nuclei: one in the egg cell and one in each of the two synergid cells adjacent to the egg cell at the micropylar end of the embryo sac, two polar nuclei near the center, and one nucleus in each of the antipodal cells at the chalazal end of the embryo sac opposite the egg cell. The vital ones for subsequent seed formation are the haploid egg cell and the two haploid polar nuclei that may or may not fuse to form a single diploid secondary central nucleus. To effect fertilization, one sperm cell nucleus fuses with the egg cell nucleus to form the diploid zygote, which develops into the embryo, while the nucleus from the other sperm cell fuses with the two polar nuclei (or the two fused central diploid nucleus) to form a triploid nucleus, from which the endosperm develops.

Thus a key feature of fertilization in angiosperms is the participation of two male nuclei to effect double fertilization. This double fusion is almost unique to angiosperms but it does occur in the gymnosperms *Ephedra* and *Gnetum* (family Gnetales); however, fusion is with other haploid nuclei so there is no triploid product. Predominantly in the gymnosperms only one pollen sperm cell is released from the microspore, but several egg cells are present within the female gametophyte, each of which may be fertilized (Sect. 2.2.1). Fertilization occurs after transfer of pollen from the male to the female cone, where it produces a short pollen tube that grows through the micropyle into the megagametophyte, releasing the sperm nucleus for fusion with the egg.

Angiosperms have developed a wide array of morphological and physiological mechanisms to enhance or restrict pollination (i.e., transfer of pollen from anthers to stigma) and fertilization. Morphological modifications, such as flower shapes and colors, have often coevolved with pollinators, including insects, birds, and animals (e.g., bats). Physiological mechanisms such as self-incompatibility are present in many species to prevent selfing and promote outcrossing. During domestication, many crops have evolved toward self-compatibility. In a number of crops, reproductive mechanisms have been modified and utilized to enable controlled cross-pollination of plants to produce seeds from two parental inbred (homozygous) lines. The plants resulting from these intraspecific hybrid (F1) seeds exhibit hybrid vigor (heterosis). Features of such plants may include improved productivity, better seed quality, superior growth characteristics, and resistance to pests.

2.2 Embryogeny and Storage Tissue Formation

Before considering the specific cellular and molecular events intimately involved in seed development, it is necessary to review briefly the morphological and anatomical aspects of embryo and storage tissue formation. The variations in patterns of development are numerous, so only a generalized picture is presented here of what occurs in a conifer (gymnosperm), the model dicot Arabidopsis and a cereal monocot (angiosperms).

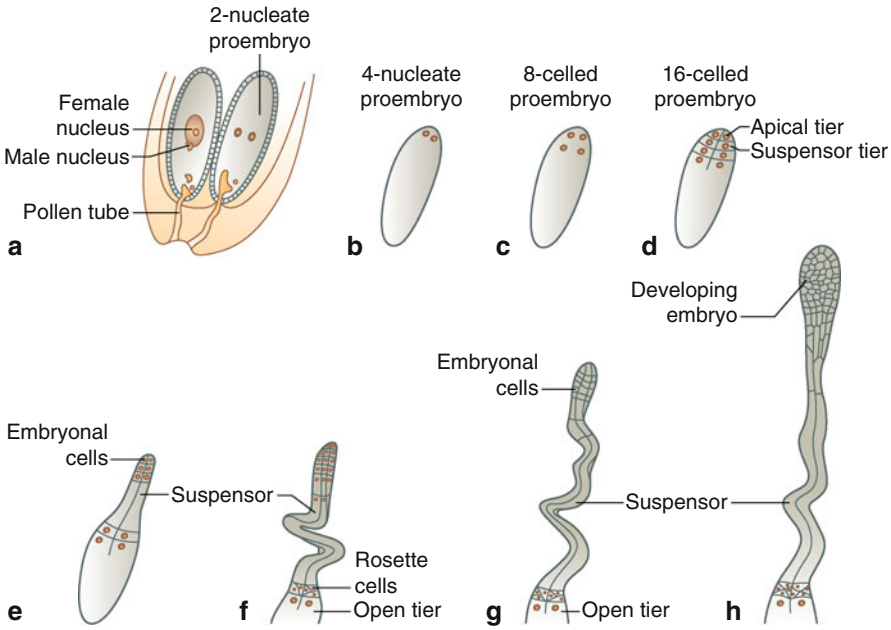


Fig. 2.1 Fertilization and embryo development in the gymnosperm *Picea*. (**a**) Fertilization of the two female nuclei, each in a separate archegonium at the base of each scale of a cone. *Left*, fertilization; *Right*, after first mitotic division. (**b**–**d**) Each nucleus then undergoes mitotic divisions to form free nuclei, followed by cell wall formation, resulting in 16-celled proembryos; only the front set of nuclei and cells is visible (development of one of the fertilized eggs is shown; the other will abort or undergo incomplete development and degenerate). (**e**–**f**) Three-tiered mature terminal embryo with subterminal suspensor cells which elongate to form the primary suspensor. (**g**–**h**) Embryonal mass divides to form the mature embryo with one to several cotyledons, which varies between *Picea* species from 4 to 15. Based on Misra (1994) and a *Canadian Forestry Service Information Report, Petawawa National Forestry Institute* entitled Forest Tree Seed Production—a Review of the Literature and Recommendations for Future Research, by Owens, J.N. and Blake, M.D. (1985) with permission of Cambridge Univ. Press

2.2.1 Embryonic Tissues

In conifers, the egg nucleus lies within the female gametophyte and is fertilized by a gamete released from the pollen tube (Fig. 2.1a). The resultant zygote then divides to produce several free nuclei, around which cell walls are laid down to form the proembryo (Fig. 2.1b–d). Subsequent cell divisions give rise to the embryonal and suspensor cells (Fig. 2.1e). These develop to form a single embryo and an elongated suspensor (Fig. 2.1f–h). Characteristic of conifers is the initial production of several embryos (polyembryony). Simple polyembryony occurs when more than one egg is fertilized in the ovule, as in spruce species (Fig. 2.1) and Douglas fir; thus each embryo is genetically different, resulting from the fusion of separate eggs and pollen grains. Cleavage polyembryony is the production of several genetically identical

embryos from a single fertilized egg, as in pine, fir, and hemlock spp. Usually, however, only one embryo develops to maturity and the others degenerate by programmed cell death (PCD) (Sect. 5.5.4). The mature embryo is characterized by the presence of one to several cotyledons depending upon the species (e.g., as many as 12 in the pine family) (Fig. 1.1), by which time the suspensors have been resorbed. Within the seed the central portion of the megagametophyte (the haploid female gametophyte) breaks down to form a cavity into which the embryo expands. Storage reserves—oil, starch, and protein—are deposited within the persistent parts of the megagametophyte (Fig. 1.1), to be used after germination.

Unlike in the gymnosperms, there is no free nuclear stage during early embryogenesis in the angiosperms; the first division of the fertilized egg cell results in an axial (distal or apical) cell and a basal cell. The pattern of development of the embryo of the dicot Arabidopsis is shown in Fig. 2.2, and of a gramineous monocot, rice, in Fig. 2.3 (see legends for details).

Although it is only a transient structure, the suspensor is formed from the basal cell in dicots following the first mitotic division of the zygote; the basal cell may also contribute some cells to the developing embryo (Fig. 2.2). The number of cells composing the suspensor varies greatly between species, from just a few in Arabidopsis, to at least 200 in scarlet runner bean. During seed development the suspensor transports nutrients and growth-regulating hormones to the growing embryo, serving as the connection between surrounding maternal and endosperm tissue. Eventually, as the seed matures, it undergoes PCD.

In monocots, the basal cell does not divide but forms the terminal (haustorial) cell of the suspensor; the embryo and the other few suspensor cells are produced from the axial cell. By definition, the embryos of the mature seeds of the dicots possess two cotyledons, whereas there is only one in monocots. The single cotyledon of the Gramineae is modified into the absorptive scutellum which lies between the endosperm and the embryo axes (Fig. 1.1). Mature gramineous embryos also include a specialized thin tissue that covers the radicle (coleorhiza) and one that is around the plumule and covers the first foliage leaf (coleoptile) (Fig. 1.1).

2.2.2 Endosperm

The true endosperm, the cells of which are triploid, is found only in the angiosperms; it is derived from the primary endosperm cell that contains the triple fusion nucleus. Two main types of endosperm development have been noted: (1) nuclear or noncellular (also called syncytial or coenocytic), where several divisions of the (free) nuclei occur prior to cell wall formation (e.g., apple, wheat, squash); and (2) cellular, where there is no free-nuclear phase, cell walls being formed after the first mitotic division (e.g., *Magnolia*, *Lobelia*). Both these types occur in monocots and dicots. A much less common form of development, found only in some monocots, is the helobial type, where there can be both free-nuclear and cellularized regions. Even rarer is the type of endosperm present in the coconut seed, with a dense cellularized peripheral region and a liquid suspension of free spherical cells.

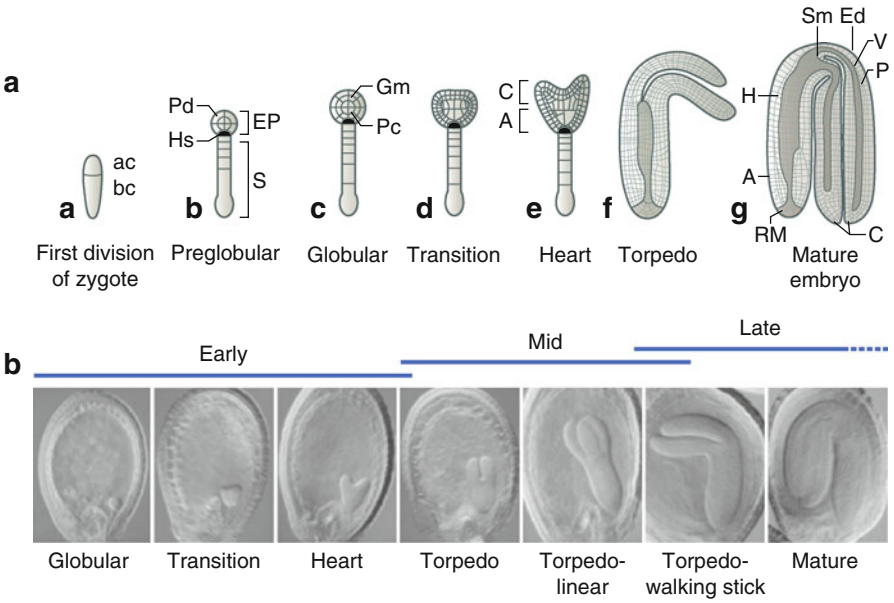


Fig. 2.2 (**a**) Stages of development of the embryo of the dicot Arabidopsis. (a) After fertilization of the egg cell to form the diploid zygote, there is an uneven mitotic division to form an apical cell (ac) and a basal cell (bc). (Larger scale than b–g). (b) Further divisions lead to the formation at the preglobular stage of the suspensor (S) from the basal cell and the proembryo (embryo proper, EP) from the apical cell. (c–g) The protoderm (Pd) develops into the epidermal layer (Ed) of the mature embryo and the hypophysis (Hs) into the root meristem (RM). The shoot meristem (Sm) differentiates from the apical-central region of the embryo. The ground meristem (Gm) of the globular stage develops into the storage parenchyma cells (P) of the cotyledons (C). The procambium (Pc) forms the vascular conducting tissue (V) within the mature embryo. The axis (A) tissues in the mature seed are the root axis (radicle), shoot axis (plumule) and hypocotyl (H). The embryo at maturity is surrounded by a one-cell-thick endosperm and a thin seed coat. From the chapter on Reproductive Development by Bewley, J.D., Hempel, F.D., McCormick S., and Zambryski. P. 2000. in: *Biochemistry and Molecular Biology of Plants*. Buchanan, B.B., Gruissem W., and Jones R.L. (eds.) pp. 988–1043. Copyright American Society of Plant Biologists. (**b**) Differential contrast interference (Nomarski) micrographs of embryogenesis in situ in developing Arabidopsis seeds, illustrating the early-, mid-, and late (overlapping) stages of development. Micrograph courtesy of J.R. Hewitt and J.L. Coppersmith. Dept. Horticulture, Oregon State Univ. Reproduced by permission of Taylor and Francis Group, LLC, a division of Informa plc

Nutrients are drawn from adjacent tissues into the endosperm during its development, and new products are also laid down therein. Thus, the growing embryo becomes enveloped in, or intimately associated with an available food source upon which it can draw during its maturation and subsequent germination/growth stages. In grasses, e.g., cereals, there is limited utilization of the endosperm during maturation, but the part that is depleted forms the intermediate layer that lies between the starchy endosperm and the scutellum of the mature embryo (Fig. 1.1). Barley is used as the example here of endosperm development in cereals (Fig. 2.4).

Endoreduplication (Sect. 4.6.2) is a ubiquitous event during cereal endosperm development as the cells cease mitosis. The reason for this is only speculated upon,

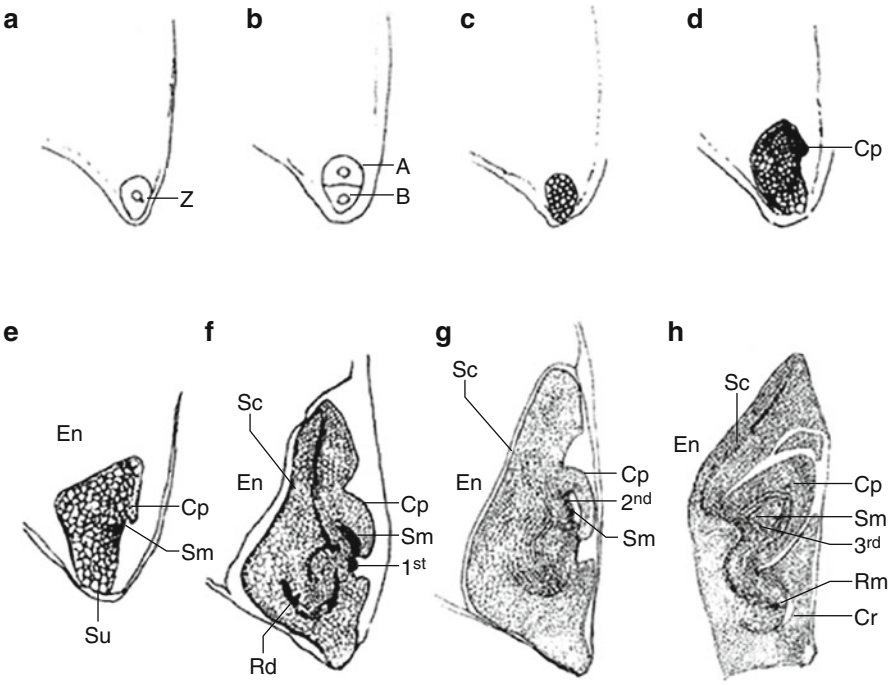


Fig. 2.3 Embryo development in the monocot cereal rice. (**a**, **b**) The first mitotic division of the zygote (z) produces an apical (A) and a basal (B) cell, both of which undergo divisions to form (**c**) a multicellular pre-embryonic mass. After further divisions (**d**, **e**) there is differentiation into a short suspensor (Su), shoot meristem (Sm), and coleoptile (Cp). As further development occurs, through (**f**) the first-leaf (1st) stage and (**g**) second-leaf stage (2nd), distinct regions become evident which are present in (**h**) the mature embryo: scutellum (Sc), radicle (Rd), root meristem (Rm), coleorhiza (Cr), and third leaf (3rd). The location of the endosperm is indicated by En. From an article entitled Development of Embryos—Cereals by Olsen, O-A in *The Encyclopedia of Seeds. Science, Technology and Uses,* Black et al. (2006). See Chap. 1 Advanced Literature references. With permission of CAB International

but it could provide more gene copies to support transcription during storage reserve synthesis, drive cell expansion without cell division, or enhance the pool of nucleotides available to the growing embryo following germination. At the completion of development and reserve deposition, cells of the starchy endosperm of cereal grains are nonliving as a consequence of controlled PCD to ensure their demise.

In non-endospermic dicot seeds, the endosperm is occluded by the developing embryo; their storage reserves are depleted and then reorganized in the cotyledons, which now become the major storage tissue and source for the growing seedling. Endosperm development has been followed extensively in Arabidopsis, the early stages of which are shown in Fig. 2.5.

The endosperm is retained as a permanent storage tissue in endospermic dicot seeds (e.g., castor bean, Fig. 1.1); in endospermic legumes (e.g., fenugreek, Fig. 1.1) this storage tissue may be surrounded by an aleurone layer. The general developmental pattern is similar to that in non-endospermic dicots, except there is

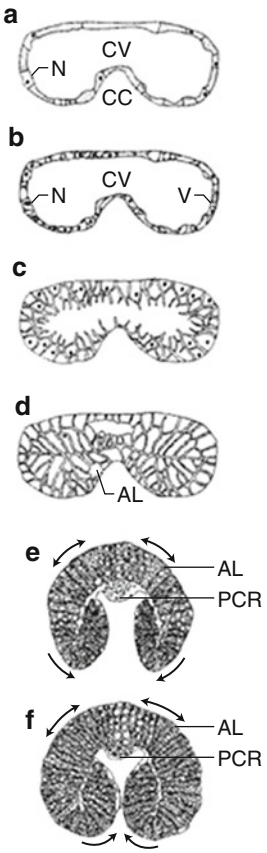


Fig. 2.4 Endosperm development in the monocot cereal barley. Development in the mid-region of the embryo sac results in (**a**) nuclear (N) divisions without cell wall formation (the nuclear endosperm) in the cytoplasm surrounding a central vacuole (CV). The central crease (CC) is evident. (**b**) Nuclei undergo further mitotic divisions and vacuoles (V) appear in the cytoplasm. (**c**–**f**) Endosperm cell walls are laid down in the periphery and cellularization continues into the CV until a large number of cells are formed (about 100,000). First cells of the aleurone layer (AL) form over the CC and spread laterally to join other localized regions of formation. (**f**) The endosperm expands over the crease as a result of cell enlargement as storage reserves (starch and protein) are deposited within them. The supply of nutrients to the grain through the placento-chalazal region (PCR) becomes occluded by the expansion to fill the crease area, and reserve synthesis is completed. Based on Olsen et al. (1992). Source as for Fig. 2.2a. Copyright American Society of Plant Biologists

only limited dissolution of the endosperm as the embryo enlarges. In some cases, as in the Umbelliferae (e.g., carrots or celery), the majority of the seed is comprised of endosperm and the embryo is at a rudimentary stage of development in the mature seed (Fig. 6.2b).

More rarely in dicots, the nucellus (maternal ovular tissues) remains to become the major nutritive tissue (e.g., perisperm) and in the gymnosperms the persistent megagametophyte fulfills this role.

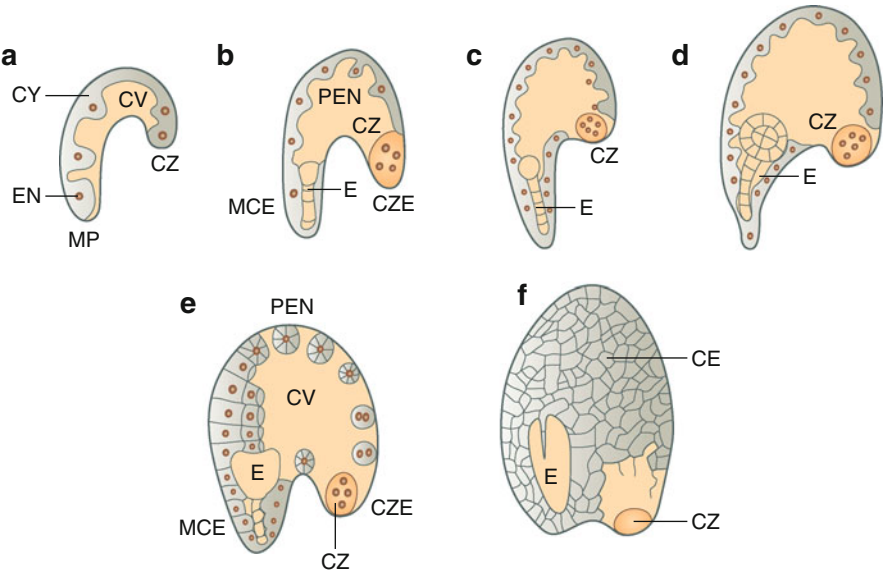


Fig. 2.5 Endosperm development in Arabidopsis. The triploid central cell undergoes (**a**) nuclear divisions without cellularization to form a multinucleate cell (coenocyte) in which the endosperm nuclei (EN) migrate from the micropylar (MP) to the chalazal region (CZ), eventually lying in cytoplasm (CY) around the periphery of a large central vacuole (CV). (**b**–**d**) The embryo (E) develops to the globular stage and is surrounded by dense nucleated cytoplasm at the micropylar end of the cell (MCE). A central or peripheral endosperm region (PEN) and chalazal endosperm region (CZE) can also be distinguished. (**e**–**f**) As the embryo assumes the globular/heart stage of development there is extensive cellularization in all but the chalazal region, spreading from the micropylar to the peripheral regions. Later, the embryo grows into the cellular endosperm (CE) as it degenerates due to programmed cell death, and at seed maturity only a single-cell layer of endosperm (sometimes called the aleurone layer) remains to completely surround the embryo. Modified from Olsen (2004). Copyright American Society of Plant Biologists

2.2.3 Testa (Seed Coat)

Development of the seed coat commences after fertilization from the inner and outer layers of the integument, lying to the outside of the ovule; therefore it is of maternal origin. Its component tissues are initially undifferentiated, but rapidly undergo programmed changes to produce a structure that is extremely variable among species (Sect. 1.2.3), or even in the same species under different environmental conditions. Thus only a brief overview of the type of coat development that occurs in the Brassicaceae will be presented. In species of this family, four layers are present in the coat, three derived from the outer integument and one from the inner. From the outer integument is formed an epidermal layer that is frequently one-cell thick and may (Arabidopsis) or may not (Chinese cabbage) produce mucilage upon imbibition of the dry seed; below this is a subepidermal layer of one to several layers of parenchyma cells (Fig. 2.6), although in some species they may be of thicker-walled collenchyma cells (white mustard) or be absent (Arabidopsis). The innermost layer

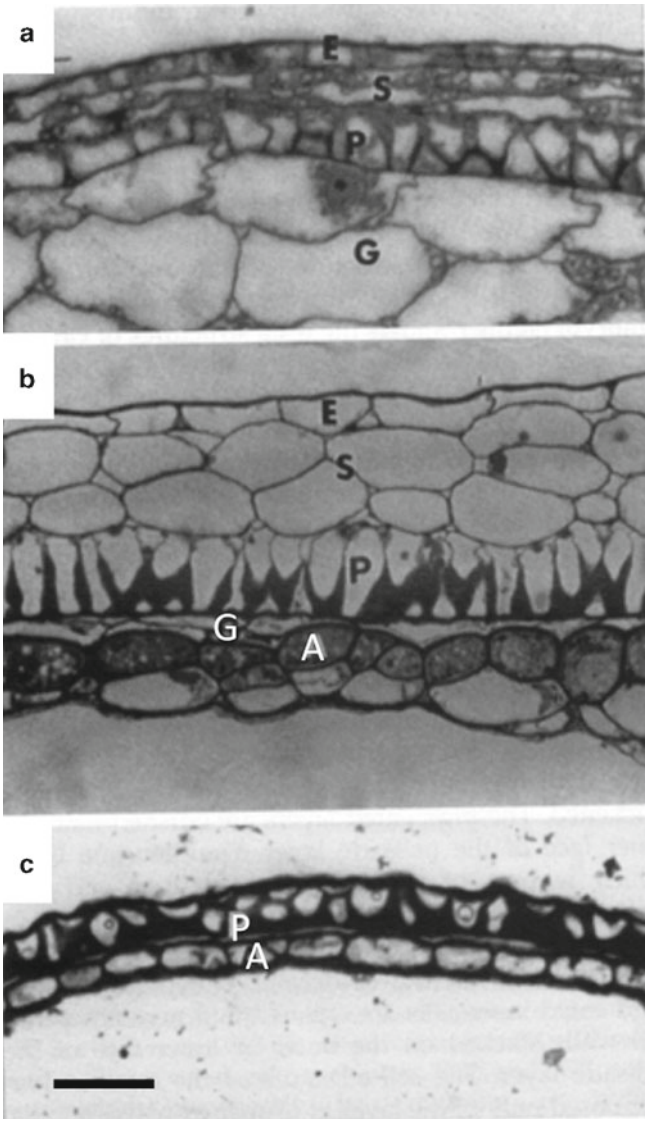


Fig. 2.6 Light micrographs showing testa structure during development of Chinese cabbage seed. (**a**) Early cotyledon (heart) stage of embryogenesis. (**b**) Early storage deposition stage in cotyledons. (**c**) Mature seed. The outer two layers of thin-walled cells are no longer present, and the palisade layer cell walls are thickened. Outer-integument-derived layers are E, epidermis; S, subepidermis; P, palisade layer. Inner-integument-derived layers are G, pigmented layer; A, single aleurone layer of the mature endosperm. Bar: 40 μm. From Ren and Bewley (1998). Courtesy of Cambridge Univ. Press

of the outer integument is of thick-walled cells, the palisade layer, which may be pigmented. A fourth layer, derived from the inner integument, is of parenchyma cells that are compressed at maturity; it is termed the endothelium or pigment layer

because the colored pigments most often accumulate there. In the mature seed, the outer layer of the residual endosperm is closely associated with the inner integument and may be called the aleurone layer (Fig. 2.6).

Several categories of seed have been distinguished depending on which layer of the inner or outer integument becomes the mechanical layer in the coat, with strengthened, usually lignified cell walls. In the Brassicaceae, the mechanical palisade layer is derived from the innermost layer of the outer integument (Fig. 2.6), and the seed is in the *endotestal* category. If the mechanical layer is derived from the outer layer of the outer integument (e.g., legume family, Fabaceae) the seed is *exotestal*, and from the middle layer (e.g., Rosaceae) it is *mesotestal*. If all or part of the outer integument develops into a fleshy tissue the seed is *sarcotestal*. If the mechanical layer is derived from the inner integument, also initially distinguishable as three layers, then the seed is *exotegmic*, or derived from the outer layer (e.g., cotton and other Malvaceae). *Mesotegmic* seeds with coats derived from the middle layer are rare, while *endotegmic* seeds with coats derived from the innermost layer of the inner integument are present in, for example, the black pepper family (Piperaceae). A relatively small number of species, including those in the Cucurbitaceae, have mechanical layers in their seed coats that are derived from more than one of these layers.

Many species at maturity have no, or only a residual seed coat; in these the covering structure is a fruit coat (pericarp) (Table 1.1) derived from the maternal ovary wall.

2.3 Regulation of Seed Development

The morphology of seeds varies among different divisions, classes, or families of seed plants (Sect. 2.2). Even within the same genus, there are prominent differences among species that are associated with plant habitat and evolutionary history. However, there is also a commonality in the basic structure of seeds and the mechanisms governing their development, with variations that aid in our understanding of the divergences in morphology.

2.3.1 Plant Hormones

Plant hormones (phytohormones) are chemical compounds that are involved in the regulation of growth and development, hence the alternative term for them of plant growth regulators. They are signal molecules that are produced within the plant and are active at very low concentrations (10^{-7}–10^{-5} M). Hormones regulate cellular and developmental processes in targeted cells, which may or may not be the cells in which they are synthesized. Plant hormones play a pivotal role in the many signal transduction chains in plants, and are particularly important in mediating the

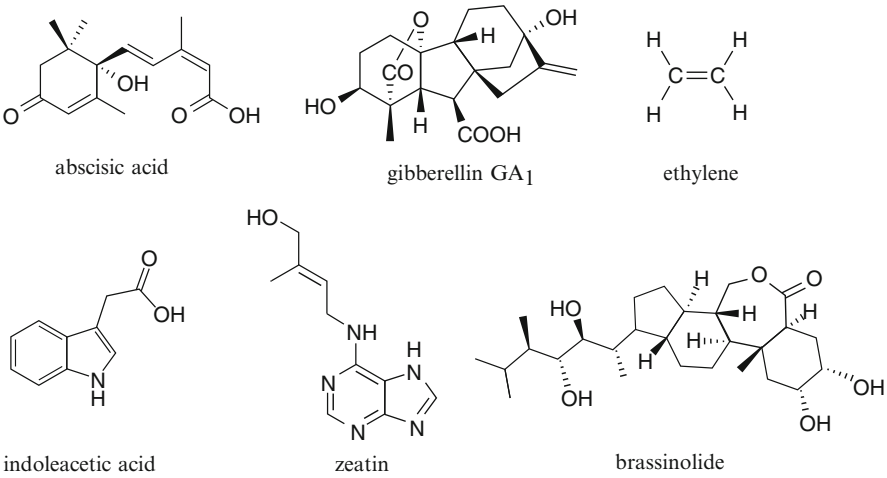


Fig. 2.7 Molecular structures of representative members of each of the six major classes of plant hormones

translation of environmental signals to internal ones (Chap. 6). A short overview of plant hormones, all of which have been reported in seeds, and their functions, is presented initially before describing seed developmental events in which they play important roles.

There are six major classes of plant hormones, as noted below, based upon their structural similarities as well as on their physiological effects. Some other plant hormones and growth regulators are not easily grouped into these classes. They may exist naturally or are synthesized by humans or other organisms. The six classes are: abscisic acid, gibberellins, auxins, cytokinins, ethylene, and the brassinosteroids (Fig. 2.7). Their roles in seed development, germination or early seedling establishment include the following.

Abscisic acid (ABA) is an isoprenoid compound formed by the cleavage of carotenoid precursors. It was named on the basis of early work on its influence on leaf abscission, although now it is more closely associated with responses to environmental stresses. Its involvement in seed development includes the following:

- Promotion of synthesis of some seed storage proteins (Sect. 3.2.4.3);
- Acquisition of desiccation tolerance (Sect. 2.5.2.2);
- Induction and maintenance of dormancy (Sects. 2.3.3, 2.4.2).

Gibberellins (GA) are a large group classified on the basis of both structure and function. All gibberellins are derived from the *ent*-gibberellane skeleton. They are named GA_1....GA_n in order of discovery. Gibberellic acid (GA_3) was the first gibberellin to be structurally characterized. There are nearly 150 GAs identified from plants, fungi, and bacteria. The gibberellins GA_1 and GA_4 are active in seeds, where they occur naturally; commercially available GA_3 is most commonly used in

experiments, although this may contain contaminating GA_1. GA activities include the following:

- Stimulation of stem elongation by promoting cell division and elongation;
- Breaking of seed dormancy to induce germination (Sect. 6.6.1.2);
- Stimulation of α-amylase production for mobilization of starch reserves (Sect. 5.5.1);
- Parthenocarpic (seedless) fruit development.

Auxins are generally considered as compounds characterized by their ability to induce cell elongation and otherwise resemble indoleacetic acid (IAA, the first auxin isolated) in physiological activity:

- Stimulating cell elongation
- Promoting root initiation and lateral root development
- Mediation of tropic responses such as bending in response to gravity and light
- Regulation of pattern formation in embryogenesis (Sect. 2.3.2).

Cytokinins (CK) resemble adenine and promote cell division, but also have other functions. The most common naturally occurring cytokinin in plants is zeatin, which was isolated from corn (*Zea mays*).

Ethylene (ETH). Unlike all other plant hormones, ethylene is a gaseous compound. Like ABA, it is the only member of its class. Of all the known plant growth substances, ethylene has the simplest structure. It may stimulate the breaking of seed dormancy (Sect. 6.6.1.3).

Brassinosteroids (BR) are polyhydroxysteroids that are present in small amounts, but play a number of roles in plant development. These include the stimulation of cell elongation and division, and gravitropism, as well as promoting resistance to stress.

Other hormones/growth regulators:

- Salicylic acid may activate genes that aid in the defense against biotic stress.
- Jasmonates are derived from fatty acids and are involved in the production of defense proteins. They may also have a role in seed germination and the control of protein storage in seeds.
- Strigolactones are associated with the inhibition of shoot branching and the induction of germination of parasitic weeds (Sect. 7.2.6).
- Karrikins are a group of plant growth regulators discovered in smoke and extracts of burned plant material that stimulate the germination of seeds at extremely low concentrations (Sect. 6.6.7.3).

2.3.2 Embryo Polarity and Patterning

The fundamental components of the embryo, such as the cotyledons, hypocotyl, and radicle, are highly conserved even between gymnosperms and angiosperms, although detailed structures (e.g., cotyledon number, embryo position) differ

Table 2.1 Embryogenesis mutants of Arabidopsis, their resultant phenotype, and the target of the genetic defect

Mutant name	Symbol	Phenotype	Gene function
dicer-like1	*dcl1*	Embryo lethal or arrest	miRNA processing, also called *emb76*, *sus1*, *caf,* or *sin1*
hyponastic leaf1	*hyl1*		miRNA processing
serrate	*se*		miRNA processing
hua enhancer1	*hen1*		miRNA methylation
argonaute	*ago*		miRNA loading
gurke	*gk*	No shoot	Acetyl-CoA carboxylase
fackel	*fk*	No hypocotyl	Sterol C-14 reductase
monopteros	*mp*	No hypocotyl and root	Auxin signaling, also called *arf5*
gnom	*gn*	No cotyledons and root	ARF guanine-nucleotide exchange factor
shoot meristemless	*stm*	No shoot meristem	KNOTTED class of homeodomain proteins
cup-shaped cotyledon	*cuc*	Cup-shaped cotyledons	NAC transcription factor
pin-formed1	*pin1*	Cotyledon defects	Auxin transporter
pin-formed7	*pin7*	Filamentous embryos with no apical cell	Auxin transporter
leafy cotyledon1	*lec1*	Desiccation intolerant, precocious germination	CCAAT-box transcription factor
leafy cotyledon2	*lec2*		B3 transcription factor
fusca3	*fus3*		B3 transcription factor
aba insensitive3	*abi3*		B3 transcription factor
pickle	*pkl*	Embryonic traits after germination	CHD3 chromatin-remodeling factor

between and within these two groups. Here, details of embryo development, which are applicable to many different species, are discussed using seeds of *Arabidopsis thaliana* as a model. Much of the information concerning the genetic regulation of embryo development has been gained from the study of mutants; examples of these and their resultant phenotypes, some of which are discussed below, are summarized in Table 2.1.

Embryogenesis can be divided into early-, mid-, and late stages, although these are overlapping (Fig. 2.2b). Histodifferentiation during early embryogenesis is regulated by the precise distribution of the plant hormone auxin between the embryo and suspensor, and this is essential for the development of embryo polarity and patterning. During the early globular stages (2–16 cells), auxin is concentrated in the embryo and is transported from the suspensor by PIN-FORMED7 (PIN7), one of several PIN transporter proteins mediating efflux of this hormone from cells. As the globular stage progresses, the highest concentrations of auxin (auxin maxima) become highly localized, and this determines the apical-basal polarity of the embryo. There is transport of auxin within the embryo towards its basal region by PIN1, and then by PIN4 to the hypophysis (Fig. 2.2a, b), the uppermost cell of the suspensor. This, together with the auxin transported from the basal suspensor cells by PIN7, results in an auxin maximum around the hypophysis from which a root meristem differentiates. Formation of the hypophysis and embryonic root depends on

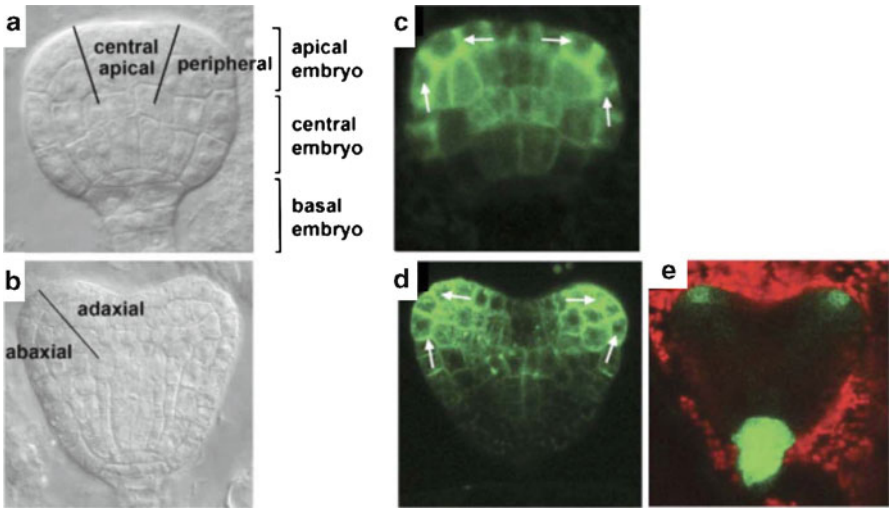


Fig. 2.8 Auxin transport and the initiation of cotyledons in the Arabidopsis embryo. (**a**) By the transition stage, the central apical domain and the peripheral domains are already determined, from which the shoot apical meristem and cotyledons will originate, respectively. (**b**) The adaxial and abaxial sides of the future cotyledons are determined around the early heart stage. (**c**, **d**) Specification of tissue domains is induced by a directional auxin flux (*arrows*) due to localized distribution of PIN auxin transporters (*green signals*) which creates auxin maxima (green signals in **e**). Green signals in (**c**, **d**) show the location of PIN proteins, while in (**e**) they represent the activation of a *DR5* auxin-responsive promoter in the root meristem and cotyledon tips. Modified from Chandler (2008). With permission of Oxford Univ. Press

MONOPTEROS (MP)/AUXIN RESPONSE FACTOR5 (ARF5), a transcription factor. The gene for this is expressed in cells adjacent to the hypophysis and induces transcription of *TARGET OF MP5* (*TMO5*) and *TMO7,* genes encoding cofactors that also play a role in specifying the formation of this structure. The TMO7 protein moves from the adjacent cells to the hypophysis precursor, and in mutants that lack this mobile signal, abnormal seeds are produced, including those containing rootless embryos. Thus, cell nonautonomous (meaning "controlled by outside factors," i.e., involving a mobile factor) signaling is critical for embryonic root formation.

Auxin distribution is also important for cotyledon patterning. After the establishment of apical-basal polarity and the root meristem in the globular-stage embryo, the central and peripheral domains of the apical part of the embryo are then specified during the transition stage (Fig. 2.8a); these will later develop into the shoot apical meristem (Sm, Fig. 2.2a, g) and cotyledons, respectively. When the embryo reaches the early-heart stage, the adaxial (closest to the axis) and abaxial (farthest from the axis) sides of the cotyledons are already determined (Fig. 2.8b). PIN1 plays a central role in the formation of auxin maxima in the peripheral regions on each side of the embryo apex, from which the cotyledons differentiate (Fig. 2.8c–e). Failure to form auxin maxima in the appropriate locations during embryogenesis results in asymmetric, fused, cup-shaped, triple, or in an extreme case, no cotyledons,

although weak phenotypes of asymmetric or fused cotyledons occur in seedlings of some species.

In addition to its physical importance in pushing the embryo into the endosperm cavity, and in the transfer of nutrients to the embryo, the suspensor is essential for maintenance of the normal embryogenesis program. In mutants defective in an embryonic identity gene (*LEAFY COTYLEDON1* [*LEC1*]), an abnormal pattern of cell division in the suspensor leads to an unusual proliferation of cells and the formation of a secondary embryo. This suggests that suspensor cells have the ability to differentiate into an embryo (totipotency), but perhaps more importantly, that the embryonic program is strictly suppressed in the suspensor cells during normal embryogenesis by an inhibitory signal from the embryo itself. Over-expression of a *LEC2* gene in non-embryonic tissues is sufficient to induce formation of a number of embryo-like structures, indicating that LECs are the master regulators of embryo identity.

Multiple transcription factors (regulatory proteins) controlling tissue- and stage-specific expression of their target genes (called "downstream genes") are involved in the maintenance of the embryogenesis program. Some of these are in turn targeted by microRNAs (miRNAs), which are small (~21–24 bases) single-stranded RNAs that downregulate specific target genes by inhibiting their transcription, by cleaving their messenger RNA (mRNA), or by repressing their translation. miRNAs play critical roles in regulating the expression of genes so that this occurs only in certain tissues or at specific developmental stages. Therefore, almost all mutants defective in miRNA biogenesis and processing exhibit embryo abnormalities, including embryo lethality.

2.3.3 ABA Content and Sensitivity to ABA During Development

Following histodifferentiation, seeds may acquire the ability to germinate. This is suppressed until the completion of development by the induction of primary dormancy. The stage in seed development and maturation at which primary dormancy sets in varies from species to species, but in the majority it is around mid-development and is often only completed close to full maturation. *Medicago lupulina* is a typical example, with an increase in germinability during development followed by a decrease due to the induction of primary dormancy (Fig. 2.9).

The onset of primary dormancy frequently coincides with a transient rise in ABA content during seed development. In most of the studied species, ABA content increases during the first half of seed development and declines during the maturation phase (e.g., tomato, Fig. 2.10a). Sometimes two ABA peaks are observed, as in seeds of Arabidopsis (Fig. 2.10b), rapeseed, cotton, and barley, usually one at around mid-development (10 days after flowering, DAF) and one at late maturation (16 DAF). The first peak of ABA that accumulates is due to its synthesis in both zygotic and maternal tissues. The maternal ABA (i.e., located in testa and fruit tissues) may play a role in the inhibition of precocious germination and in early developmental processes, whereas the ABA that is synthesized during late maturation

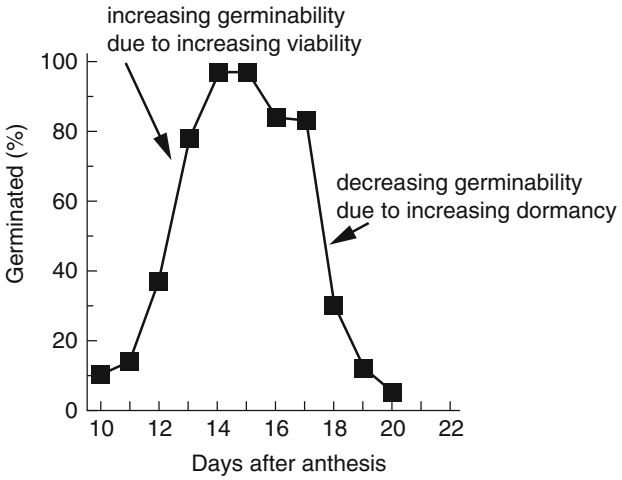


Fig. 2.9 Onset of dormancy in *Medicago lupulina* seeds during their development in the pod. Germinability of seeds was determined at different ages (i.e., days after anthesis—dehiscence of the anther, which is very closely followed by pollination and fertilization). After approximately 15 days, germinability declines owing to the development of dormancy, which is complete in 20-day-old seeds. Adapted from Sidhu and Cavers (1977)

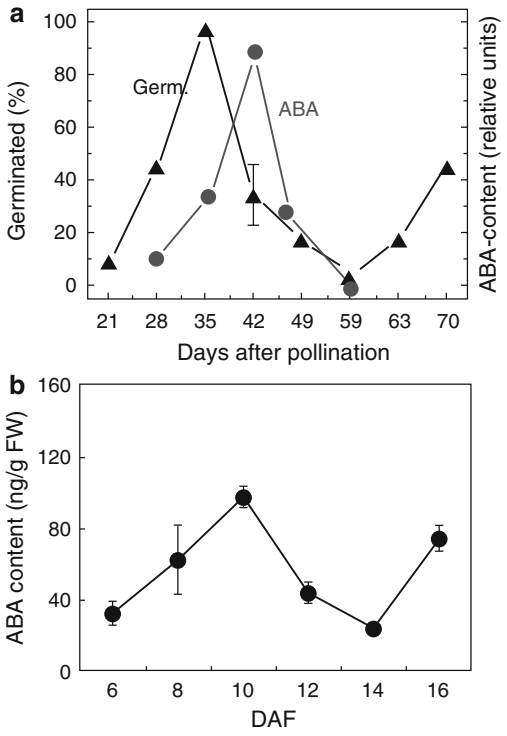


Fig. 2.10 ABA content during seed development. (**a**) ABA content (●) and germinability (▲) of tomato seeds during development. From Hilhorst (1995). With permission of Cambridge Univ. Press. (**b**) ABA content of developing seeds of *Arabidopsis thaliana*, showing two peaks of synthesis. DAF, days after flowering. Based on data in Okamoto et al. (2006). Copyright American Society of Plant Biologists

originates from zygotic tissues and is related to the induction and maintenance of dormancy.

ABA may be present in all seed and fruit tissues and since the rise in its content coincides with many developmental processes other than dormancy, it has been implicated in storage protein synthesis, suppression of precocious germination, induction of desiccation tolerance, and synthesis of late embryogenesis abundant proteins (Sects. 2.4.2, 2.5). Single-gene mutations leading to defects in the synthesis or sensitivity to ABA in Arabidopsis and tomato result in the formation of nondormant seeds. Crosses between wild-type and mutant plants have shown that maternal ABA indeed does not affect dormancy. Thus, a (transient) increase in embryonic ABA content during seed development is required to induce dormancy.

Besides ABA content, sensitivity to ABA may play an equally important role in the expression of dormancy. For example, the ABA-insensitive *abi1*, *abi2,* and *abi3* Arabidopsis mutants display varying reductions in seed dormancy. On the contrary, seeds of the ABA-supersensitive *era1* mutant possess an enhanced dormancy phenotype. Several cultivars of wheat and maize display vivipary, also known as preharvest sprouting (Sect. 2.4.2). In many cases these sprouting-susceptible cultivars have a reduced sensitivity to ABA. Molecular analysis of Arabidopsis and maize mutants exhibiting vivipary has led to the identification of ABA-responsive genes that are the cause of these phenotypic characteristics: *ABA INSENSITIVE3* (*ABI3*) and *VIVIPAROUS1* (*VP1*), respectively. These genes encode transcription factors of the B3 domain family that activate the transcription of ABA-inducible genes that are more generally involved in the control of seed development, in addition to dormancy. They play a role in maintaining the developmental state in seeds and in suppressing an (untimely) transition to the vegetative or growth stage. As a result, developing embryos from mutants lacking these master genes show characteristics of premature germination and growth.

Sensitivity to ABA gradually declines during seed development, as illustrated in developing alfalfa embryos (Fig. 2.11). Interestingly, the peak in ABA content occurs at a developmental stage (VI) when the sensitivity to ABA is reduced to half of its initial value; thus, ABA content alone is not a good indicator of germination ability, for this also depends on the sensitivity of the seed or embryo to this hormone.

2.3.4 Regulation of the Seed Maturation Program

Mutant analyses have revealed a number of genotypes with altered dormancy characteristics but frequently with normal ABA contents throughout seed development, e.g., the *abi3*, *leafy cotyledon (lec1*, *lec2)*, and *fusca (fus3)* mutants of Arabidopsis and the *viviparous* (*vp*) mutants of maize (Sect. 2.4.2). All of these have seed phenotypes that are typical of the vegetative rather than the reproductive state, reduced desiccation tolerance, expression of germination-related genes and absence of dormancy. *LEC1, LEC2, ABI3 (VP1),* and *FUS3* are regulatory genes with apparently overlapping functions in the overall control of seed maturation. LEC1 is a homolog of the HAP3

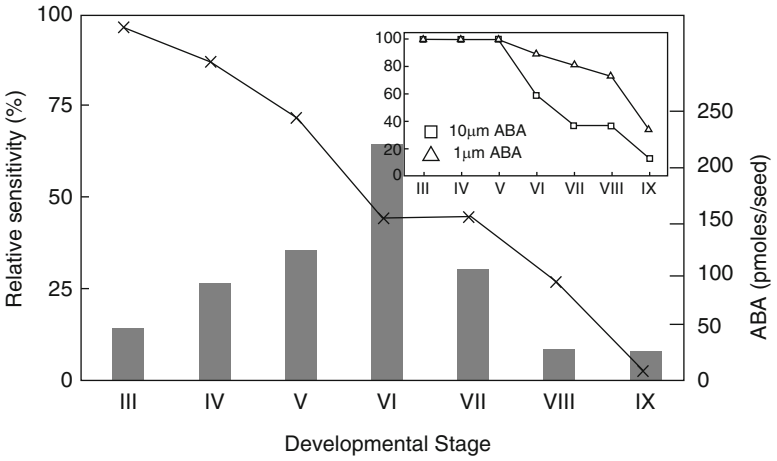


Fig. 2.11 Sensitivity to ABA of developing alfalfa embryos (x), expressed in terms of a germination index. The sensitivities to 0.1 μM of ABA, and to 1.0 and 10 μM (inset) are shown. The bar graphs show the ABA content of intact alfalfa seeds at different stages of seed development. Adapted from Xu and Bewley (1991, 1995)

subunit of the CCAAT-binding transcription factor whereas LEC2, FUS3, and ABI3 (VP1 in maize) all have B3 DNA-binding domains. Because maturation is defective in the *lec1*, *lec2*, *fus3,* and *abi3* mutants, dormancy is not induced and the mutant seeds often germinate precociously (Table 2.1). More detailed analysis of the *fus3, lec1, lec2,* and *abi3* mutants has shown that the occurrence of premature germination differs among them. The *LEC1* and *FUS3* loci likely regulate developmental arrest because mutations in these genes cause a continuation of growth in immature embryos. Since dormancy induction occurs later in seed development than this developmental arrest, ABA-controlled dormancy (via ABA content and ABA-responsive genes) to prevent subsequent germination is additive to the developmental arrest controlled by *LEC1* and *FUS3*. Furthermore, the dependence of germination on GAs is retained in the *fus3* mutant but not in *lec1*. This suggests that the germination potential of seeds of these mutants is affected in different ways.

There is a temporal and spatial pattern to the expression of *LEC1, LEC2, ABI3,* and *FUS3* during Arabidopsis seed development (Fig. 2.12). *LEC1* is expressed earliest in the embryo and suspensor and subsequently in the endosperm. *LEC2* is expressed in a similar pattern, but appears later and its transcripts are not present in the mature seed. *FUS3* is also transcribed early in embryo and endosperm development, but its mRNA remains present throughout maturity, particularly in the remaining single-celled layer of the mature endosperm. *ABI3* transcripts are present at low levels early in development, but remain high in the mature embryo. While the defects resulting from mutations in these genes indicate their importance for normal seed development, there is no direct relationship between *LEC1, FUS3,* and *ABI3* gene expression during seed development, and subsequent ability to germinate of

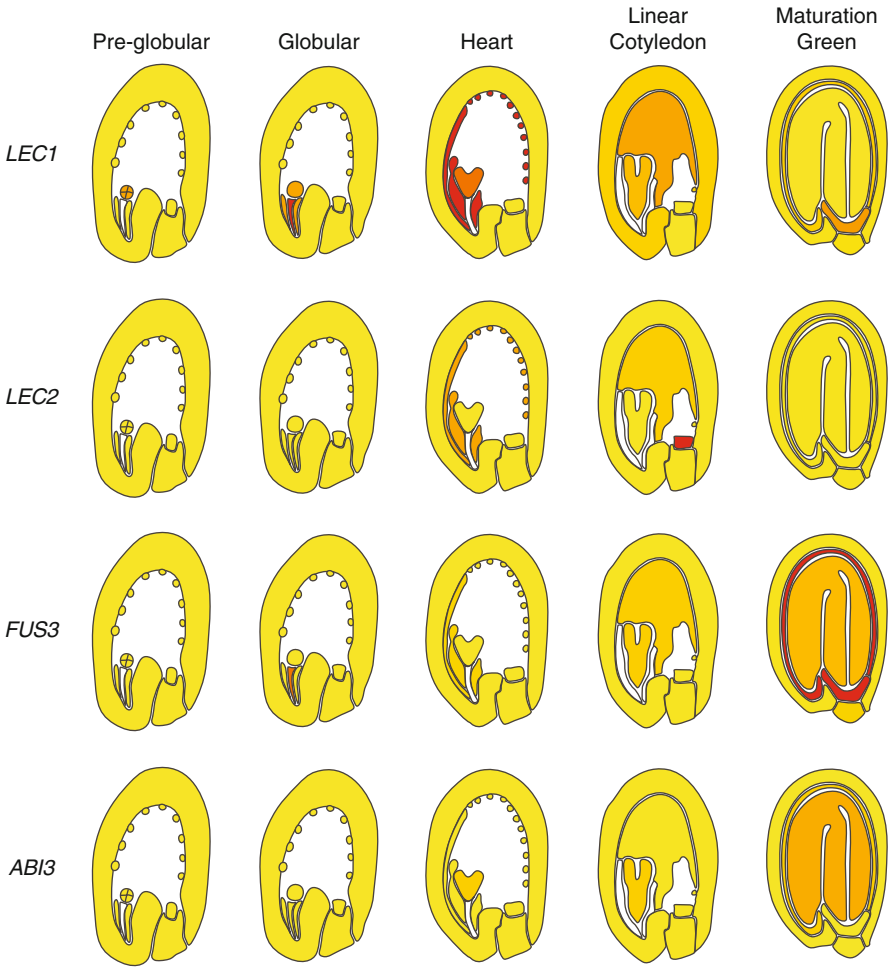


Fig. 2.12 Temporal and spatial expression patterns of *LEC1*, *LEC2*, *FUS3,* and *ABI3* genes encoding transcription factors involved in the regulation of embryogenesis and seed maturation. Different stages of embryogenesis are shown, with the relative intensity of color (*yellow*: low or absent, to *red*: high) indicating mRNA abundance in the indicated tissues. Figures from Arabidopsis eFP Browser (www.bar.utoronto.ca, Winter et al. 2007) based on data from Le et al. (2010)

several accessions of Arabidopsis with variable degrees of dormancy. This indicates that other factors, such as the environment during seed maturation, can also influence the balance between ABA and GA that regulates germination (Sect. 6.5). All of these factors, both internal and external, interact to govern multiple and overlapping processes during seed development and maturation that influence the balance between germination and dormancy (Fig. 2.13).

Correspondingly, developmental arrest and dormancy are considered separate phenomena in tomato seeds. After the completion of embryogenesis both wild-type (cv. Moneymaker) and ABA-deficient *sitiens* (*sit*^{w}) mutant seeds enter a state of

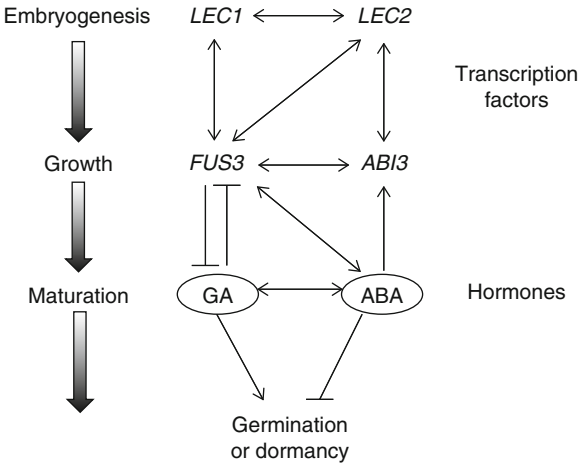


Fig. 2.13 Interactions in the regulatory network involving *LEC1*, *LEC2*, *FUS3,* and *ABI3* genes in the control of seed development and maturation. Positive interactions or activation are indicated by arrows and repression by bars. The transcription factors coordinate development during embryogenesis and seed growth but normally prevent precocious germination during maturation. They, along with the maternal environment during maturation, also influence the balance between GA and ABA which can determine the depth of primary dormancy

developmental arrest. The *sit*^{w} mutant is lacking abscisic aldehyde oxidase (AAO3) that catalyzes the final step in ABA synthesis (Fig. 6.13a). Developmental arrest in the wild type is followed by the induction of dormancy, in parallel with a rise in ABA content, but this does not occur in the *sit*^{w} mutant and developmental arrest is not maintained. This may result in vivipary within overripe fruits of the *sit*^{w} mutant (Sect. 2.4.2). Interestingly, at this stage seeds of both genotypes have almost undetectable amounts of ABA, yet germination is suppressed in the wild type. The osmotic environments of the locular tissues in which the seeds are embedded in the fruits of both genotypes are comparable so, apparently, the mutant seeds possess a greater "growth potential." Also, mature mutant seeds display a stronger resistance to osmotic inhibition (Fig. 2.14). Since there are no appreciable differences in sensitivity to ABA between the mutant and wild-type seeds, these results may be explained by the observed aberrations in the testa of the mutant. This consists of only one cell layer in *sit*^{w} seeds, compared to 4 or 5 in the wild type, and is a pleiotropic effect of the mutation. In this sense the *sit*^{w} mutant bears resemblance to the *transparent testa* (*tt*) mutants of Arabidopsis (Sect. 6.3.2.3). The thinner testa of the *sit*^{w} mutant apparently provides a lesser restraint to expansion of the embryo than that of the wild type.

2.3.5 Epigenetic Control of Endosperm Development

While the embryo is the vital next generation of plant, the major role of the endosperm is to provide nutrition to the embryo during its development and/or

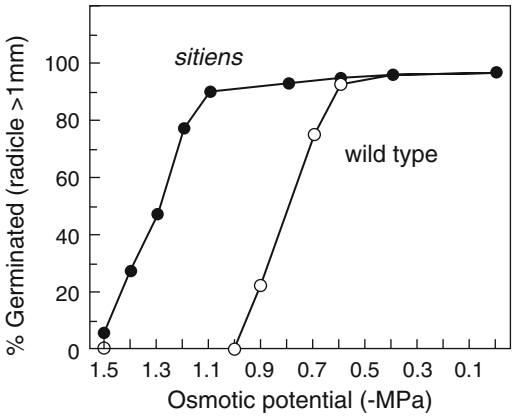


Fig. 2.14 Effect of osmotic potential on germination of wild-type and ABA-deficient *sitiens* mutant seeds of tomato. Dormancy of the wild type was removed by after-ripening. Seeds of wild type (○) and *sitiens* mutant (●) were incubated on osmotica (solutions of polyethylene glycol) of different strengths. Germination (radicle > 1 mm) was recorded after 12 days. Note that radicles of *sitiens* seeds elongate in osmotic potentials as low as −1.2 MPa which is completely inhibitory to those of wild-type seeds. After Groot and Karssen (1992)

following germination until the seedling becomes autotrophic. In legumes, the endosperm is formed during embryogenesis but is consumed during embryo development, with little or none remaining in the mature seed. Cereal grains (caryopses) contain a substantial endosperm at maturity, although with the exception of the aleurone layer it is nonliving (Sects. 1.2.2, 2.2.2), as a consequence of PCD (Sect. 5.5.4). However, even in those seeds from which the endosperm is absent at maturity, the interactions between it and the embryo and testa during embryogenesis play a critical role in the developmental program.

Development of the endosperm is initiated after double fertilization (Sect. 2.1), and continues concomitantly with embryo development (Sect. 2.2.1). However, these two genetically controlled programs—embryo and endosperm development—are separable. There are Arabidopsis mutants (e.g., *fertilization-independent endosperm* [*fie*] and *fertilization-independent seed* [*fis*]) that undergo endosperm formation without fertilization or embryo formation. Nuclear division and cellularization occur spontaneously in the central cell, which clearly contains the complete information to form the endosperm, albeit one that is diploid rather than triploid. Interestingly, the testa also develops normally in these mutants, suggesting that its development is independent of embryogenesis and that endosperm development can stimulate the integuments to develop. In other words, fertilization is a trigger that removes the repression of subsequent developmental programs. The phenomena of repression and derepression are also important in understanding the regulation of seed germination (Sects. 4.6, 6.6.1).

The *fie* mutation causes embryo abortion, which suggests this gene is also important for embryogenesis. When *fie* mutants are crossed with wild type, half of the progeny exhibits embryo abortion, but only when the mutants are used as the female

parent. This sex-specific expression of the mutant phenotype is associated with the silencing of the *FIE* gene, particularly that of paternal origin (from the pollen). This is called the *Parent-of-Origin Effect* or *imprinting*. Plants (and also animals) that contain identical genetic information can express different phenotypes; this variation, due to factors other than changes in DNA sequence, is called epigenetics. Some genes that are expressed only maternally suppress endosperm development, and other genes that are expressed only paternally promote endosperm development. These genes are controlled by silencing through methylation and by activation through demethylation of DNA. Chromatin remodeling by the modification of histones, nuclear proteins associated with DNA in chromosomes, is also important for epigenetic control of seed development.

A well-known example of genetic imprinting is the R-gene control of anthocyanin production in the aleurone layer of developing maize endosperms. When the RR female (red endosperm) is crossed with the rr male (colorless endosperm) all of the kernels are red, but when the reciprocal cross of RR male and rr female is made the kernels are mottled due to irregular anthocyanin distribution. This is not due to a gene dosage effect (e.g., RR:r or rr:R diploid female nuclei to male haploid nucleus during fertilization of the polar nuclei, Sect. 2.1), but is due to the mode of inheritance of the R allele. Kernels are mottled regardless of the number of R alleles that are inherited paternally but always solid red when the R allele is inherited maternally. Alleles of other genes in maize are likewise imprinted when inherited maternally, e.g., for the storage prolamin α-zein.

2.3.6 Testa Development and Its Interaction with the Endosperm and Embryo

Different layers of the integuments, which become the testa of a mature seed, play distinct roles during development. In Arabidopsis seeds, the column-like cells of the outermost layer of the outer integuments (columellae) provide the testa surface texture (Fig. 2.15a, b), which results from the elevation of radial cell walls to increase coat strength. Pectin components such as rhamnogalacturan are synthesized in the *trans*-Golgi network in the epidermal cells in the outer integuments, being transported to the apoplast in vesicles for deposition in discrete pockets in the outer-cell-layer walls; the pectin surrounds the columellae and becomes dry at maturity. This forms mucilage upon imbibition and acts as a natural hydrogel, which influences seed water relations during germination (Sect. 4.3.1). Mutations that reduce mucilage synthesis include *mum4/rhm2*, seeds of which synthesize less than half the normal amount of the pectin-components rhamnose and galacturonic acid, several *tt* (*transparent testa*) mutants, and *ap2* that eliminates mucilage synthesis. The latter two mutations are effective because they result in interference with the normal interactions between transcription factors that regulate the synthesis of enzymes associated with pectin/mucilage synthesis.

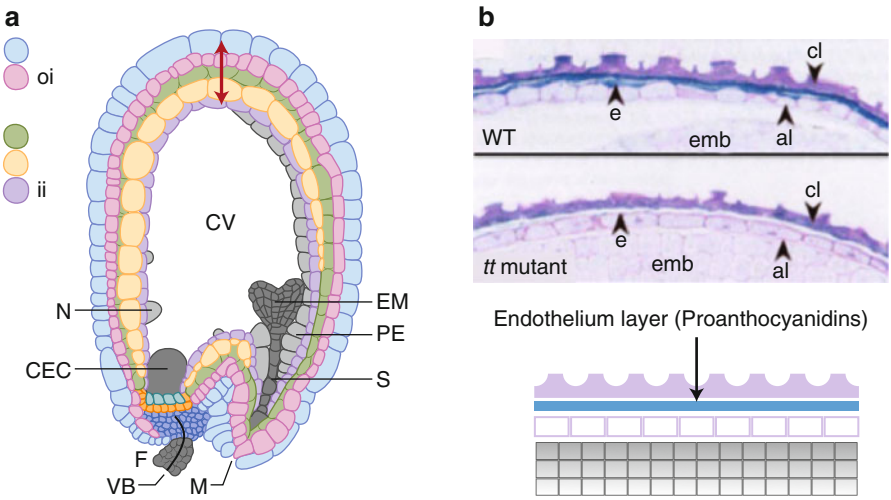


Fig. 2.15 Development of the integuments to form the testa in Arabidopsis seeds. (**a**) The testa derives from the outer (oi) and inner (ii) integuments. The outermost layer of the outer integuments forms the columellae cells, with elevated cell walls. Drawn by I. Debeaujon, INRA, Versailles. (**b**) The innermost layer of the inner integuments (endothelium) of wild-type Arabidopsis seed accumulates proanthocyanidins (stained in *blue*) while the *transparent testa* (*tt*) mutant lacks the pigments. A schematic representation of the seed coat is shown below (**b**). al, aleurone layer (endosperm); CEC, chalazal endosperm cyst; cl, columellae; CV, central vacuole; EM, emb, embryo; F, funiculus; M, micropyle; N, endosperm nodule; PE, peripheral endosperm; S, suspensor; VB, vascular bundle. The *red arrow* shows the extent of the integuments. Modified from Lepiniec et al. (2006) and Debeaujon et al. (2001). Copyright American Society of Plant Biologists

The innermost layer of the inner integuments (endothelium) accumulates proanthocyanidins (PA), flavonoid pigments, during the early stages of seed development (Fig. 2.15b). These pigments, originally produced as colorless compounds, are oxidized during maturation drying and confer a dark brown color to the mature seed. Mutants lacking enzymes necessary for PA biosynthesis or the transporter proteins for PA accumulation in the vacuoles produce pale yellow or colorless seeds, the *tt* mutants, which have little dormancy. The importance of testa pigmentation in seed dormancy control will be examined in Sect. 6.3.2.3.

Mutations to the regulatory proteins controlling PA biosynthesis and deposition, such as *TRANSPARENT TESTA GLABRA2* (*TTG2*) (this encodes a WRKY transcription factor, so called because of the presence of a conserved WRKY [tryptophan-arginine-lysine-tyrosine]-containing domain) also causes the *tt* phenotypes. *ttg2* mutant seeds are yellow and smaller than wild-type ones, due to side effects of the mutation on the endosperm. Development of the testa and endosperm occurs independently from that of the embryo, as observed also in the *fie* and *fis* mutants, and, in principle, also independently of each other. The proliferation of integument cells is under maternal control, while endosperm development is under zygotic control.

However, there is also some coordination between the testa and endosperm during seed development; e.g., impaired cell elongation in the integuments in the *ttg2* mutant limits endosperm development and results in smaller seeds.

Many mutants defective in *DICER-LIKE1* (*DCL1*), encoding an miRNA-processing enzyme, are embryo-lethal because of arrested embryo development. The lethality is caused not only by changes to characteristics of the embryo but also by abnormal development of the integuments. While the outer integumental cell layers totally cover the inner integuments and the embryo sac in wild-type ovules, in some mutants defective in *DCL1* this does not occur because of uncoordinated growth of both integument layers. Hence, embryo arrest in the mutants is not only through the defects in the zygote but also through maternal effects imposed by the testa. Therefore, the testa interacts with both the endosperm and embryo to influence seed development.

2.3.7 Somatic Embryogenesis and Apomixis

Somatic embryogenesis is the formation of embryos asexually, i.e., circumventing the necessity for gamete production and fusion. This can occur naturally, but is also a widespread experimental tool used for the production of many embryos from the somatic (vegetative) tissues of a single plant.

In nature, somatic embryogenesis can occur spontaneously: e.g., embryos may form and develop into plantlets on leaf margins (*Kalanchoë* spp.), but more commonly asexual embryos are produced from tissues of the ovule by mitosis. This is termed gametophytic apomixis and occurs in about 400 species of plants, commonly in species of the Asteraceae (formerly Compositae), Poaceae, and Rosaceae. Diplosporous apomixis, as occurs in the Poaceae (e.g., Kentucky blue grass) and some Asteraceae (e.g., dandelion, hawkweed), results from the formation of an embryo sac in which there are no meiotic divisions. The resultant egg cell is diploid, is not fertilized, and develops directly into an embryo. Apospory involves the formation of an embryo sac from the nucellus, a maternal tissue; the egg cell again retains the same ploidy as the parent and is not fertilized, e.g., in millet and other panicoid grasses. Sporophytic or adventitious embryony is a rare form of apomixis and occurs in some *Citrus* spp.; here there is no embryo sac formation, but instead the diploid embryo is formed directly from cells of the nucellus or integument. More than one type of apomixis can occur in a species (mixed apomixis), as can a combination of normal fertilization and apomixis to produce zygotic (sexual) and somatic (asexual) embryos, even in the same flower. This latter phenomenon confounded Mendel when, using hawkweed, he tried to repeat the results of his inheritance experiments on pea, but found an excessive, inexplicable maternal influence on the progeny. This enigma was a factor in discouraging him from continuing his research.

In gametophytic apomixis, formation of the endosperm may or may not be fertilization-induced (i.e., it can be diploid or triploid), but in sporophytic apomixic seeds this tissue is diploid.

There is considerable practical interest in apomixis, as it would be a way of fixing desirable heterozygous genotypes during seed propagation. For example, seeds of F1 hybrid varieties must be produced each year by controlled crossing of two distinct inbred parents. In the next generation (F2), the chromosomes from the two parents segregate independently among the progeny, which then do not have the uniformity and hybrid vigor of the F1 generation. If apomixis could be induced in the F1 plants, the resulting seeds would have the identical hybrid genotype, enabling seed production of hybrid varieties without the necessity of remaking the parental cross each year. Similarly, many horticultural plants that are highly heterozygous and must be propagated by vegetative cuttings or buds could potentially be propagated by seed if apomixis could be controllably induced.

Experimentally, somatic embryogenesis has been achieved in a large number of cultivated and wild species. Embryos can be induced to form in sterile culture from explants (small sections) of vegetative tissues, e.g., nucellus, hypocotyl, cotyledons, leaves, or petioles (direct embryogenesis), or the explant can be placed on a medium to encourage the formation of callus, which fragments when subsequently shaken in suspension culture, with the resultant formation of many embryos from the released cells or cell clusters. The conditions required for induction and production of somatic embryos varies among species, and even among cultivars of the same species; the optimal nutrient and hormonal conditions in the media used for both the induction of embryogenesis and the subsequent conversion of embryos to seedlings must be determined empirically for each.

Somatic embryogenesis has several important applications and has been used successfully as a gateway technology in the production and propagation of genetically transformed lines to produce new breeding stock. Tissues or protoplasts into which new genetic traits have been introduced by *Agrobacterium* transformation, particle bombardment, or micromanipulation can be grown into embryos and the resultant plants bred on a large scale for seed production. Superior genotypes of plants, especially those which are slow-growing, are infertile, produce sterile hybrids, or are difficult to prevent from cross-pollinating can be propagated through tissue culture to produce many embryos that retain the desired characteristics. Somatic embryogenesis has potential in the forestry industry to clonally propagate superior trees of species that take many years to mature to seed-producing age, and in the regeneration of rare and endangered species for which sexual reproduction is problematic. Despite the potential benefits of somatic embryogenesis, however, there are few successful commercial products available, because the technology is labor-intensive, expensive, and unpredictably variable in success. In addition, genetic variation can occur during somatic embryo production (somaclonal variation), and the mutations may introduce undesirable consequences. Phenotypic variation due to incomplete patterning during development is also a common problem, e.g., somatic embryos frequently lack well-developed cotyledons and contain fewer storage reserves, thus reducing the success of their conversion to seedlings.

The use of haploid tissues (pollen or egg cells) to produce embryos in culture is an alternative and more successful method. Their production from pollen (androgenesis) is the preferred route; haploid embryos may become diploid spontaneously

or this double haploid state may be induced by treatment with colchicine. This results in homozygous plants with uniform genetic backgrounds in a single generation, greatly reducing the time required for producing inbreds, which otherwise requires repeated selfing and selection for six or more generations. Methods of producing doubled haploids from sexual crosses without requiring embryogenesis in tissue culture have been developed, based on inducer lines that result in lack of transmission of all chromosomes of the inducer parent. Chromosomes of the haploid progeny from these crosses can then be doubled to create homozygous diploids. These methods have greatly facilitated the use of doubled haploids in breeding and hybrid seed production programs.

Haploid cultures of different lines or related species are a source for protoplasts that can be fused to create interspecific and intergeneric hybrids of commercial value. This application has been used for the production of exotic flowering species, and in the melding of such desirable traits as yield potential or stress and disease resistance between one species and another (as between oilseed *Brassica* spp.).

2.4 Germinability During Development

In the previous sections the cellular and biochemical/synthetic events integral to seed development were discussed; these are clearly regulated in a coordinated manner so that the progeny of a particular species are phenotypically very similar at maturation. Development proceeds in an environment in which seeds are hydrated, yet they do not germinate. What, then, prevents seeds from germinating during their development? And how are the controls that maintain seeds in a developmental mode eventually overcome to permit germination? Given that seeds of many species are dry at maturity and become considerably shrunken in size, especially those from temperate and arid environments, what mechanisms are in place to ensure that the seed survives in this state of low moisture content, one that is fatal to its vegetative parent plant?

2.4.1 Ability to Germinate During Development

While seeds do not usually germinate during development on the parent plant, their ability to do so is often acquired long before it is completed (see also Sect. 2.5). In some cases, even quite immature seeds can germinate when removed from the fruit and incubated in water, but germination is normally prevented during seed maturation (Fig. 2.9). In other cases, dissection of the embryo from the seed is required to enable germination. Alfalfa (lucerne) seeds, for example, do not germinate when excised from the silique (pod) until late during development, a time when they are undergoing maturation drying. Isolated embryos, however, will germinate much earlier if placed on a nutrient medium (Fig. 2.16a), presumably because they

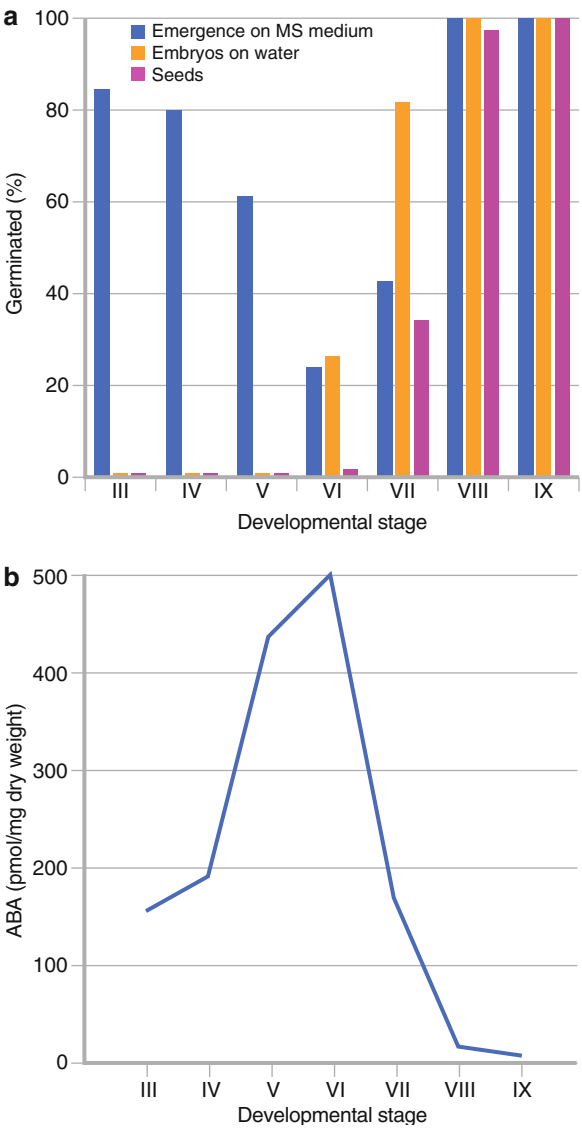


Fig. 2.16 (**a**) Germination of seeds and embryos of alfalfa during their development. At various stages of development, seeds were removed from the pod and placed on water (*purple bars*), or embryos were dissected from them and placed on a nutrient medium (*blue bars*) or on water (*orange bars*). (**b**) ABA content of the endosperm at different developmental stages (that of the embryo and testa is about 20% of that of the endosperm). Note that the germination capacity of embryos on nutrient medium decreases as the ABA content increases, and that the capacity of embryos and seeds to germinate increases with the decline in ABA content. From Xu et al. (1990)

lack sufficient reserves to support germination. Thus, constraints on germination of the embryo are imposed by the surrounding seed coat and endosperm. It takes longer for very young embryos to complete germination than older ones, and the former are more dependent on the nutrient medium for survival than the latter, which germinate when placed only on water (Fig. 2.16a). Thus, constraints on growth of the embryo are imposed by the surrounding seed coat and endosperm. The capacity to grow when removed from the surrounding tissues can also develop sequentially

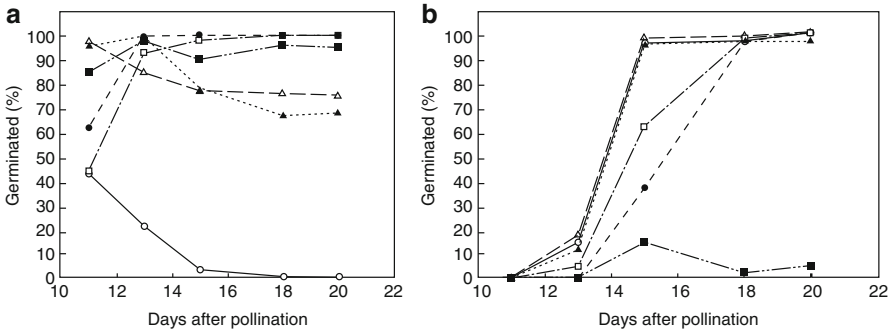


Fig. 2.17 (**a**) Germination of fresh (not dried) developing Arabidopsis seeds on water after removal from the siliques at various times after pollination of (○) wild-type, (●) *aba*, (Δ) *abi3*, (▲) *abi1*, (□) *aba/abi1,* and (■) *aba/abi3* genotypes. While germination capacity declined during development of wild-type seeds, seeds impaired in the synthesis of, or response to, ABA germinated throughout development. (**b**) Germination of seeds of the same genotypes after desiccation and rehydration in 100 μM gibberellin solution (GA_{4+7}). Following dehydration, seeds of all genotypes except *aba/abi3* germinated after acquiring desiccation tolerance between 15 and 18 DAP. Seeds of *aba/abi3* did not develop desiccation tolerance and therefore did not germinate after drying. From Koornneef et al. (1989). Copyright American Society of Plant Biologists

in different embryo tissues. For example in developing muskmelon embryos, the cotyledons, hypocotyls, radicles, and epicotyls sequentially gain the capacity to grow (when removed from the enclosing tissues) between 15 and 35 days after anthesis (DAA).

Germinability even of isolated alfalfa embryos, however, declines during development at the same time that their ABA content increases to its highest (Fig. 2.16a, b), suggesting that its accumulation is associated with this inhibition of fresh seed germinability. As noted previously (Sect. 2.3.3), ABA plays an important role in preventing germination during early seed development; the application of ABA to isolated developing embryos prevents their germination, and metabolically they remain in a developmental mode. This role is further supported by studies on mutants of Arabidopsis that either fail to synthesize ABA (ABA-deficient mutants, *aba*), or fail to respond to ABA (ABA-insensitive mutants, *abi*) (Sect. 2.3.3). Developing wild-type Arabidopsis seeds germinate to 40% on water after being dissected from the siliques at 11 DAP, but this declines to zero over the following 4 days (Fig. 2.17a), similar to the pattern for alfalfa embryos. However, fresh (not dried) seeds of *aba* and *abi* mutants (and the double *aba/abi* mutants) germinate 45–98% already at 11 DAP, and this germination capacity remains high thereafter. Thus, the inhibition of fresh-seed germination during development does not occur when ABA biosynthesis or sensitivity is impaired, and genetic studies show this is an effect of embryonic, not maternal ABA. Desiccation tolerance, i.e., germination of dried seeds, does not occur until 14-18 DAP, or not at all in double mutants of stronger *abi* alleles (*aba/abi3*) (Fig. 2.17b). Similar mutants occur in other species, including tomato (*sitiens* is ABA-deficient) and maize (Sect. 2.4.2); these show a

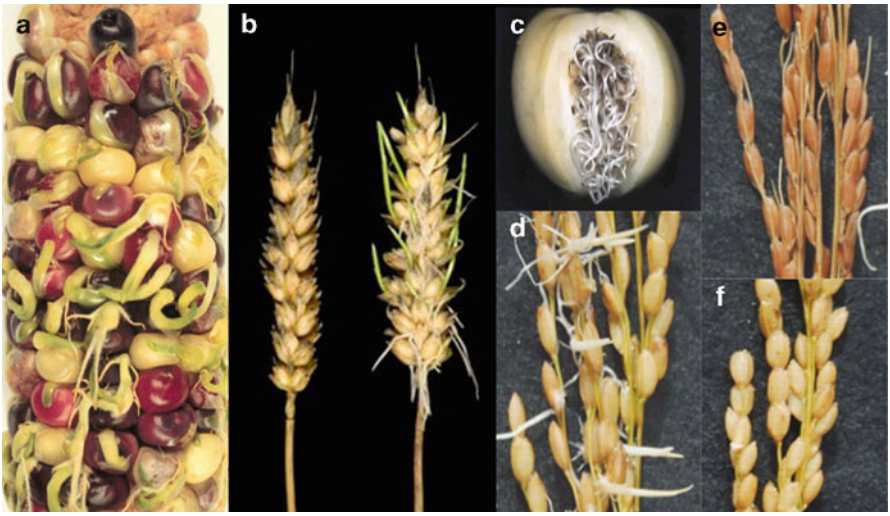


Fig. 2.18 Vivipary or precocious germination of developing seeds of (**a**) maize, (**b**) wheat, (**c**) tomato, and (**d**–**f**) rice. (**a**) is *vp14* ABA-deficient maize. (**c**) shows extreme vivipary in the non-ripening *rin* tomato mutant, in which the fruits do not decay. For rice, (**d**) vivipary-susceptible *japonca* cv. Nipponbare (Npb), (**e**) vivipary-resistant *indica* cv. Kasalath (Kas), and (**f**) the near isogenic Npb line carrying a dormancy QTL (*Sdr4-k*) from Kas are shown. Photos are by K. Koch and D. McCarty, Univ. Florida, Gainesville (see also McCarty et al. 1989) (maize), K. Loeffler, Cornell Univ. (wheat), M.J. Ahrens, while at Univ. California, Davis (tomato) and Sugimoto et al. (2010) (rice), courtesy of the National Academy of Sciences, USA

similar germination behavior to Arabidopsis seeds. As the content of and/or sensitivity to ABA declines late in seed development (e.g., Fig. 2.16b), the reduction in seed water potential (ψ_{seed}) during maturation drying becomes the major constraint on germination of nondormant seeds.

2.4.2 Precocious Germination: Vivipary and Preharvest Sprouting

The acquisition of germination capacity (Sect. 2.4.1) during seed development can result in vivipary, i.e., germination on the maternal plant prior to dispersal (Fig. 2.18). In some species, such as the mangrove *Rhizophora mangle*, this is a normal part of the seed development program, where seeds germinate while still attached to the mother plant and seedlings are shed, stick into the mud below, and continue to grow. In most species, vivipary is termed "precocious germination," which can occur when developing seeds with little dormancy experience rainfall or humid conditions. Such preharvest sprouting (PHS) during cereal (e.g., wheat, barley, rice, sorghum) production is a serious problem because it reduces grain quality and results in

significant economic losses. Considerable effort has been devoted to understanding the physiological and genetic mechanisms underlying vivipary and to developing technologies to prevent it.

Vivipary occurs in mutants lacking genes that suppress germination during seed development. For example, developing seeds of mutants with defects in the ABA biosynthetic pathway, or its upstream carotenoid biosynthetic pathway, can exhibit vivipary because both are essential to maintain sufficient ABA to prevent sprouting (e.g., maize, Fig. 2.18a). The maize *viviparous 14* (*vp14*) mutant has a defect in a gene encoding 9-*cis*-epoxycarotenoid dioxygenase (NCED), an enzyme that catalyzes the oxidative cleavage of an epoxycarotenoid to produce xanthoxin, a precursor of ABA, which is a rate-limiting (most critical) step in the ABA biosynthetic pathway (Fig. 6.13a). Another maize viviparous mutant (*vp1*) is capable of synthesizing ABA, but it lacks sensitivity to it due to a defect in a transcription factor involved in signal transduction of this hormone; i.e., *vp1* is an *abi* (*aba-insensitive*) mutant. The maize *VP1* gene is an ortholog of Arabidopsis *ABI3* (Sect. 2.3.3). In fact, when the wild-type maize *VP1* gene is expressed transgenically in *abi3* mutant Arabidopsis plants (a "complementation" experiment in which the mutant gene is replaced), it is able to restore ABA sensitivity to the seeds and prevent vivipary. Similar mutants of both ABA biosynthesis and sensitivity have been isolated from Arabidopsis and other species. These results provide compelling evidence that both ABA biosynthesis and perception are required to suppress germination during seed development.

In some species, such as wheat or sorghum, the sensitivity of the seeds to ABA naturally declines at maturity, making them relatively insensitive to inhibition of germination by the hormone and thus contributing to PHS. In other species, such as tomatoes or melons, the seeds develop inside of a fleshy fruit, a hydrated environment in which they potentially can germinate. While ABA is also involved in preventing precocious germination in these species, the osmotic potential of the fruit apoplast is also important. For example, if water is injected into the central cavity of an intact muskmelon fruit, the seeds inside will germinate, indicating that the low osmotic potential inside the fruit due to cellular breakdown associated with ripening contributes to preventing germination. Similarly, ABA-deficient tomato seeds generally do not exhibit vivipary until fruits are overripe. Tomato cultivars for machine harvesting have been bred to hold mature fruit on the plant without decay for once-over destructive harvests. Early cultivars with this trait, however, experienced problems with precocious germination in the fruit due to their more advanced maturity. An extreme case of this can be seen for the fruit of the *rin* tomato mutant, which mature but do not ripen or rot. While the fruits can be held for several months at room temperature without decay, continuing metabolism apparently consumes sugars that normally contribute to the fruit osmotic potential, enabling seed germination to occur inside the fruit (Fig. 2.18c).

Seed dormancy has been lost during domestication of cereals, including wheat (Fig. 2.18b) and rice, which makes the cultivated varieties susceptible to PHS. *Sdr4* (*Seed dormancy 4*), a quantitative trait locus (QTL), determines the susceptibility of rice grains to PHS. Seeds of the *japonica* cv. Nipponbare (Npb) are prone to vivipary (*Sdr4-n*), while seeds of the *indica* cv. Kasalath (Kas) are relatively resistant to it

(*Sdr4-k*) (Fig. 2.18d, e). When the near-isogenic line (NIL) is generated, where the *Sdr4-k* locus has been incorporated into the Npb genome, Npb grains became resistant to PHS (Fig. 2.18f). These results suggest that *Sdr4* has been modified by selection during rice domestication. The promoter region of *Sdr4* contains the seed-specific RY motif, ABA-responsive element (ABRE) and coupling element (CE) required for the regulation by B3 transcription factors (Sect. 2.3.3). Consistent with this, *Sdr4* expression is regulated by OsVP1 (*Oryza sativa* VP1), the rice ortholog of ABI3.

Vivipary, as in the case of PHS of cereals, could cause problems in food production, especially as weather and rainfall patterns change in association with global warming and climate change. Modern biotechnology can be utilized to recover the vivipary-resistant phenotypes, or restore dormancy that has been lost during domestication. Knowledge of the biochemical pathways of hormone metabolism (i.e., biosynthesis and deactivation) has made it possible to manipulate the hormone content of seeds. For example, a proof of concept for this type of approach has been provided through a modification of the *NCED6* gene, encoding a rate-limiting enzyme in ABA biosynthesis (Sect. 6.6.1.1) that is expressed during seed development in Arabidopsis. Over-induction of *NCED6* with a chemically-inducible gene expression system called the "Plant Gene Switch System (PGSS)" increases ABA in seeds more than 20-fold, and successfully suppresses experimentally-induced precocious germination of developing seeds in the siliques (pods). The chemical inducer used to trigger gene expression in this system is an environment-friendly chemical (methoxyfenozide), thus making the technology commercially applicable. This system provides a robust technology for not only suppressing but also promoting seed germination by engineering the pathways of hormone metabolism.

Another biotechnological approach to suppress PHS in wheat has been tested in field trials. This approach does not change hormone content but reduces expression of a thioredoxin (Trx) gene. Trx is an enzyme that reduces disulfide (S–S) bonds in diverse seed proteins (storage proteins, enzymes, and enzyme inhibitors [Sect. 5.8.2]). Through mechanisms as yet unidentified, specific forms of Trx promote seed germination. Expression of an antisense *PTrxh* gene from sunolgrass, which is very similar to wheat *Trxh*, using the α-gliadin promoter, reduces the expression of *Trxh* in wheat grains, and results in more efficient suppression of PHS compared to null segregants (wild-type grains segregating from heterozygous transgenics). Successful application of biotechnology will potentially solve PHS problems in the major cultivation areas for white-grained wheat varieties, such as in China, where 83% of the wheat production region is reported to be subject to sprouting damage.

2.4.3 Role of Preharvest Drying in Development of Germinability

While the occurrence of precocious germination under humid conditions indicates that seeds can become germinable prior to maturation drying, there are contrasting results concerning whether some degree of drying is essential in switching seeds

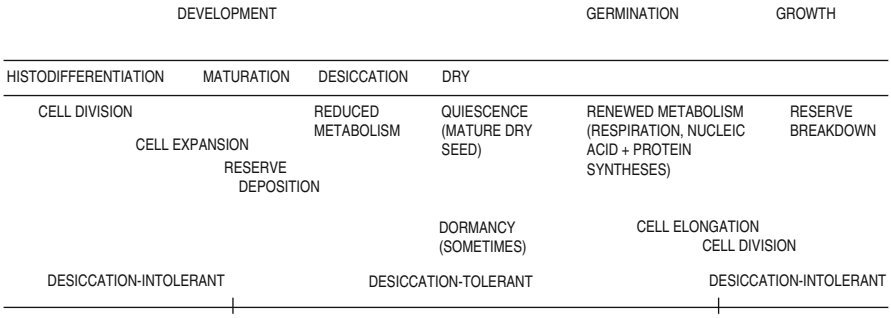


Fig. 2.19 An overview of events associated with seed development, germination, and early seedling growth. From Kermode et al. (1986). Courtesy of the American Society for Horticultural Science

from a developmental to a germinative mode. The relationships of desiccation tolerance and maturation drying to normal development and germination are shown in Fig. 2.19. Prior to desiccation, the seed is undergoing developmental and mostly anabolic metabolism associated with formation of the embryo and its surrounding structures and the deposition of the major storage reserves. Following desiccation and rehydration, developmentally-related events cease and metabolism in support of germination commences. After completion of germination, metabolism is largely catabolic as the major reserves are mobilized and utilized in support of early seedling growth. Thus, for most desiccation-tolerant (termed "orthodox") seeds, maturation drying clearly switches the seed to a germinative mode upon subsequent hydration.

However, desiccation-intolerant (termed "recalcitrant") seeds can effect this switch without dehydration, as can orthodox seeds developing inside of fleshy fruits such as tomatoes and melons, which also develop the capacity to germinate without maturation drying. In addition, as noted previously, some orthodox seeds can switch to germinative metabolism when removed from the fruit early in development prior to drying or when insufficient ABA is present to prevent precocious germination (Fig. 2.17). On the other hand, some seeds (e.g., castor bean), when isolated prematurely and incubated on water, are unable to germinate unless they have first been dried (Fig. 2.20). However, the situation is more complex than this, because removal of the developing castor bean seeds and incubation in 100% relative humidity for a period of time with only slight water loss also results in increased germination capacity. Some studies of gene expression patterns have indicated that severing of the funicular vascular connection between the developing seed and the mother plant, rather than dehydration per se, is the trigger for the switch from development to germination. That is, signals from the mother plant may be involved in maintaining the developmental mode, and loss of those signals (i.e., by funicular abscission) shifts the seed into the late maturation mode of gene expression prior to drying. Premature removal of the seed from the fruit inevitably severs the maternal connection, and if such seeds are provided with water, they may progress directly into the germinative mode. Since all experiments with excised developing seeds sever the

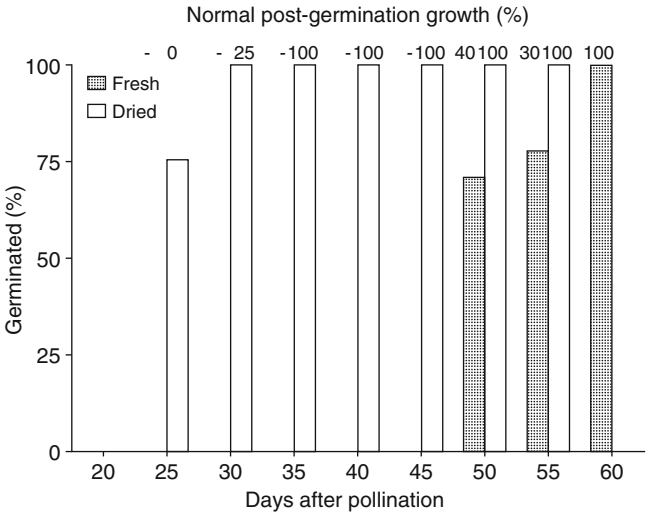


Fig. 2.20 Germination of dried or fresh intact seeds of developing castor bean harvested at various days after pollination (DAP) and placed on water. Fresh seeds that are not subjected to premature drying fail to complete germination until 50 DAP; only 30–40% of these seeds achieve normal post-germination growth (i.e., do not attain the normal radicle lengths of mature germinated seeds). Those seeds dried prematurely from 35 DAP onwards complete germination and exhibit normal seedling growth. Based on data in Kermode and Bewley (1985)

connection to the mother plant, specific experimental procedures (such as high humidity incubation) are needed to separate a post-abscission effect per se from a requirement for drying.

Even for the same species, such as *Phaseolus* bean and soybean, some studies have reported that fresh developing seeds are unable to germinate without prior dehydration, while other studies found that the germinability of fresh seeds increases prior to the development of desiccation tolerance (Fig. 2.27). In fact, one report found that *Phaseolus* bean seeds near the end of the seed filling period are capable of germinating within the fruit if water is injected into the pods, even with the funicular connection intact. Thus, maintenance of reduced ψ_{seed} in developing fruits is important in preventing precocious germination (Sect. 2.4.1). It is also possible that provision of excess water, whether in vivo or to detached seeds, causes loss of ABA or other inhibitors from the seed, promoting germination. On the other hand, the condition of "high water potential inhibition" has been reported, in which seeds germinate better at slightly reduced water potential (e.g., −0.2 MPa) than they do in pure water. It is possible that this condition is preventing the germination of fresh immature seeds incubated in pure water. The reasons for the discrepancies among various reports concerning whether drying is essential to switch from a developmental to a germinative mode are not evident. While the germination capacity of developing seeds that can survive desiccation is nearly always improved by drying, whether some degree of drying is required for the switch to germinative metabolism, or under what conditions drying is required, needs further investigation.

2.5 Maturation Drying and the "Switch" to Germination

During development, the water content of seeds gradually declines as it is displaced by the deposition of insoluble storage reserves (Fig. 3.1); however, it is assumed that the water content of the cytoplasm is sufficient for the required synthetic activities to be maintained, and ψ_{seed} remains in close equilibrium with the ψ of the associated tissues of the mother plant. As maturation approaches completion, desiccation (maturation drying) is the normal terminal event for orthodox seeds (Fig. 3.1), after which they pass into a metabolically quiescent state (which is distinct from dormancy, Chap. 6). Seeds may remain in this dry state from several days to many years and retain their viability (Chap. 8).

Little is known about how seeds lose water when on the parent plant during the final stages of maturation, although it is presumably due to evaporation without replacement. For many, desiccation likely commences when the supply of water to the enclosing fruit becomes restricted, resulting in its senescence, and/or by severing of the vascular water supply from this parent tissue to the seed (Fig. 3.2). The deposition of pectins in the lumina of the xylem elements conducting water to the ear of cereals may lead to a decline in its supply to the maturing grains. For seeds developing inside fleshy fruits, seed moisture content and ψ_{seed} decrease moderately (e.g., to ~−2.0 MPa) in association with changes during fruit ripening (e.g., cellular breakdown and increase in apoplastic solutes), and final seed dehydration only occurs after the fruits decay or are eaten and seeds are dispersed by birds or animals.

2.5.1 The Acquisition of Desiccation Tolerance

During development, seeds become tolerant of desiccation considerably earlier than maturation drying itself; e.g., developing seeds of castor bean, excised from the capsule and placed on water do not germinate until some 50–55 days after pollination (DAP), when maturation drying has commenced (Fig. 2.20). However, if the excised seeds are first desiccated, and then placed on water, germination is achieved as early as 25–30 DAP. It appears, then, that desiccation tolerance is achieved less than halfway through development, and within a period of 5 days. A similar rapid acquisition of tolerance occurs in developing seeds of other species (e.g., *Phaseolus* bean, maize, mustard and rapeseed). The ability of seeds to tolerate desiccation improves progressively during development; at early stages, survival occurs only if the castor bean seed is dried slowly, over several days, whereas at later times there is tolerance of a rapid water loss. This is probably a consequence of physiological and morphological changes that take place gradually as development proceeds, including the synthesis of specific protective substances in the later stages (Sect. 2.5.2). Also, as seeds mature they not only become more tolerant of desiccation, but upon rehydration they have an increased capacity to form normal seedlings (Fig. 2.20).

Seeds of some species, including those of certain Gramineae, can withstand rapid desiccation relatively early during their development. Wild oat grains, for

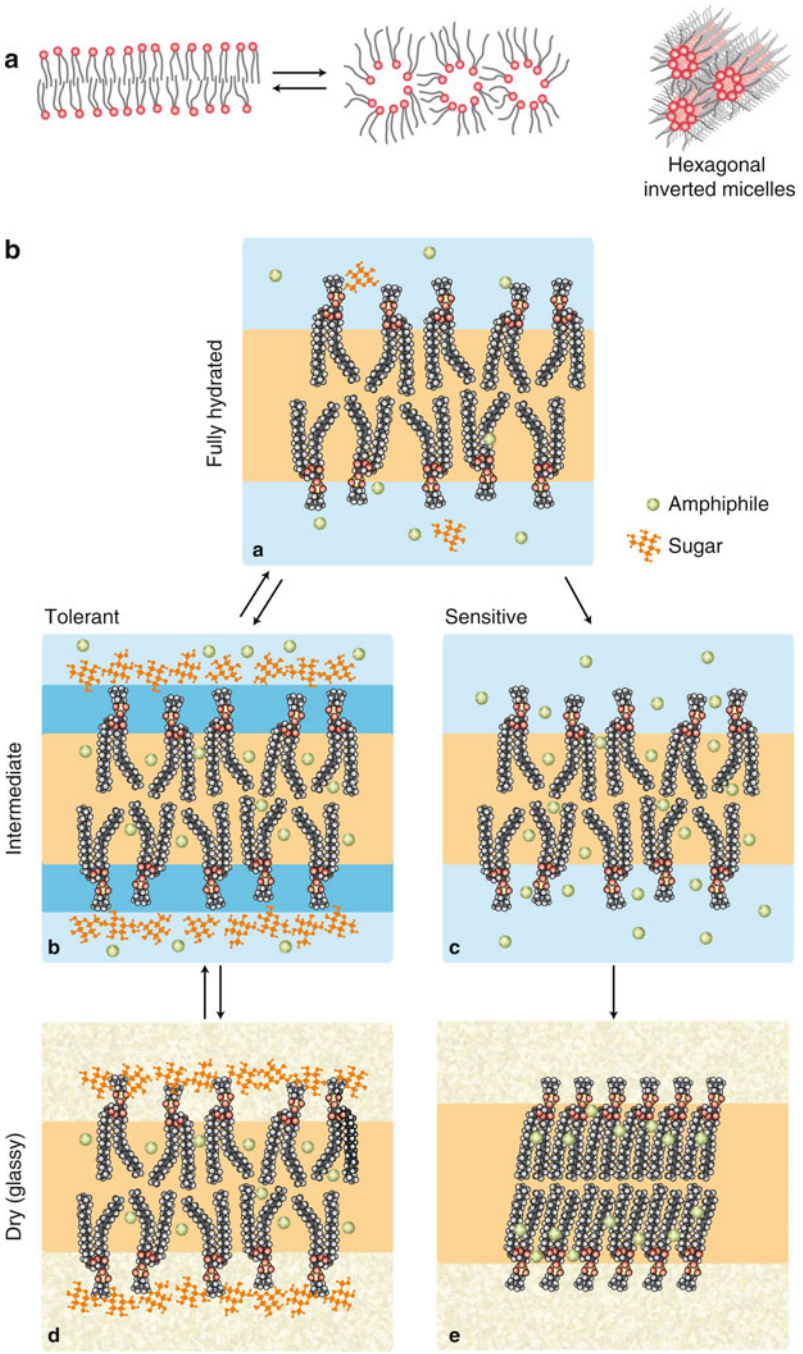
example, can survive air drying after only 5–10 days of development, and germinate upon subsequent rehydration; maturation drying normally occurs some 15–20 days later. The onset of desiccation tolerance in developing seeds may or may not coincide with their (or their embryos') ability to germinate, depending upon the species. In barley, for example, isolated embryos can germinate at about 10 DAP, but they cannot withstand desiccation until at least 5 days later. The developing aleurone layer in maize can withstand desiccation earlier than the embryo, but tolerance develops in both long before maturation is normally completed.

2.5.2 Protective Mechanisms Associated with Drying

To survive in the dry state (<~10% moisture content on a dry-weight basis), a seed has to avoid damage to its cellular components, both during water loss and upon subsequent rehydration; damage does occur, but is limited to a reparable level due to various cellular adaptations. Cellular membranes and large macromolecules such as proteins and nucleic acids are particularly vulnerable because the hydrophilic and hydrophobic interactions of their components with water are essential in maintaining their functional 3-dimensional structures. Protective mechanisms are initiated during seed maturation to preserve the integrity of these cellular components as water is removed.

2.5.2.1 Membranes, Proteins and Water Replacement

Cellular membranes are composed of a bilayer of phospholipids, with the hydrophilic polar head groups oriented outward and the hydrophobic fatty acyl chains oriented inward (Fig. 2.21a). This arrangement forms spontaneously in water, as it represents the lowest free energy state. However, as water is removed, a point is reached where there is insufficient to hydrate the entire surface, and the lowest energy state is one where micelles form with the hydrophilic head groups oriented inward to surround the remaining water, and the acyl chains are directed outward; with further drying, these micelles form a hexagonal arrangement (Fig. 2.21a). If this were to occur during desiccation, the continuity of the membranes would be disrupted, leakage could occur upon imbibition, and cellular functions that depend upon membrane compartmentalization would be disrupted. Similarly, the 3-dimensional structure of proteins also depends upon hydrophilic/hydrophobic interactions in an aqueous environment. Removal of water from proteins can disrupt their structure and cause loss of functionality. To prevent this from occurring, a mechanism known as "water replacement" occurs prior to and during dehydration. Desiccation-tolerant cells accumulate nonreducing sugars (e.g., sucrose, trehalose) and oligosaccharides (e.g., raffinose family, RFOs) that can interact with hydrophilic functional groups on the membranes and proteins. As water is removed, these sugars (and other "compatible solutes" such as proline and glycinebetaine) accumulate and


a
Hexagonal
inverted micelles
b
Fully hydrated
a
Amphiphile
Sugar
Tolerant
Sensitive
Intermediate
b
c
Dry (glassy)
d
e

replace water in maintaining the structural relationships and integrity (Fig. 2.21b). These solutes also counteract the destabilizing effects of ions and amino acids such as arginine that become concentrated in the cytoplasm as a result of water loss. In addition, amphiphiles, or molecules that can associate with either hydrophobic or hydrophilic regions depending upon their hydration state, can move from the cytoplasm into the lipid regions of membranes or the hydrophobic domains of proteins to stabilize their structure during drying. Upon rehydration, these amphiphiles move back into the cytoplasm as free solutes. Together with the sugars and compatible solutes, they replace the effect of water in maintaining structural integrity in dry systems.

While most orthodox seeds accumulate nonreducing sugars (e.g., sucrose and/or RFOs and cyclitols) in the late stages of maturation, and studies of model membrane systems strongly suggest that these have a functional role in protection against desiccation damage via water replacement, in vivo demonstration of this in seeds is lacking. Seeds of a number of species (e.g., maize, soybean, cauliflower and some tree species) can develop desiccation tolerance either in the absence of specific sugars or oligosaccharides, or their accumulation is unrelated to increased tolerance. An alternative suggestion is that the role of these sugars is to increase the longevity of seeds in the dry state by promoting the formation of the "glassy state," or a physical state of high viscosity in dry tissues; the role of the glassy state in dry seeds is discussed in Sect. 2.6.3.

Fig. 2.21 Changes in the cell membranes in tissues that are tolerant of, or sensitive to, desiccation as inferred from studies on in vitro model systems. (**a**) The arrangement of lipids in a hydrated state in vitro is for the two hydrophobic fatty acid (acyl) chains to be oriented inwards towards each other, with the hydrophilic polar head groups pointing outwards. During dehydration a state is reached where there is insufficient water to hydrate the entire surface, and the lowest energy state is one where micelles form with the hydrophilic head groups oriented inward to surround the remaining water, and the acyl chains are directed outward; with further drying, these micelles form a hexagonal arrangement. (**b**) Application of the model to explain membrane changes in a drying cell. (a) When fully hydrated, phospholipids are in the liquid crystalline state and the membrane (a phospholipid bilayer with intrinsic proteins) remains fluid. (b–e) Upon drying amphiphilic compounds (possibly phenolic acids and flavonoids which are water-attracting and -repelling) increase in concentration and preferentially migrate into the membrane. These cause membrane disturbances in both tolerant and sensitive cells. Preferentially excluded solutes such as nonreducing sugars and late embryogenesis abundant (LEA) proteins are present in high amounts in tolerant tissues. (b) At intermediate water contents, i.e., during drying but before this is completed, these solutes maintain a certain hydration level (hydration shell: *dark blue area*) around the phospholipid head groups, thereby preventing membrane damage in tolerant cells. (d) In the dry state, the hydration shell is no longer present, but the water molecules are replaced by specific sugars and proteins (water replacement hypothesis), which maintain the spacing between the phospholipid molecules, and thus the membrane retains its fluid liquid-crystalline shape. (c) In contrast, sensitive cells do not produce large amounts of sugars and proteins and the loss of water from the hydration shell (*light blue area*) results in (e) the packing of the membrane phospholipids and a transition from a liquid-crystalline to a gel phase. In this phase, membrane fluidity declines, and as cytoplasmic glass (a fluid of extreme viscosity) formation occurs, appropriate molecular rearrangements are prevented. From the gel phase, the functional and physical integrity of the membrane cannot be restored, resulting in its permanent disruption, and through which there is permanent leakage of solutes from the cytoplasm upon rehydration. From Hoekstra et al. (2001). Courtesy of Elsevier

Table 2.2 Selected examples of genes for which transcripts change during maturation and desiccation of *Arabidopsis thaliana* seeds
The protein product or influenced pathway is shown

Upregulated during maturation and desiccation (12%)
Heat shock and LEA proteins
Downregulated during maturation and desiccation (12%)
TAG metabolism (fatty acid synthesis and elongation)
Carbohydrate metabolism
Nucleotide and amino acid metabolism
Transporters (sugars)
Upregulated during desiccation (42%)
DNA repair and deoxynucleotide metabolism
Organelle protein synthesis
Respiratory pathways
Downregulated during desiccation (32%)
Abiotic stress
Starch and cell wall synthesis
Storage protein synthesis and glycosylation
DNA synthesis
TAG metabolism (fatty acid synthesis and elongation)
Hormone synthesis and signaling

The percentage figures relate to proportion of the 6,963 genes documented that fall into a particular category. Based on information in Angelovici et al. (2010)

2.5.2.2 Gene Expression and Protein Synthesis

A large number of changes in gene expression and resultant metabolism occur in seeds either just prior to, or during, drying. At these times the seeds undergo changes that terminate development, prepare for drying and maintenance in the desiccated state, preserve cellular components required for continued metabolism following drying, ready the seeds for germination and, in some species impose the dormant state. Examples of transcripts whose occurrence changes during Arabidopsis seed maturation and as a consequence of drying are shown in Table 2.2. Notable is the decline in mRNAs related to the synthesis of the major storage reserves, particularly during drying; these presumably are degraded as part of the normal turnover of messages within the seed cells and are not replaced by transcription. Drying directly influences the expression of genes for the storage proteins napin, vicilin and β-phaseolin: the function of their promoter regions, upstream of the coding region of the gene, is permanently suppressed (downregulated) by drying, thus preventing transcription upon subsequent imbibition of the seed. The permanent suppression by drying of gene expression for a legumin protein (LEG B) is shown in Fig. 2.22.

Other genes increase in expression as maturation and drying proceeds, e.g., for late embryogenesis abundant (LEA) proteins and for heat-shock proteins (HSPs); these proteins are related to tolerance of desiccation, although their synthesis increases earlier than water loss per se. Increased expression of some messages at

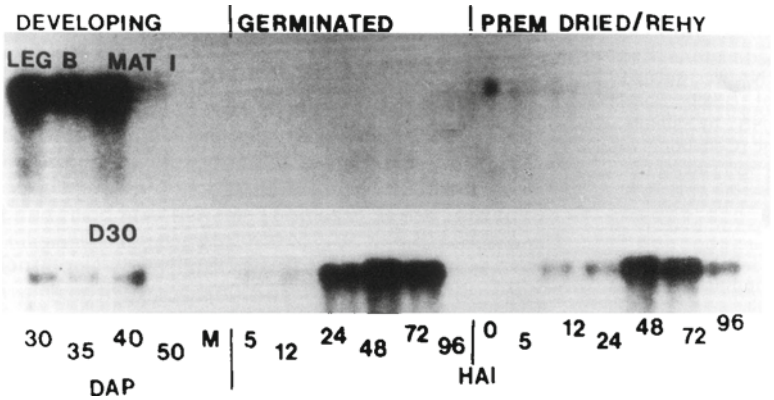


Fig. 2.22 Northern blot analysis to show the presence of a developmental messenger RNA (*Leg B Mat 1)* and a post-germination message (*D30*) in the endosperm during development and germination/growth of castor bean seeds. Mature (M) seeds were germinated for up to 96 h after imbibition (HAI). Immature seeds were prematurely dried at 40 days after pollination (DAP). mRNAs were extracted from developing seeds, dry seeds (0 HAI), following premature or maturation drying, and from seeds and young seedlings incubated for up to 96 HAI following maturation drying (germinated) or premature drying (prem dried/rehy). From Han et al. (1997)

the time of water loss likely is related to the requirement for their translation products early during subsequent germination.

The synthesis and accumulation of LEA proteins, encoded by the *LEA* genes, during seed development correlates well with the acquisition of desiccation tolerance. Their synthesis commences during mid-development and continues thereafter so that their abundance in the mature seed, in the cytoplasm and nucleus, can account for up to 5% of total protein and about 30% of non-storage ones. They rapidly decline during and following germination. LEA proteins have considerable heterogeneity and have been classified into five groups based on similarities in their sequences; those in Group 2 are also termed dehydrins. An important feature of dehydrins and some other LEA proteins is their extreme hydrophilicity due to a high content of lysine and glycine and virtual absence of hydrophobic amino acids. This gives them high solubility and the ability to bind water, possibly forming a stabilizing envelope of ordered water in association with membranes and macromolecules under conditions of cell water deficit, perhaps in conjunction with compatible solutes. Dehydrins also do not denature upon drying or heating, and form a disordered structure that may prevent physical collapse of associated proteins during dehydration. Some Group 3 LEA proteins may also act as protectants by binding ions as these become increasingly concentrated in the cytoplasm during drying. In general, despite much correlative and in vitro evidence for a role for LEAs in desiccation tolerance, it has been difficult to demonstrate their essentiality, such as by gene silencing or mutation experiments. This could be due to the redundancy of function among multiple LEA proteins and their abundance in maturing seeds. For example, seeds of *Atem6* mutants of Arabidopsis lacking the Group 1 LEA protein

AtEM6 (*Arabidopsis thaliana* EARLY METHIONINE-LABELLED 6) undergo maturation drying earlier than those of the wild type, yet they are still able to acquire and retain desiccation tolerance and viability, possibly due to redundancy of function with another LEA, the EM1 protein. However, deficiency of EM6 apparently induces other pleiotropic effects, such as early seed maturation drying, indicating possible additional functions for LEA proteins during seed development. Many studies have concentrated on only one or a few of the LEA proteins present in seeds; that others may be important cannot be ruled out, especially since they vary in their structures and potential functional significance.

ABA, which plays a crucial regulatory role during seed development (Sect. 2.4.1), also can influence the production of LEA proteins. Seeds of *aba/abi3* double mutants of Arabidopsis (ABA-deficient and -insensitive) lack several LEA proteins compared to the wild type, and are also desiccation intolerant. Tolerance of drying can be conferred on the developing seeds by treating the parent plants with an analogue of ABA or by exposing excised developing seeds directly to ABA and sucrose. One particular Group 1 LEA protein, which has homology with the Em (early methionine-labeled) protein in wheat embryos, is much reduced in concentration in seeds of *abi3* and slightly so in *aba*. In mutants of maize, the many *LEA* genes appear to be differentially affected by ABA. Embryos of the ABA-deficient viviparous mutants *vp2* and *vp5* (Sect. 2.4.2) produce the RAB (*R*esponsive to *AB*A) 17 and RAB 28 LEA proteins, but the latter is absent from those of the ABA-insensitive *vp1*. The maize Em LEA protein, in contrast, is not found in either *vp5* or *vp1* but, surprisingly, some LEAs are induced in the insensitive *vp1* mutant by applied ABA. LEA protein production may not be regulated solely by ABA because osmotic stress in wheat embryos induces Em protein formation even when ABA production is prevented by an inhibitor; also, some barley *LEA* genes respond differently to ABA and water stress. It is possible, therefore, that LEA protein production is regulated by different mechanisms, involving ABA in some but not in all cases. Seeds of some recalcitrant species, i.e., ones that are incapable of withstanding desiccation, accumulate dehydrins in response to ABA even while being fatally dried. Thus there is no clear correlation between the ability or inability of seeds to produce certain LEA proteins and their tolerance of desiccation.

While there is evidence that LEA proteins play some role in desiccation tolerance, their biological function in developing seeds in vivo is still unclear. They likely have a protective role within cells during desiccation and in the dry state, but probably alone they are not sufficient and must operate in collaboration with other compounds and mechanisms. In some cases, over-expression of LEA proteins has also increased the accumulation of other protective compounds, such as proline, polyamines, and sugars, suggesting that LEAs can have indirect effects on other desiccation-tolerance mechanisms, or that there are feedback systems to coordinate the expression of multiple stress adaptations.

A second class of proteins that has been implicated in the acquisition of seed desiccation tolerance is the small heat shock proteins (smHSPs). The expression of their genes during seed development often coincides with that of *LEA*s, and both respond positively to ABA. Because smHSPs are thought to function in heat stress by

maintaining or restoring protein structures and preventing irreversible denaturation, they could perform a similar role with respect to desiccation tolerance. The specific role that smHSPs play in desiccation is not known, but they likely are a part of the mix of small and large molecules that interact to ensure that cells experience limited damage as water is lost from the seed.

2.5.2.3 Other Changes in Metabolism Associated with Drying

In addition to the accumulation of sugars and compatible solutes and the synthesis of LEAs and smHSPs, other changes in metabolism are associated with the acquisition of desiccation tolerance. Damage to intolerant tissues due to desiccation is often associated with oxidative damage, such as peroxidation of lipids. This is thought to arise from the generation of reactive oxygen species (ROS) as a result of the impairment of electron transport chains during drying. In general, respiration rates of desiccation-tolerant tissues decrease during drying, while those of desiccation-intolerant tissues increase. The higher respiration rates of intolerant tissues may result in increased ROS generation and subsequent oxidative injury. Alternatively, different components of respiratory and metabolic pathways may be differentially sensitive to water loss, resulting in "metabolic imbalances" that result in the generation of damaging ROS and free radicals. Thus, it is hypothesized that a regulated shut-down of metabolism must occur during dehydration to prevent these adverse consequences. How this occurs or is achieved is unknown. Many genes related to antioxidant defense are upregulated in desiccation-tolerant tissues, but few of these are specific to seeds. However, peroxiredoxin proteins that have antioxidant function are expressed in seeds and other desiccation-tolerant plants and may have a specific role in desiccation tolerance. Antioxidants such as tocopherols accumulate in maturing seeds and can serve as amphipathic molecules, entering hydrophobic domains such as membranes during dehydration and presumably reducing oxidative damage there. The sucrose-non-fermenting-related kinase (SnRK1) complex plays a key role in regulating anabolic versus catabolic metabolism and stress responses in microorganisms, plants and animals, and therefore could be a master regulator of metabolism in maturing seeds. Removal of a regulatory protein subunit of SnRK1 (SNF4b) in *Medicago truncatula* seeds impairs the accumulation of oligosaccharides but does not prevent the development of desiccation tolerance, although seed longevity is reduced. This illustrates the difficulty in many cases of separating desiccation tolerance per se from reduced longevity in the dry state, as these two properties are often closely associated (Chap. 8).

2.5.3 Gene Expression Changes Upon Rehydration

Upon imbibition of dry seeds, metabolic events associated with germination commence, even in those which are dormant (discussed in detail in Chaps. 4 and 6).

This makes sense, because desiccation clearly marks an irreversible end to development, even if it occurs prematurely, and dried seeds have no other option upon rehydration than to either germinate or remain dormant. That drying promotes these events is well-illustrated in studies on the endosperm of castor bean, in which premature drying leads, upon subsequent imbibition, to a change in the pattern of protein synthesis from one that is distinctly developmental to one that is identical to that of germinating and germinated seeds following normal maturation drying. This in turn is attributable to changes in gene expression. Globulin (legumin, LEG B) storage protein mRNAs present in the developing seed endosperm decline during maturation drying, between 40 and 50 DAP, and are not resynthesized during subsequent germination (Fig. 2.22). Seeds that are prematurely dried at 40 DAP exhibit a similar decline in storage protein mRNA within the endosperm, so that in the dry state (0 HAI [hours after the start of imbibition], prem dried/rehy, Fig. 2.22) little is detectable by northern blot analysis. There is no increase in this mRNA upon rehydration of the seeds. In contrast, the message for an unidentified growth-related protein (D30) is expressed in low amounts during development, but is abundantly present in the endosperms by 48 HAI. Likewise, following premature drying at 40 DAP, transcription of this mRNA is strongly evident by 48 HAI, showing that it is induced following desiccation (Fig. 2.22). Removal of the castor bean seeds from the capsule and imbibition in water without drying does not elicit the same changes in gene expression.

Premature drying of developing seeds also elicits the production of enzymes required for the mobilization of stored reserves. In soybean and castor bean seeds, for example, enzymes for the mobilization and conversion of the stored lipid reserves are almost absent during seed development, but increase greatly after premature drying, to a similar extent as in the germinated mature seed (Fig. 2.23a). Mobilization of the starch reserves in germinated cereal grains requires the production and secretion of α-amylase by the aleurone layer, which occurs in response to GA (Sect. 5.5.3). The ability of the aleurone layer to produce this enzyme is not normally acquired until the onset of maturation drying, but premature desiccation of wheat (Fig. 2.23b), barley, triticale and maize is able to induce its synthesis following germination. The effect of drying is to increase the sensitivity of the aleurone layer to GA, which in barley provokes the expression of appropriate α-amylase genes (*Amy1* and *2*); these are not expressed in nondried aleurone layers. Increased responsiveness to GA may be the result of desiccation-induced alterations to hormone receptors or signaling pathway components. It has been suggested that depletion of ABA (or a decline in sensitivity to ABA) in the aleurone layer due to drying increases the response of this tissue to GA. In maize, however, inhibition of ABA synthesis in the aleurone layer does not permit it to respond to GA without the imposition of a drying treatment.

As noted in Sect. 2.4.1, seeds can switch from a developmental mode to a germinative mode without a requirement for drying. In particular, seeds that develop within a fleshy fruit, e.g., melons and tomatoes, do not dry prior to dispersal. When mature, the seeds within a ripe tomato fruit have a water content of ~40% (dry weight basis). Tomato seeds taken from the fruit during development and placed on

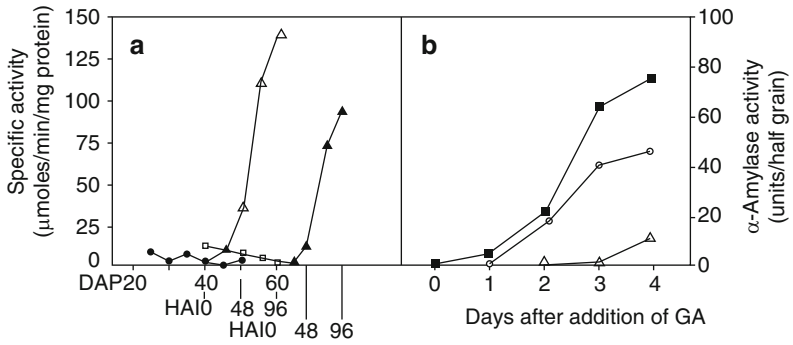


Fig. 2.23 (**a**) Increases in isocitrate lyase activity (an enzyme involved in the conversion of fatty acids to sugars, Sect. 5.7) in castor bean endosperms elicited by maturation drying (▲) and premature drying at 40 DAP (Δ). Developing seeds (●) do not produce the enzyme, nor do seeds removed from the capsule at 40 DAP and placed on water (□) for 96 h, i.e., not desiccated. DAP, days after pollination. HAI, hours after the start of imbibition. (**b**) Time course of α-amylase production by half-grains of mature (■) and immature wheat grains dried prematurely at 25 DAP (○) or maintained moist in an atmosphere of 100% relative humidity (Δ). The half-grains were incubated in GA; only those subjected to drying produced substantial amounts of the enzyme. From data in (**a**) Kermode and Bewley (1985b) and (**b**) Cornford et al. (1986)

water, or a nutrient medium, germinate without any requirement for drying, so removal of the seeds from the constraints of the fruit is sufficient. An analysis of the expression of genes for developmental, germinative, and post-germinative proteins shows that the behavior of seeds taken from the fruit and placed on water (fresh seeds) and of those dried at the same stage of development (dried seeds) before imbibition are very similar during subsequent germination and growth. The decline in the expression of the developmental protein oleosin (associated with oil body stability, Sect. 3.2.3) is similar in the germinating and germinated fresh and dried seeds (Fig. 2.24a, b). Likewise, an increase in transcript and protein for the germination/post-germination enzyme isocitrate lyase (ICL, important in the conversion of oils to sugars during mobilization, Sect. 5.7) occurs in both simultaneously (Fig. 2.24c, d). Thus, while the seeds from wet fruits are desiccation tolerant, the switch from a developmental to a germinative mode is achieved simply by separation of the seed from the surrounding fruit tissues and hydration to increase ψ_{seed} sufficiently to allow germination to proceed.

2.6 Late Maturation Events and Seed Drying

In agronomic crops, the emphasis on seed production is associated with dry weight accumulation and yield, so much attention has been focused on physiological (or mass) maturity (PM), which is the point in development when dry weight accumulation is maximal (Fig. 3.1). It has also been posited that this is when seed quality with

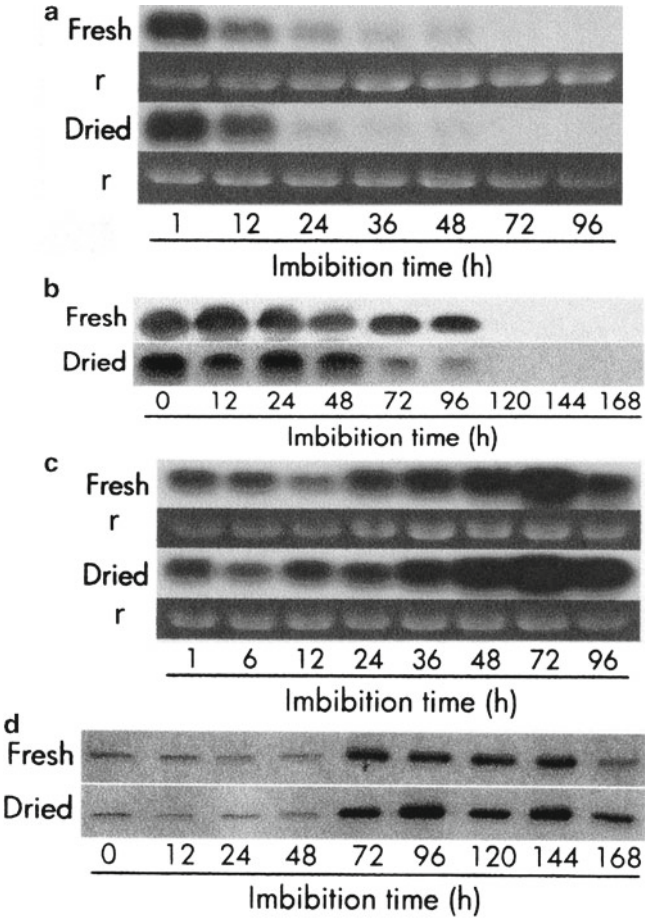


Fig. 2.24 Transcripts and proteins in germinating and germinated seeds of tomato, taken fresh from the fruit and placed on water during late development, or dried and then imbibed. (**a**, **b**) Transcripts and protein, respectively, for oleosin, as shown by northern blots to detect mRNA, or western blots to detect the protein using a specific antibody. (**c**, **d**) Northern and western blots for isocitrate lyase, respectively. 78% and 85% of wet and dried seeds, respectively, had germinated by 72 h after the start of imbibition (HAI); wet seeds commenced germination after 36 HAI, and dried seeds a few hours prior to this. r, control lanes on northern blots (**a**, **c**) to show equal loading of RNA. Previously unpublished data from the PhD thesis of C.F. Machado, Univ. Guelph (2007)

respect to germination and vigor is also maximized, and that from this point onward seeds can only lose quality. That is, the final stages of maturation and drying have not been considered to be important to development of seed quality. However, considerable work has now established that seed vigor and potential longevity continue to increase after PM, and that the last stages of seed maturation are important for maximizing seed quality. The developmental events that contribute to these late maturation changes, as well as the biophysical state of dry seeds, are considered here.

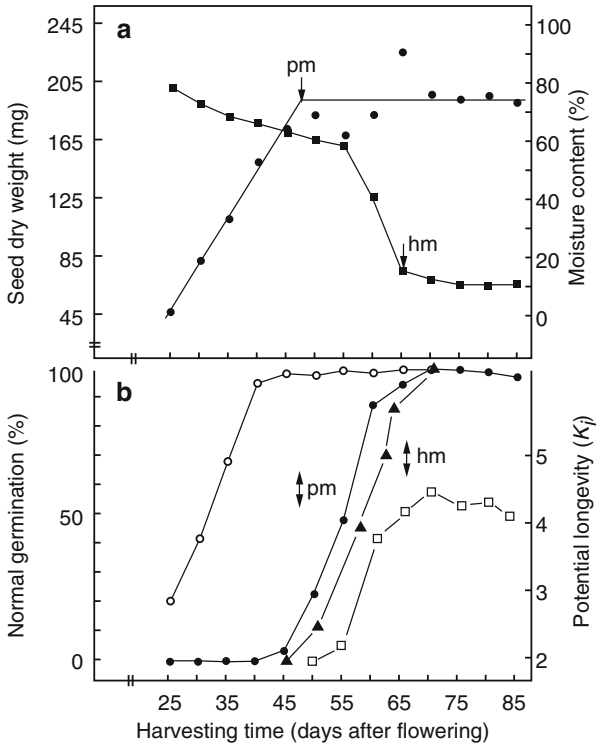


Fig. 2.25 Changes in (**a**) seed dry weight (●) and moisture content (% wet basis, ■) and (**b**) the capacity of freshly harvested (o) and dried (to 10–11% moisture content, ●) seeds to germinate normally within 14 days (o, ●) or for dried seeds to germinate within 3 days after imbibition (▲). Also shown in **b** are changes in the potential longevity (K_i) of seeds harvested and dried at different times of development and then aged at 12% moisture content and 40°C (□). K_i is the predicted probit of the initial viability in the seed viability equation (Sect. 8.2.1). The times of maximum dry weight accumulation (physiological maturity, pm) and harvest maturity (hm) are indicated. Modified from Zanakis et al. (1994a, b). With permission of Cambridge Univ. Press

2.6.1 Physiological Maturity Versus Harvest Maturity

Physiological maturity (also termed mass maturity) is the point during development when seed dry weight has reached its maximum value. As shown for soybean (Fig. 2.25), this occurs at 45 DAF, while the seeds are still desiccation intolerant. Desiccation tolerance develops between 45 and 55 DAF, just prior to the rapid loss of water associated with maturation drying (Fig. 2.25a). This is followed closely by an increase in the ability of seeds to germinate quickly upon rehydration (which is associated with increased seed vigor), and subsequently by the development of maximum potential longevity in storage (Fig. 2.25b). In this case, several components of seed quality, including desiccation tolerance, vigor, and longevity, develop after

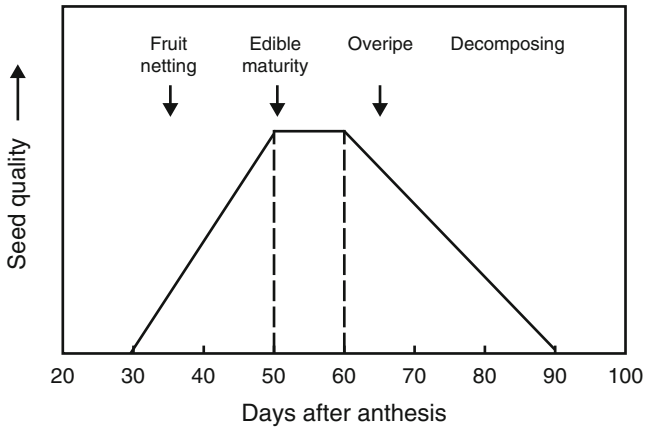


Fig. 2.26 Pattern of development and loss of seed quality (i.e., viability, germination rate, seedling growth, tolerance to stress) in muskmelon as the enclosing fruit ripens and decays. Seed quality increases as seeds become germinable, vigorous, and desiccation tolerant between 30 and 50 days after anthesis (DAA), as the fruits reach edible maturity. Seed quality is maximal during and shortly after fruit ripening, then declines again in overripe and decomposing fruits. The optimal harvest period is therefore between 50 and 60 DAA. Modified from Oluoch and Welbaum (1996)

physiological maturity and before harvest maturity, a period in which seed moisture content is declining. The earliest harvest maturity is considered to be 65 DAF, when the seeds have dried below 20% moisture content (wet weight basis) (Fig. 2.25a). Thus, there is a period of 20 days of development between the attainment of maximum dry weight and of maximum seed quality at or slightly after harvest maturity. Similar studies in a number of species (*Phaseolus* beans, rape, Arabidopsis, tomato, pepper, melon) have confirmed that seed quality continues to increase after physiological maturity. Once the seeds have dried below about 20% moisture content, developmental metabolism has ceased and deterioration may begin, as seeds are particularly susceptible to aging at moisture contents between about 10 and 25% (Sect. 2.6.3). For this reason, many crop seeds are harvested at relatively high moisture contents and dried quickly but carefully to prevent deterioration in the field and to preserve maximum quality.

Even though they do not experience rapid drying during late maturation in the fruit, extending the harvest date for seeds in fleshy fruits, including tomato, melon, and cucumber, also can result in loss of seed quality. In muskmelon, seeds are fully germinable by 35 DAA, but maximum quality is not achieved until 50 DAA, which coincides with ripening and edible maturity of the fruit (Fig. 2.26). If seeds are allowed to remain in the overripe and decomposing fruit, seed quality declines, with an increase in seeds exhibiting "osmotic distension" or swelling of the perisperm envelope enclosing the embryo following washing or imbibition (see also Sect. 8.4.2). Swelling of the perisperm can split the testa, resulting in a condition known as "fishmouth" in cucurbit seeds that resembles precocious germination, but is actually

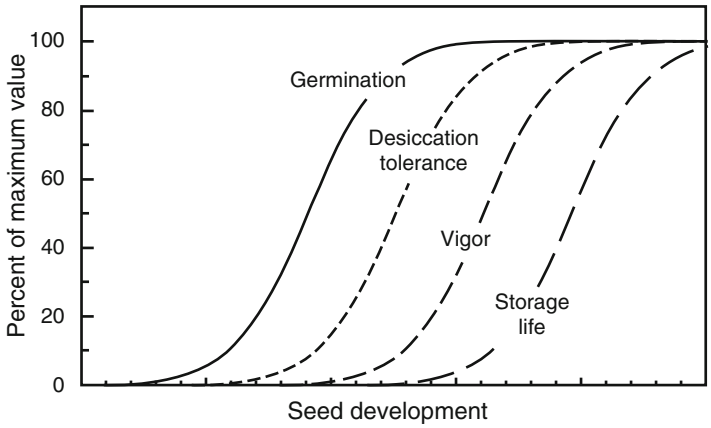


Fig. 2.27 Generalized pattern of development of seed quality components. The capacity for germination generally develops first, followed by desiccation tolerance (in orthodox seeds). Vigor (rapid germination, stress tolerance) and potential longevity in storage increase in the last stages of development, just prior to or associated with maturation drying. The relative relationships concerning when each of these quality components develops vary among species. In addition, the spread in time over which each component achieves its maximum value also varies, depending upon whether an individual seed or a population of seeds is being considered

an indication of embryo damage, leakage of solutes within the selectively permeable perisperm envelope, and osmotic distension. In general, ripening or senescence of the fruit is a good indication that seeds are at harvest maturity, as this is associated in nature with seed dispersal.

2.6.2 Seed Development and Seed Quality

The pattern of development of seed quality is summarized in Fig. 2.27. In general, the ability of seeds to germinate (at least under some conditions) develops early, prior to maximum dry weight. This is followed by the development of desiccation tolerance and maximum viability after dehydration (Sect. 2.5.1). Seed vigor, indicated by more rapid germination or greater tolerance of stressful conditions, lags behind viability, and maximum storage life is not attained until the end of the full developmental period. The relative relationships of the curves in Fig. 2.27 can be closer or farther apart, depending upon the species. In addition, sigmoid curves are shown, indicating that an individual seed may develop these attributes gradually (e.g., among different tissues), and on a population basis, so all seeds in a fruit or on a plant do not attain a given developmental stage simultaneously. Thus, any bulk harvest of seeds will contain seeds from a range of developmental stages and therefore of different quality levels. For many wild species, seeds are shed as they mature (termed shattering for dry seeds/fruits) to facilitate dispersal. Absence of shattering

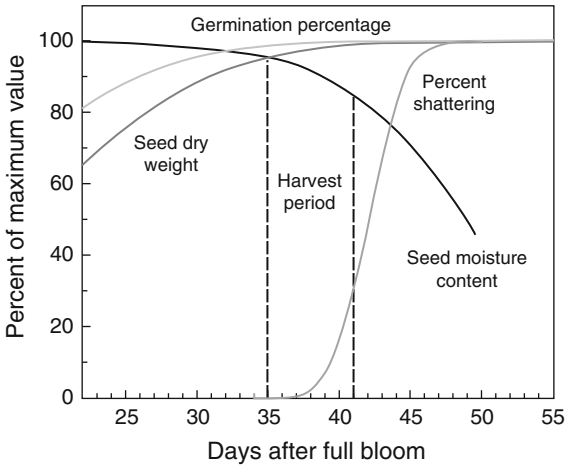


Fig. 2.28 Development and shattering (shedding) of onion seeds in relation to seed yield. Seed dry weight and germination percentage increase until about 40 days after full bloom, while seed loss due to shattering begins somewhat before this. Thus, the optimal harvest period must be timed for the period when seed quality is as high as possible without unacceptable losses due to shattering. Because seed moisture content declines consistently in relation to seed maturation, the seed moisture content can be a reliable guide to determine the harvest period. Modified from Steiner and Akintobi (1986). Courtesy of the American Society of Agronomy-Crop Science Society of America-Soil Science Society of America

and retention of the mature seeds on the mother plant is closely associated with domestication, as occurs in most domesticated cereals and legumes. This increases yield by preventing seed loss, but also results in a situation in which seeds at multiple developmental stages are present on a given plant. In species that eventually senesce completely, the slower developing seeds can complete development while at the same time the non-shattering trait prevents loss of the earlier developing seeds, thus reducing the range of seed quality at harvest. However, indeterminate species, which continue to produce new flowers and set seeds even as earlier fruits and seeds are maturing, often retain the shattering characteristic, making it critical to identify the optimal harvest period when the largest percentage of seeds are mature, but losses due to shattering are minimized (Fig. 2.28). In species of the Umbelliferae, such as carrot, seed quality varies among seeds developed on sequentially maturing umbels. Bulk harvests of seeds can contain a wide range of developmental stages, as is evident in a diversity of embryo sizes within the seeds, resulting in slow and nonuniform germination. In practice, seed companies utilize a number of strategies to concentrate seed set and manage seed maturity in order to maximize seed quality while achieving economical yields.

One strategy that could be employed to increase both seed yield and quality is to prevent shattering in crops that exhibit the trait, such as canola and other Brassicas. Several genes have been identified, including *SHATTERPROOF*, *FRUITFUL*, and *ALCATRAZ*, which when mutated or silenced result in abnormal development of the dehiscence zone of the siliques, preventing their separation and retaining the seeds

within the fruits until harvest. Similar alterations of seed dehiscence zones have occurred during domestication to result in the non-shattering traits common in domesticated crops, so it is likely that similar genes are present in most species.

2.6.3 Maturation Drying and Biophysical Aspects of Dry Seeds

Seed water content gradually declines during development as storage reserves are deposited in storage vacuoles, displacing water (Fig. 3.1). At some point after physiological maturity, orthodox seeds undergo more rapid dehydration that accompanies the end of seed maturation (Fig. 2.25a). As discussed in Sect. 2.5, maturing seeds have prepared for this dehydration by synthesizing an array of small molecules and proteins that enable them to preserve the structural integrity of critical organelles, membranes, and proteins so that they can persist during the dry state and resume biological functions upon rehydration. This loss of water changes the hydration status of cellular components and results in different physical states that affect the types of biochemical and chemical processes that can occur. In particular, avoidance of oxidative damage during drying and in the dry state is important for seed survival during long-term storage. This will be considered further in Chap. 8 with respect to retention of seed viability in storage.

The relationships among seed water content, biophysical measures of seed water potential, biochemical and chemical processes, water-binding sites, and the state of water in seeds are illustrated in Fig. 2.29. The highest hydration level (V) extends from pure water (0 MPa) to a water potential of about −1.5 MPa, still in equilibrium with a relative humidity of >99%. This is the range of hydration at which turgor pressure is possible and cellular metabolism is not restricted by lack of water. Developing seeds are in the middle to lower end of this hydration level until just before the rapid dehydration that ends development. Imbibition to return to this level of hydration is required for germination to occur following dehydration. In hydration level V, water is available in solution and ψ_{seed} is reduced mainly by the presence of osmotic solutes, or by any water stress experienced by the mother plant. Thus, all of the potential water-binding sites at the molecular level are saturated and solvent water is available for biochemical activities.

Between approximately −1.5 and −5.0 MPa, some biochemical activities can continue. Protein and nucleic acid synthesis can continue into this hydration level, but at decreasing rates as ψ_{seed} falls. However, integrated metabolic activity is reduced and can be damaging if seeds are held in this hydration level for extended periods (Sect. 2.5.2.3); however, it is generally traversed relatively quickly during maturation drying (Fig. 2.25a). Bulk liquid water is still present in hydration level IV, but is being withdrawn and pulled into capillaries and structures that can generate sufficient tension to retain the water. The osmotic solute concentration increases as the water is removed and the physical state of water in the tissues becomes "syrupy," i.e., somewhat viscous. At the lower end of hydration level IV, biochemical activities have been reduced to low rates compared to those in hydrated tissues.

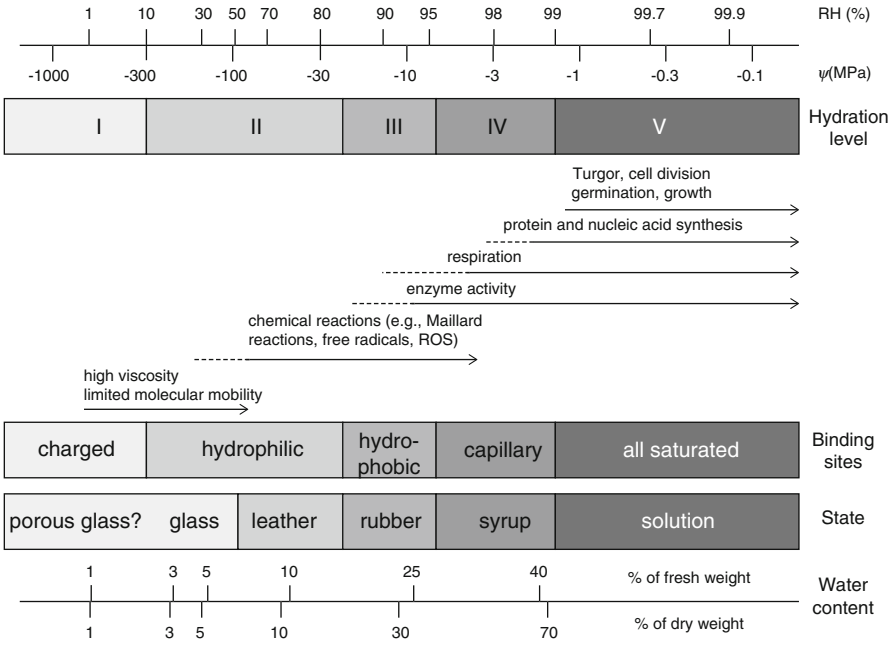


Fig. 2.29 Hydration levels and associated metabolic/chemical activities in seeds. The upper scale shows relative humidities (RH) and corresponding water potentials (ψ). Five regions of hydration have been proposed extending approximately over the ranges of RH or ψ shown. The types of metabolic, biochemical, and chemical activities that can occur in each hydration level are shown, with the arrows indicating the approximate ranges. The water-binding sites that are occupied in each hydration level are indicated; as free water is removed, the remaining water is bound to increasingly hydrophilic molecular sites. The physical state of water in each hydration level is indicated, from solution water to increasingly viscous states until the glassy state is formed, which has very high viscosity allowing limited molecular mobility. The seed moisture contents corresponding to the hydration levels are shown at the bottom, on both fresh and dry weight bases; these values are only approximate and will vary considerably with the oil content of the seed, temperature, and other factors (Fig. 2.31). Based on Vertucci and Farrant (1995) and Walters et al. (2002)

As dehydration continues, seeds move into hydration level III, spanning approximately −5 to −12 MPa, or approximate 30–20% moisture content (fw basis) and still in equilibrium with >90% RH. In this hydration level, integrated biochemistry has largely ceased, although some enzymatic processes can continue, albeit at much reduced rates. Nonenzymatic chemical reactions can also proceed at this hydration level. For example, nonenzymatic reactions in which reducing sugars are added to proteins can occur, forming Maillard products that have been associated with seed deterioration. Free radicals can also be generated, such as by lipid peroxidation, and chemical changes due to free radicals can continue to propagate in the tissues. Liquid or solution water is now largely absent, and remaining water is bound to sites on both hydrophobic and hydrophilic molecules. Multiple layers (or hydration shells) of water molecules may still be present around proteins and membrane polar head groups.

Water replacement by sugars is occurring (Sect. 2.5.2.1) and desiccation-intolerant tissues will suffer damage when dried to this extent. The tissue enters a "rubbery" state, indicating that its viscosity has increased but it still remains somewhat pliable and not yet brittle. This is a dangerous hydration level for seeds, as it is too low for active repair of chemical or structural damage, but still high enough for damaging chemical processes to continue. It is a range in which seeds deteriorate and lose viability rapidly (Chap. 8), particularly at warm temperatures that accelerate the damaging oxidative reactions.

With further drying, <−15 MPa or <20% moisture content, seeds enter hydration level II. This is the range, from equilibrium with RH of 90% to ~10%, that dispersed orthodox seeds will generally experience, depending upon the prevailing RH of their environment. At the upper end of hydration level II, chemical reactions can continue to occur, while the rates of these reactions decrease at the lower range (~−200 MPa), and seed longevity increases logarithmically as the moisture content decreases (Chap. 8). Water is now lost from hydrophobic sites and is bound only to hydrophilic and ionic sites that can strongly attract it. The physical state of the tissues can be "leathery," or more viscous than the rubbery state, and at the lower end can form the "glassy" state. A glass is defined as a fluid with such extreme viscosity that it has mechanical properties similar to a solid; cellular contents in a glass are highly restricted in their ability to move and are essentially locked into place. This vitrified, or inert, metastable glassy state, contributes to the longevity of dry seeds by greatly slowing the rates of damaging chemical reactions due to the restricted molecular mobility. Compounds accumulated during seed maturation, such as sucrose, RFOs, and cyclitols (Sect. 1.3.1) may be involved in forming and maintaining the glassy state; they can facilitate glass formation in in vitro systems. The extent to which they are required for glassy state formation in vivo is still subject to debate, but they are likely to be contributors. The LEA proteins and smHSPs may also promote glass formation, perhaps in conjunction with sugars.

With more extreme drying, seeds may enter hydration level I, characterized by RH values less than 10% or moisture contents less than about 3%. In this state, even hydrophilic molecules are no longer hydrated and water is present only in association with charged molecules or ions. The tissues remain in a glassy state, but even this structure may change to other forms and biophysical properties can change. There is debate as to whether ultra-drying to these low levels is in itself damaging to seeds or shortens their longevity in storage. While there is a limit to the increase in longevity with decreasing seed moisture content, some experiments show no detrimental effects, while others have found reduced longevity following ultra-drying. In general practice, seeds are not dried to such low moisture contents for storage; equilibrium with 15–25% RH is a recommended condition for long-term storage (Chap. 8). It is critical that seeds dried to such low moisture contents are first rehydrated through the vapor phase to avoid imbibitional damage (Sect. 4.3.2).

The relationship between seed moisture content and equilibrium relative humidity at a given temperature is called a moisture sorption isotherm, which also illustrates the three lower hydration levels (Fig. 2.30). Seeds exhibit characteristic sorption isotherms that have been analyzed mathematically and biophysically as representing

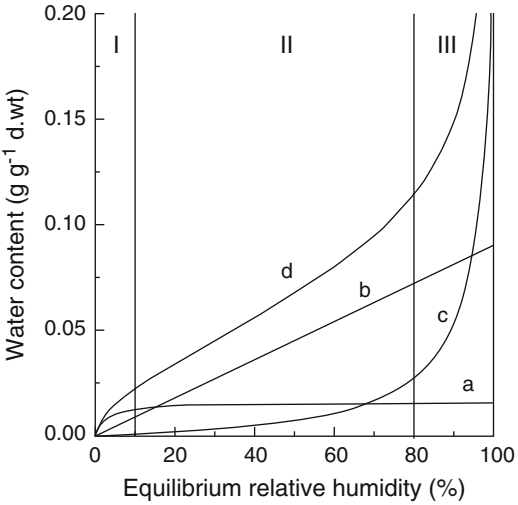


Fig. 2.30 A seed moisture sorption isotherm and its components. The relationship of seed moisture content to equilibrium relative humidity is called a moisture sorption isotherm. According to the D'Arcy-Watt model of sorption isotherms, the complete curve (d) is comprised of three major subcomponents. Strong binding sites, such as charged ionic groups, hold water very tightly and are the last to lose bound water; they also saturate at low hydration levels (I) (curve a). Between 10 and 80–90% RH, weak binding of water in polar, hydrophilic sites associated with hydration level II increases seed moisture content linearly with increasing RH (curve b). At high RH (<80%), multimolecular layers of water form and water content increases steeply as RH increases in hydration level III (curve c). The sum of the contributions of these components results in the observed sorption isotherm (curve d)

the sum of three major underlying components (termed the D'Arcy-Watt model). At the lowest RH and moisture contents, equivalent to hydration level I, water is bound tightly to ionic groups, and these sites saturate at low water contents. Between 10 and ~80% RH, moisture content increases linearly as water binds to more weakly binding hydrophilic (polar) sites (hydration level II). Above 70–80% RH, multimolecular layers of water begin to form that can bridge hydrophobic regions and water content increases steeply with RH in hydration levels III and IV (hydration level V is generally achieved only by contact with liquid water). The sum of these three components results in the characteristic reverse sigmoid shape of seed moisture sorption isotherms (Fig. 2.30).

The seed water content at a given equilibrium RH depends upon several factors. The one with the greatest influence is the seed composition, particularly the oil content. As water is excluded from the interior of the hydrophobic oil bodies, the water content of the seed at a given RH decreases as the oil content increases (Fig. 2.31a). Thus, cereals and other starchy seeds have relatively high moisture contents while lettuce or groundnut (peanut) seeds have comparably low moisture contents even in high RH. Physiologically and biophysically, however, the types of chemical and biochemical reactions that can occur in seeds are governed by the

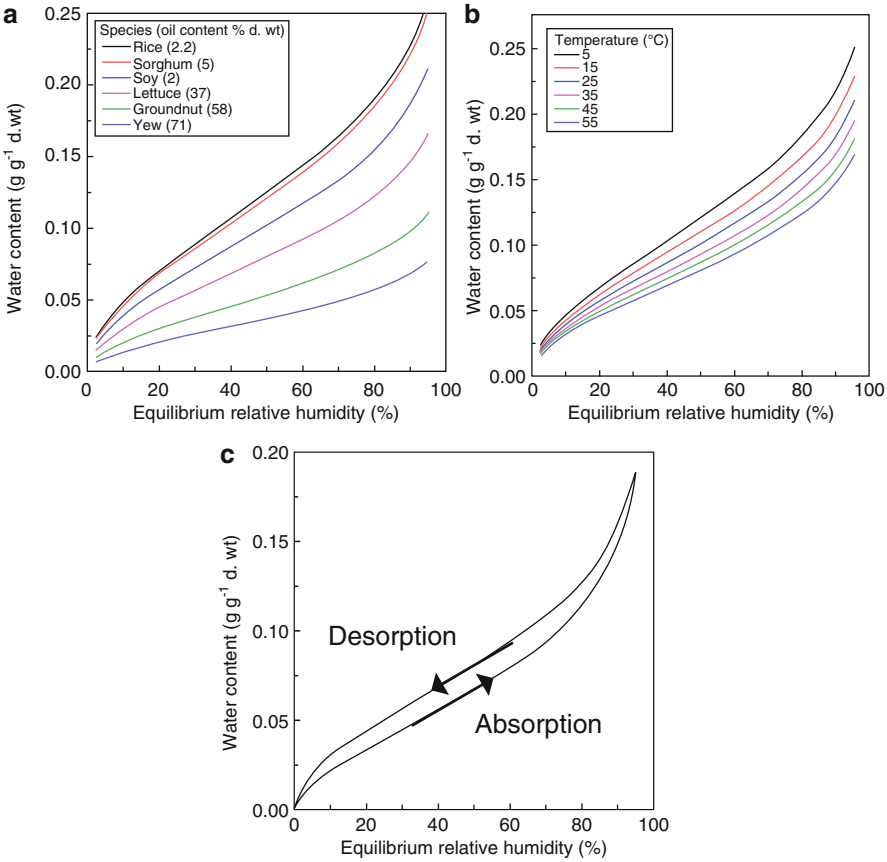


Fig. 2.31 Effects of seed composition, temperature, and hysteresis on moisture sorption isotherms. (**a**) The relationship between seed moisture content and equilibrium RH is markedly affected by seed oil content, as water is excluded from the oil bodies. Thus, as the oil content (% dry weight of seed) increases, the seed moisture content at a given RH decreases, as illustrated for seeds of several species. (**b**) The temperature at which the seeds are equilibrated also affects sorption isotherms, with the seed moisture content at a given RH decreasing as the temperature increases. (**c**) Whether the seed is losing water (*desorption curve*) or gaining water (*absorption curve*) also affects the isotherms, a phenomenon known as hysteresis. The difference in seed moisture content depending upon whether it is dried to a given RH or gains water from a lower RH can be as much as 0.5–1% on a dry weight basis. Figures compiled by F.R. Hay, International Rice Research Institute, The Philippines

hydration levels (RH or water activity) rather than by the absolute moisture content. That is, the volume of the seed that is not oil is hydrated to a similar extent in all seeds at the same RH or moisture content. Moisture sorption isotherms also are sensitive to temperature. As the temperature of equilibration increases, the seed moisture content at a given RH decreases (Fig. 2.31b). Finally, whether the seed is losing (desorption) or gaining water (absorption) also affects the isotherm. Isotherms

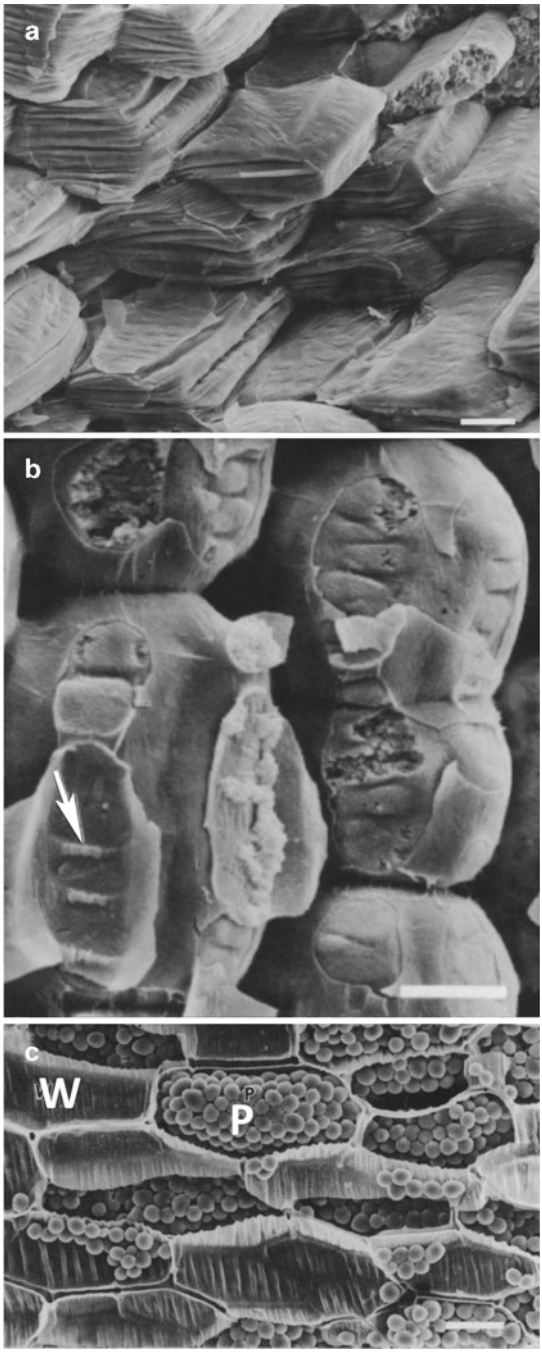


Fig. 2.32 Structure of cells in dry seeds. (**a**) Cotyledon cells of *Yucca angustifolia* seed that was fixed anhydrously using OsO_4 vapor to preserve structure in the absence of water. Note the collapse of the cells and folding of the cell walls to accommodate the volume reduction due to dehydration. The storage organelles within some cells are also visible, which limit the extent of collapse. (**b**) Aqueously fixed cotyledon cells in comparison with those in *panel a*, illustrating the volume expansion and stretching of the cell walls upon hydration. Thickenings occur on the inner cell wall in areas where the cells are in contact (*arrow*). (**c**) Cotyledon cells of zucchini prepared by acetone extraction, which removes the lipid reserves but leaves the protein storage vacuoles (P). Folding of the cell walls (W) and filling of the cells with storage vacuoles is evident. From Webb and Arnott (1982)

exhibit hysteresis, meaning that when seeds lose water and then regain it, their RH/moisture content relationship does not follow the same path (Fig. 2.31c). Moisture contents at a given RH are higher on a desorption isotherm than they are on an absorption isotherm. This represents a nonequilibrium situation, and theoretically seeds on the two isotherms would, over time, move toward a true equilibrium position. However, the time scales over which this occurs are very long, partly due to the high viscosity or vitrification of the tissue, which slows the structural adjustments toward equilibrium that presumably underlie the hysteresis in the isotherms. These relationships among RH, moisture content, seed composition, temperature, and hysteresis have practical importance in the drying and storing of seeds (Chap. 8).

As is suggested by the hysteresis between desorption and sorption isotherms, structural aspects of seeds affect their ability to bind water. Seed volume is also influenced, as mature dry seeds that are hydrated and then redried to the same initial moisture content do not shrink to their original volume. They do so only if they are dried to near hydration level I and then brought back up to their original moisture. Little is known about these structural aspects of dry seeds at the molecular level, although biophysical approaches are being applied to examine them. The importance of physical structure, and its maintenance in order to preserve viability upon rehydration, is evident from the appearance of cells in dry seeds that have been fixed and microscopically observed without contact with water (Fig. 2.32a). The cell walls fold and collapse against the cellular contents as the volume is reduced, in striking contrast to the taunt, stretched appearance of a hydrated cell (Fig. 2.32b). Mature seed tissues are filled with storage materials (Fig. 2.32c), so volume change upon water loss is less than for a typical vacuolated cell; this minimization of volume changes and their disruptive effects on cellular structure is an important component of desiccation tolerance.

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Chapter 3
Synthesis of Storage Reserves

Abstract The synthesis of stored reserves occurs during the seed growth period following histodifferentiation. The reserves are located within the embryo itself or in an alternative storage tissue, e.g., the endosperm of cereal grains and the cotyledons of dicot seed crops. Sucrose is the major source of carbon for reserve synthesis, and amino acids the nitrogen source; both are imported from the parent plant. The quality and yield of the storage reserves is considerably influenced by the prevailing environmental conditions before and during their synthesis. The biosynthetic pathways for starch, hemicelluloses, oils, and proteins are well documented, and there is an increasing understanding of the cellular pathways by which these reserves are imported into and sequestered within their unique storage organelles. Improvements to the quantity and nutritional quality of the reserves by genetic engineering rely upon a comprehensive understanding of their composition, synthesis, and regulation.

Keywords Synthesis of starch • Hemicelluloses • Oil • Protein • Phytin • Assimilate supply

3.1 Assimilates for Grain and Seed Filling

The extent of grain or seed filling in crops harvested for food is very important agronomically, and much research has gone into determining the factors that influence yield. Breeding for higher yielding seed crops has been undertaken for many years, and factors such as resistance to diseases, tolerance of stresses, efficiency of water and nutrient use, adaptation to plant density and shading, and translocation of carbon- and nitrogen-containing compounds, enter into consideration when trying to produce an ideal line. This large and intensively studied area of crop physiology can be given only cursory coverage here, and will largely be confined to the sources of carbon (C) and nitrogen (N) for the storage materials laid down within the seed, their translocation from the vegetative plant to the seed, and the effects of the environment on seed yield.

J.D. Bewley et al., *Seeds: Physiology of Development, Germination and Dormancy, 3rd Edition*, DOI 10.1007/978-1-4614-4693-4_3,

3.1.1 Sources of Nutrients for Storage Reserve Synthesis

Seed development proceeds by cell division and histodifferentiation to form the embryonic and endosperm tissues, followed by an influx of water that drives cell expansion, then deposition of storage reserves, and finally maturation drying as the seed ceases developmental events and becomes quiescent (Fig. 3.1). Filling of the cells that compose the storage organs, the endosperm in cereals and usually the cotyledons in seeds of dicot crops, occurs as the major storage reserves, starch, oils, and proteins, are synthesized.

Sucrose is the primary source of C for the developing seed. Much of this is provided by current photosynthesis in the leaves, although often there is accumulation of starch and other carbohydrates in the vegetative parts of the parent plant which are remobilized during seed filling. The contribution of these is very variable; for example in cereals it may provide up to 20% of the final dry weight of the grain, but generally in legumes the C assimilated before flower opening (anthesis) is proportionately much less readily available to the developing seeds than that produced by current photosynthesis. In some legumes, e.g., certain cultivars of soybean and field and garden pea, the translocated sucrose produced by photosynthesis in the leaves and pods may be stored temporarily as starch in the pod prior to remobilization and transfer to the developing seeds. In species that are cultivated for their edible pods, e.g., runner bean, the fleshy pod serves both as a photosynthetic capsule for recycling CO_2 respired by the seeds and as a permanent depository for ostensibly seed-bound sugars.

The current source of N for reserve deposition, like that of C, is variable. Cereals can accumulate up to 90% of their final N in the vegetative tissues before anthesis, and this is remobilized to the developing grains as those parts senesce. In legumes, about 75% of the N accumulated in the lupin seed is derived from symbiotic N-fixing activity after anthesis, whereas seeds of cowpea gain 69% of their N requirement from that fixed before anthesis, which is remobilized to the fruit from protein reserves in the leaflet.

The photosynthetic rates exhibited by different parts of the plant are quite variable among species. In wheat and barley, net photosynthesis in the flag leaf (that below but closest to the ear) and ear itself is relatively high, and these regions provide the major nutrient source to the grain, although each may make quantitatively different contributions in different crops and cultivars. In oats and rice, the flag leaf and penultimate leaf appear to be of equal importance in supplying assimilates for grain filling. Sugars produced by the leaves above the ear in corn are transported efficiently into the kernel, but that from leaves below the ears is poor. In general, the upper, or uppermost, leaves in cereals direct their assimilates mostly to the grains and the stem (the ears exclusively to the grain), and the lower leaves to the roots and tillers (seed-bearing stalks).

Storage reserve synthesis in legume seeds depends heavily on C fixed during fruiting itself, and thus seed yield is likely to be extremely sensitive to adverse environmental factors that reduce photosynthesis. The major source of C for the developing pea seed varies with time of development. During most of seed growth,

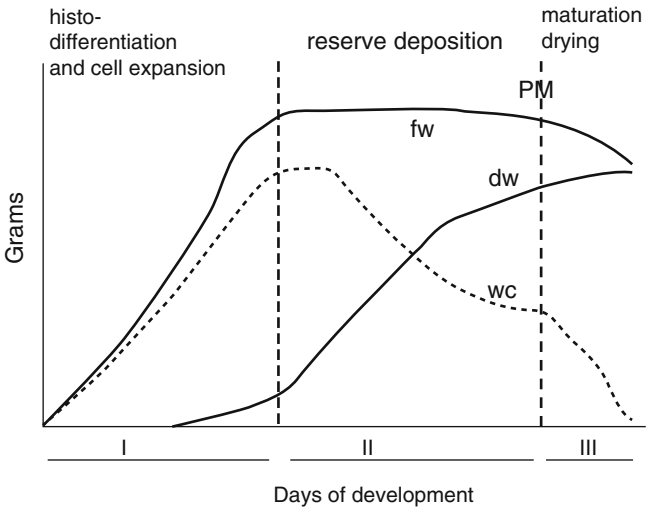

Fig. 3.1 Pattern of seed development to show the changes in whole-seed fresh weight (fw), dry weight (dw), and water content (wc). Three phases of development are: Phase I, gain in fresh weight due to cell division and expansion; Phase II, gain in dw due to expansion of the storage-reserve-containing cells and deposition of reserves therein. This results in a decline in wc as the insoluble reserves displace water from the cytoplasm; Phase III, loss of fw as the seeds undergo maturation drying. The time taken to complete each phase varies between species, cultivars, and with the ambient environmental conditions. Physiological maturity (PM), or mass maturity, is the time at which reserve deposition effectively ceases, and is at a wc that is considerably higher than when the seed is harvested

the pod, adjacent leaflets and stipule supply about two-thirds of the C required by the seeds borne at that node. In late development, they acquire most of their C from sources outside of the blossom node; at this time the seed may draw upon C remobilized from senescing tissues of the shoot and roots. Although green, the seeds themselves photosynthesize only weakly and lose as much C as CO_2 by respiration as they gain by photosynthesis; this CO_2 is often refixed in the pod. Other legumes exhibit different patterns in relation to their sources of C during fruit/seed set, for in some, e.g., cowpea, there is early senescence of the subtending leaflets and sources other than the blossom node provide most of the assimilates for seed filling.

Sucrose is the transported (translocated) form of sugar from the sites of photosynthesis or storage in the vegetative plant to the developing seeds of legumes and grains of cereals. In both, the major forms of N transported to them are the amino acids asparagine and glutamine; in some species there is also alanine. In cereals the ultimate source of N is generally fertilizer, but in legumes it is acquired as a result of fixation of gaseous N in root nodules. In soybean and cowpea about 10–15% of the transported N is as ureides (allantoin and allantoic acid); there are enzymes present in the seed coats of these species to convert them to asparagine and glutamine. Temporary storage of C may occur in the legume pod when its supply from the vegetative tissues outstrips demand by the seed, with the conversion of sucrose to starch, and amino acids may be converted to O-acetylhomoserine or homoserine.

3.1.2 *Import of Nutrients into the Developing Seed*

There is no direct vascular connection between the parent vegetative plant and the embryo within the seed. The vascular tissue, or veins, of which phloem is the major transporter of nutrients, and xylem of water, ends in the fruit coat or seed coat of dicots and in the placento-chalazal region of cereal grains. Nutrients (assimilates) are transported from the parent plant through the phloem to the seed coat or placental region, released into the extracellular space (apoplast) between the parental and filial tissues, and then imported by the developing endosperm and embryo.

The key features of this translocation pathway are shown in Fig. 3.2. In temperate cereals such as wheat and barley, assimilates are supplied via the vascular tissue in the furrow (crease) that runs the length of the grain. They must first pass through the placento-chalazal region, then through the nucellar projection, and finally through the aleurone layer before entering the starchy endosperm (Fig. 3.2a). Rice grains do not have a crease, so nutrients are transported into the developing grain in a single vascular bundle embedded in the pericarp. In maize and several other tropical cereals (e.g., sorghum, millet) assimilates are unloaded from the phloem terminals located at the base of the grain (the pedicel), where specialized transfer cells facilitate movement from this maternal tissue into the base of the developing endosperm (Fig. 3.2c). Transfer cells have ingrowths of the cell wall (Fig. 3.2d) and hence an increased plasma membrane surface area for the absorption or export of solutes, and are rich in mitochondria to provide energy for membrane transporters.

Assimilates required by legume seeds for reserve deposition in the cotyledons are translocated from the parent plant via a vascular strand that branches from the vascular tissue running through the pod. This strand then passes through the funiculus and into the seed coat (Fig. 3.2b). Passage of assimilates through the funiculus, and from the seed coat into the cotyledons is aided by the presence of transfer cells. The phloem in the seed coat, through which the assimilates are distributed, may consist of only one or two vascular strands (e.g., pea), or may exhibit an extensive, reticulate network (e.g., soybean). Movement of assimilates symplastically (i.e., within cells connected by plasmodesmata) cannot occur between the seed coat (maternal tissue) and the embryo; thus they have to pass from the phloem in the coat into an apoplastic space (i.e., outside of the cytosol-bounding plasma membrane) between the two generations. They are then taken up by the embryo, redistributed symplastically and utilized in reserve synthesis. There may be transfer cells in the outer layers of the exporting seed coat cells of several legumes (e.g., pea, *Vicia faba*, runner bean), but not always in the import cells of the cotyledons of the embryo (e.g., runner bean). Even in species where transfer cells do not occur (e.g., *Phaseolus* bean, rice) the cells of the maternal-filial interface are specialized for nutrient transport.

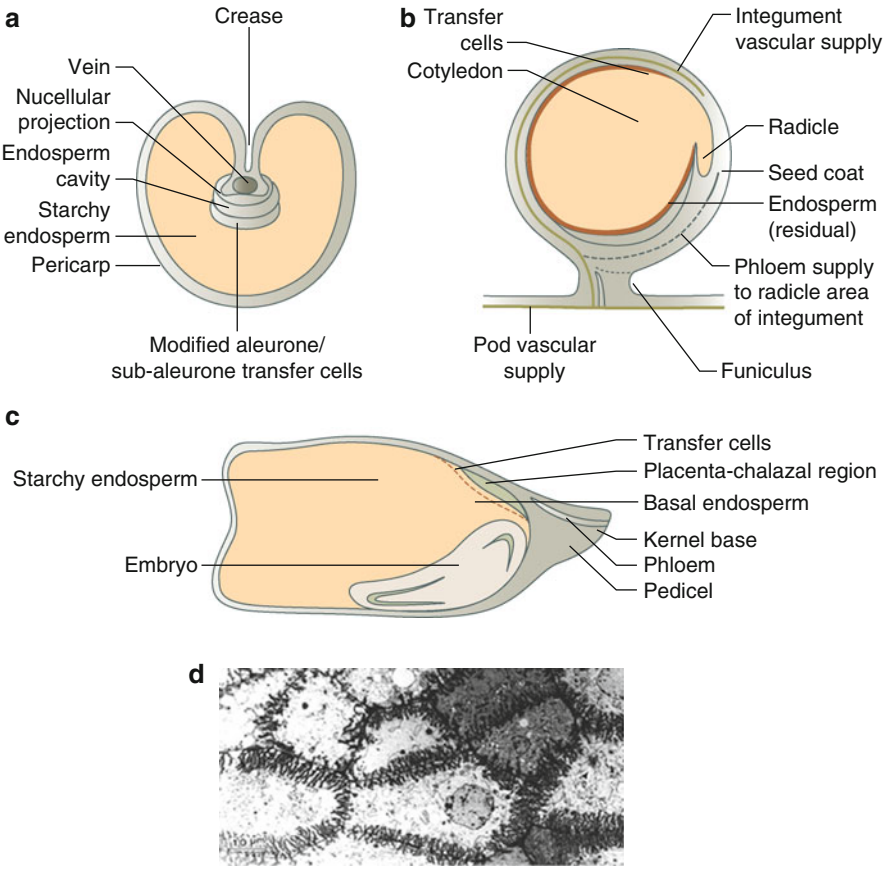

Fig. 3.2 (**a**) Cross sections through a developing wheat or barley grain at the midpoint between the apex and base to show the relationship between the vascular tissue and the starchy endosperm. Assimilates transported from the parent plant via the phloem are unloaded along the length of the furrow and pass symplastically through maternal tissues to the cells of the nucellar projection. There, transfer cells redirect assimilates to the apoplast (endosperm cavity); they now diffuse to the endosperm and are taken up into the outer layer where cells of the aleurone layer are modified into transfer cells. Transport now occurs symplastically to the starchy endosperm cells, where the assimilates are used for the synthesis of reserves. (**b**) Mid-saggital section of a developing seed of the garden pea to show the presence of vascular tissue in the seed coat. Assimilates pass from the phloem symplastically to the cells of the coat and are released into the apoplastic space into which the embryo has grown. From there they are taken up by the embryo and redistributed symplastically for use in reserve synthesis. (**c**) Assimilates entering the pedicel in maize are transferred symplastically from the phloem to the placento-chalazal region, which is a maternal tissue. There they are released from the cells and diffuse apoplastically to the endosperm transfer cells where they re-enter the symplast and are translocated throughout the endosperm. (**d**) Light micrograph showing ingrowths of the cell wall of transfer cells in the maize pedicel that result in an increased surface area of the wall and the plasma membrane, thus enhancing the ability of the cells to take up nutrients. From http://www.agron.missouri.edu/mnl/62/68davis.html

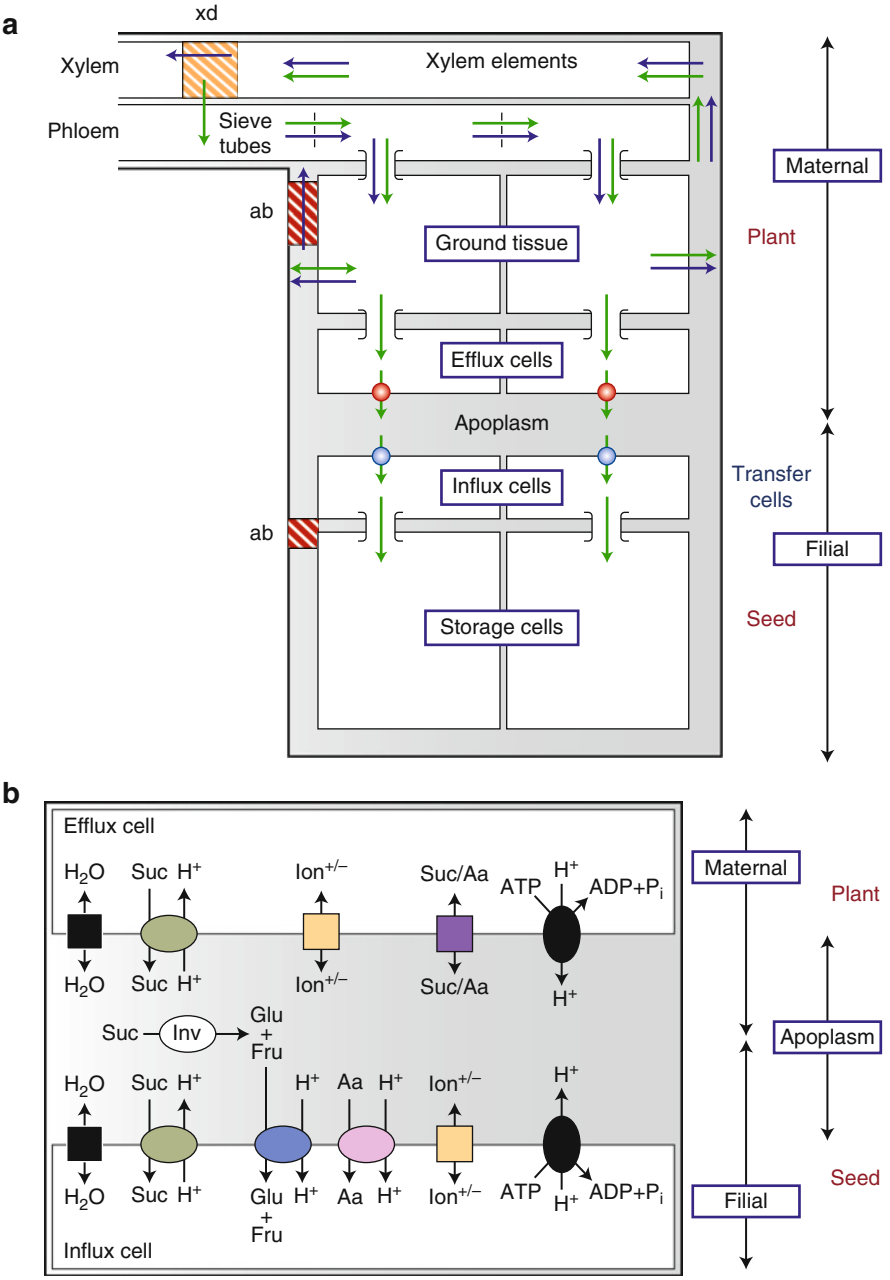

Fig. 3.3 (**a**) Diagram to demonstrate how nutrients and water are transported into the developing seed. Nutrients (⟵) and water (⟵) enter the seed maternal tissues via the phloem.

The pathway by which nutrients pass from the parental to the filial tissues in both cereals and legumes is modeled in Fig. 3.3 (details are given in the figure legend). Complex coordination is required for the import of assimilates into the phloem, their movement and subsequent release into maternal tissues and cells juxtaposed to the seed, export from these maternal cells, and finally import into the filial cells. This is necessary to ensure a steady flow of precursors for eventual conversion to storage carbohydrates, oils, and proteins.

The amides, glutamine and asparagine, may be deaminated in the seed coat to glutamic and aspartic acids, respectively, followed by conversion to other amino acids before import into the seed. But this deamination and subsequent production of the other approximately 18 amino acids present in storage proteins occurs more readily in the filial cells during the time when reserve synthesis is high. The amino group of the amide-containing amino acids provides the nitrogenous component for newly synthesized amino acids, and carbon skeletons are furnished by the translocated carbohydrates.

Invertases are present within the apoplast of the pedicel at the base of maize grain (Fig. 3.2c), which cleave sucrose to glucose and fructose; these products are taken up actively into the endosperm cells and resynthesized to sucrose prior to utilization in starch biosynthesis and other metabolic pathways. This hydrolysis of sucrose in the maternal tissue is an integral part of the transport mechanism of maize, and

Fig. 3.3 (continued) They are unloaded symplastically from the phloem via plasmodesmata ( ) into adjacent cells. They then move symplastically to cells at the interface between the maternal tissue (efflux cells that are often transfer cells) and the apoplast, into which they are released by transporters in the plasma membrane ( ). Retrieval of the nutrients from the apoplast involves transporters also, which are present in membranes of the influx cells (again often transfer cells) on the surface of the filial embryo. These are then redistributed symplastically to where the reserves are synthesized. Excess water is returned to the maternal plant via the xylem, while loss of nutrients from the seed is prevented by xylem discontinuities (xd) and apoplastic barriers (ab). (**b**) An illustration of some important nutrient transporters in the plasma membranes of the maternal efflux cells and the filial influx cells of developing seeds. Sucrose (Suc) is released from the maternal cells into the apoplasm via a sucrose/H^+ ion antiporter or through a poorly selective channel that also transports amino acids (Aa). The Suc may be imported directly into the filial cells by sucrose/proton symporter, or after hydrolysis to glucose and fructose (Glu, Fru) by an extracellular invertase (Inv), in which case they are taken up by a hexose/proton symporter. Apoplastic Aa are taken up into the filial cells by an Aa/proton symporter. There are also poorly selective ion channels, water-transporting channels (aquaporins), and proton pumping ATPases that may act to build up a proton gradient across the plasma membrane. From an article entitled Development of seeds—Nutrients and water import, by Throne-Holst, M., Offler, C.E. and Patrick, J.W. in *The Encyclopedia of Seeds. Science, Technology and Uses*, Black et al. (2006), see Advanced Literature references in Chap. 1. Courtesy of CAB International; also based in part on Patrick and Offler (2001)

mutants (*miniature*-1) lacking invertase exhibit impaired phloem unloading and aberrant development of both the endosperm and the pedicel itself. One possible reason for the damage to the pedicel is that, because of the lack of invertase, sucrose accumulates and upsets the osmotic balance of the cells. Because the phloem system operates by pressure-driven flow, unloading of solutes from the phloem cells will lower their turgor and maintain a pressure gradient for transport to those unloading sites. The conversion of sucrose to glucose and fructose in the apoplast doubles the osmotic effect of unloaded sucrose and assists in osmotically drawing water from the phloem and reducing its internal pressure. Maintaining a high apoplastic solute content in unloading tissues may be an important component determining the relative strengths among competing sinks. In wheat, rice, and barley, sucrose itself is absorbed from the apoplast of the maternal tissues into the developing endosperm. Transport of assimilates into the developing cereal grains usually ceases when they become swollen with the deposited reserves, and the regions in which the phloem is located are crushed and nonfunctional.

In broad (faba) bean a cell wall-associated invertase present in the chalazal vein and testa during early seed development converts the entering sucrose to hexoses, and these promote cell division, enhancing development of the embryo. As the seed develops further, cell wall invertase activity declines, as does expression of its gene, fewer hexoses are produced, cell division ceases because histodifferentiation is completed (Fig. 3.1), and the major imported sugar, which is now sucrose, is utilized for the synthesis of starch reserves. Concurrently, the gene for invertase in the cotyledons increases in expression. This change from a high-hexose to a high-sucrose environment is not the driving force for development of the embryo and reserve synthesis in rapeseed (canola), however. In rice, genes for three invertases are expressed during grain development in a similar temporal pattern, but each is spatially different in its distribution in the coat, embryo, or endosperm. At early- and mid-stages of development their expression is high, declining as the grain matures and reserve synthesis declines. It is likely that the different invertases play complementary/synergetic roles in assimilate unloading and loading during grain filling.

3.1.3 Factors Affecting Seed Production and Quality

Of interest for growers of food crops is the quality and quantity (yield) of seed obtained in a growing season. Yield for seed crops is the product of seed number multiplied by seed weight, so the number of ovules produced, the percentage of these that are fertilized and complete development, and the final seed weight all contribute to it. Most plants produce many more ovules than they can support through to maturity. Some are not fertilized, while others may be aborted early in development if insufficient assimilates are provided soon after fertilization. In addition, plants may shed partially developed seeds/fruits to adjust sink load to source size, as in many fruit trees. Management practices such as inflorescence/fruit

thinning enhance size of the remaining seeds/fruits by reducing competition for the available photosynthetic resources. Seed growth rate and seed-fill duration are genetically controlled in most crop species, but they are also affected by the environment. Most genetic differences in mature seed size are manifested as differences in total cell number and seed growth rate; e.g., large seeds have more cells and a higher growth rate than small seeds. Suppression of expression of some embryo-specific transcription factors early in seed development, such as APETALA2 and REVOLUTA, can increase seed size, associated with increased cell division and embryo size in the case of APETALA2. However, variation in mature seed size can also be a function of seed-fill duration, due to a complex relationship between seed metabolic activity (the strength of a seed to act as a sink for assimilates) and the parent plant (its ability to act as a source tissue for these assimilates).

The environment can influence seed growth directly by altering the ability of the seed to accumulate dry matter (sink effect) or, perhaps more predominantly, indirectly by changing the ability of the parent plant to supply assimilates (source effect). Stresses such as water deficits, low or high temperature, nutrient deprivation, and shading can occur at any time during seed development. Sometimes more than one stress may be experienced by plants, e.g., high temperature and water stress, and the impact of these on seed development may be magnified and be greater than the sum of the individual stresses. Duration of the stress is also an important consideration as well as the time (stage) during seed development that it occurs. Bearing in mind all these variations and permutations in the stresses that plant may experience, it is not surprising that the huge literature on this subject contains quite a number of seemingly contradictory conclusions. A few generalizations are made here, but there will be exceptions.

(1) *Light environment* impinging on the crop canopy plays a major role in determining photosynthetic activity of a given plant part. The developing ears of barley and wheat are fully exposed to sunlight and can realize their full photosynthetic potential. In contrast, the ears of certain improved rice cultivars have the tendency to bend and be positioned below the flag leaf; hence they are heavily shaded and make an insignificant contribution to the total assimilates utilized in grain filling. It is notoriously difficult to estimate precisely the photosynthetic contribution by different parts of the plant in a crop canopy under field conditions, but factors such as leaf area index, leaf age and longevity, angle of incidence to the sun, shading, ear structure (awned or awnless), and amount of carbohydrate stored in the parent plant, all play roles in final seed fill, as well as environmental variables like temperature and availability of nutrients and waters. A reduction in canopy photosynthesis during seed or grain filling, once their number is fixed in the head or pod, will reduce mature seed size and yield; this is reversible if light conditions improve. The importance of light interception and plant modifications to maximize its use in photosynthesis can be seen in the fact that the approximate doubling of maize yield in the past 40 years has been due largely to increasing plant populations per unit area, from ~50,000 plants/hectare (ha) in the early 1970s to ~80,000 plants/ha in 2010 in high-yield environments. In general, increasing plant populations will increase yield

until mutual shading by adjacent plants decreases seed production per plant due to assimilate limitation. Breeding for increased seed yield in maize has resulted in plant types that can be crowded closer together, yet still intercept sufficient light to fill the grains while not becoming too tall and lodging (falling down).

(2) *Longevity* of the green tissue in the parent plant during grain development may be a factor in yield. Rice leaves remain green almost until grain maturity, whereas the ear turns yellow fairly early during ripening. In wheat, however, the leaves become yellow (i.e., senesce) before the ear does, and the latter may be the more important in providing photosynthate at the late stages of maturity. At this time, however, respiration by the grain may exceed the net import of sugars and other substrates, resulting in a small loss in grain weight. A specific gene in wheat (*Gpc-B1*) encoding a transcription factor (NAM-B1) accelerates leaf senescence and increases nutrient remobilization from leaves to developing grains. This gene is active in ancestral wheat genotypes, but is nonfunctional in all modern wheat cultivars; introducing the functional allele into these increases grain protein, zinc and iron content without reducing grain yield. In soybean, on the other hand, seed yield is more dependent upon current photosynthesis during seed filling, and delaying leaf senescence may be more beneficial for seed yield.

(3) *Position* of the seed on the plant, pod, head, ear, etc., may affect seed growth rate and seed-fill duration. Seeds receiving an insufficient nutrient supply during early growth may abort, whereas at later stages this results in smaller seeds. Seeds from late-developing flowers may be smaller because of a shorter filling period. Those seeds furthest away from the source of assimilates, e.g., at the distal end of a pod (legume) or cob (maize) may remain small because the amount reaching them is insufficient to support maximum seed growth potential.

(4) *Water stress* has little direct effect on seed development and filling, but has major indirect effects, such as reduction of photosynthesis and acceleration of leaf senescence. Drought reduces the number of primordia that are produced during development of the inflorescence of cereals, resulting in a reduction in the total number of grains. If the stress occurs at the time of anthesis and fertilization, pollen production is affected, and the ability to form receptive stigmas, e.g., silks in maize, can also suffer. Water stress during anthesis (flower opening) can also result in abortion of ovules after fertilization, which is due to insufficient sucrose supply (Fig. 3.4). Cell division (hence cell number) and cell enlargement following fertilization determine the size and storage capacity of the grain, and both can be adversely affected by water and heat stress. The occurrence of water stress at certain stages of development of wild oat grains can lead to a reduction in their dormancy at maturity.

Drought during the early stages of soybean seed development results in a decrease in the number of pods per plant because of the induction of abortion and abscission when the pod fails to expand. At later stages this water stress can affect the photosynthetic activity of the vegetative plant, and reduced production of assimilates for the developing seed results in reduced yield. However, water stress can have differential effects on seed composition: protein content in soybean seeds developed under stress is proportionately increased because its accumulation is reduced by about half as much as that of oil (9% vs. 20% reduction). This is explained by the

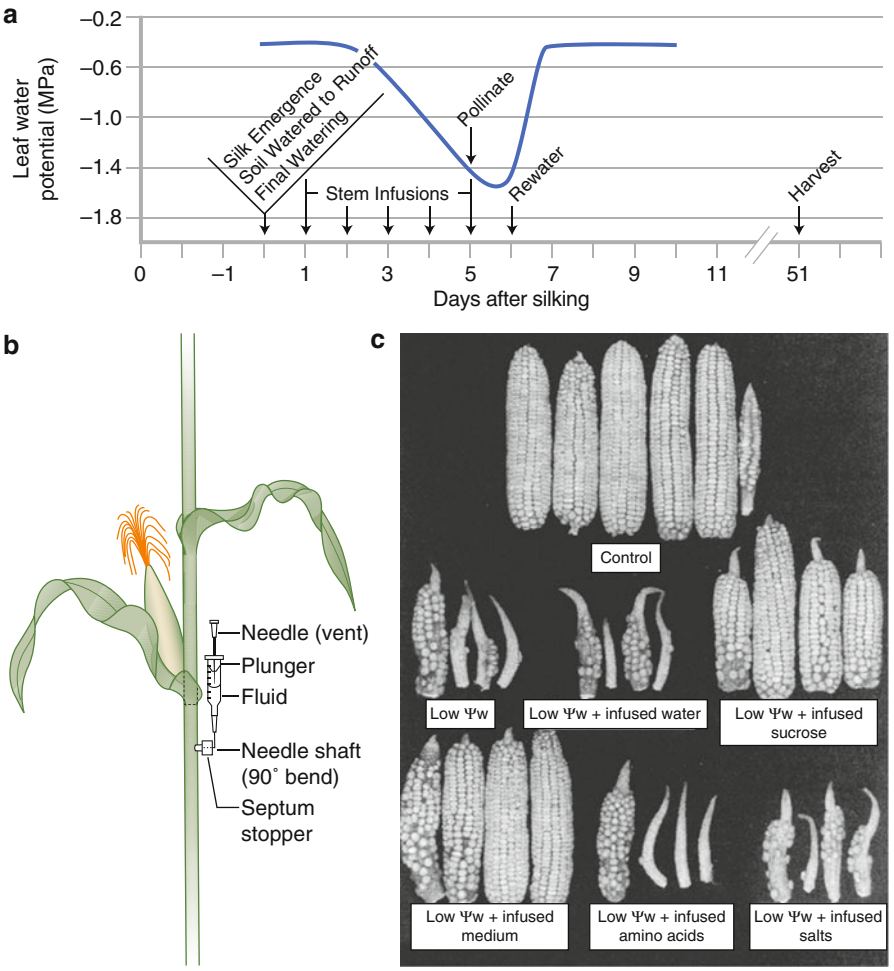

Fig. 3.4 Demonstration that reduced assimilate supply during water stress is responsible for kernel abortion in maize. (**a**) Experimental plan: water was withheld from maize plants at the beginning of silking (emergence of the styles and stigmas from the ear), resulting in decreasing leaf water potential. Twice daily for 5 days, up to the time of pollination, the stems were infused with water or other solutions to test their effect on embryo abortion following subsequent fertilization. (**b**) The stem infusion technique, where small amounts of water or other solutions were introduced into the stems of intact plants below the ear. (**c**) Consequences of water stress on seed abortion due to water stress before pollination, and its reversal by sucrose. The control ears are from plants that did not experience water stress. The Low Ψ_w (water potential) ears show the severe effect of brief water stress at pollination in causing seed abortion. Infusing water only (Low Ψ_w + infused water) did not reverse this, as the amount of water supplied is minimal compared to plant water use. However, infusing a sucrose solution (Low Ψ_w + infused sucrose) almost completely restored fertility and kernel production. Similarly, complete nutrient medium including sucrose was effective, whereas the addition of only amino acids or only nutrient salts was ineffective. These experiments, and others using shading, leaf removal, etc., have clearly demonstrated that the assimilate supply immediately before fertilization has a major influence later on the retention or abortion of embryos. From Zinselmeier et al. (1995) and Boyle et al. (1991). Courtesy of the American Society of Agronomy-Crop Science Society of America-Soil Science Society of America

dependence of N supplies to the seed on remobilization from vegetative tissues and ongoing supplies from the roots, whereas carbohydrate assimilates for oil biosynthesis are more dependent upon current photosynthesis, which is reduced under water stress due to stomatal closure. Seed viability and vigor may also be adversely affected by water stress late in seed development.

Water stress can also accelerate leaf senescence, which can impact assimilate supply for seed fill. Cytokinins, plant hormones associated with cell division, can delay senescence of plant tissues and increase sink strength to attract assimilates. Expression in rice of the *IPT* gene encoding an enzyme that enhances cytokinin synthesis (isopentenyltransferase), and which is under the control of a senescence-responsive promoter (P_{SARK}), results in a marked decrease in leaf senesce and maintenance of seed yield in plants subjected to either pre- or post-anthesis water deficit (Fig. 3.5). This is because cytokinin prevents the degradation of photosynthetic protein complexes in the leaves during drought, enabling recovery of photosynthetic activity following rewatering to provide assimilates for grain filling.

(5) *Temperatures* that are too high or too low can affect directly the growth rate of a seed through altering its metabolic activity; there are also indirect influences such as reduction of photosynthesis by the parent plant, thus lessening the amount of sugar imported into the seed. The duration of grain filling is shortened at high (over 30°C) temperatures in wheat; this is probably a consequence of accelerated development, and because the rate of grain filling does not increase proportionately to the larger number of cells that are produced there is diminished grain weight (yield) at maturity. As the temperature experienced by an ear of wheat increases from 15 to 30°C, the proportion of protein storage reserves laid down increases relative to starch deposition, although there is a decline in the synthesis of both.

Low-temperature stress during development of some oil-crop seeds results in both quantitative and qualitative changes in their major reserve, with implications for the quality of extracted oil. A nonlethal frost during late stages of development of rapeseed prevents the testa from losing its green color. Chlorophyll contaminates the oil during commercial extraction, and processing to remove it reduces profitability.

3.2 Deposition of Reserves Within Storage Tissues

Most mature seeds contain at least two or three stored reserves in appreciable quantities (Table 1.2), and to a large extent they are synthesized concomitantly during seed development. However, for the sake of clarity and convenience, the synthesis and deposition of each of the major reserves is discussed singly—carbohydrates, oils (triacylglycerols, TAGs), and proteins—and the important minor reserve, phytin.

An interesting question, however, is what determines the types and proportions of the reserves laid down. Since seeds produce at least two different major reserves in the same tissue, or in some cases three in different tissues, they obviously possess

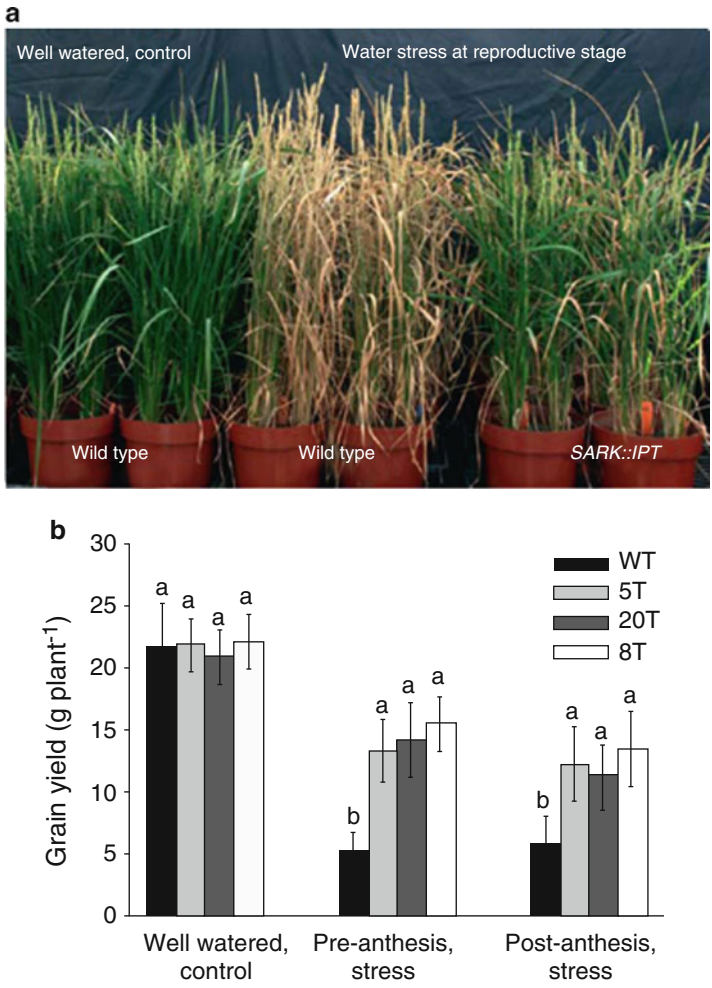

Fig. 3.5 Effects of pre- and post-anthesis water stress on recovery and subsequent grain production by rice plants. (**a**) Wild-type plants exhibit severe leaf senescence and yield loss due to water stress followed by rewatering (compare well-watered control to water-stressed wild-type plants). Transgenic plants expressing a gene enhancing cytokinin biosynthesis (*SARK*::*IPT*), on the other hand, survive drought stress with less leaf senescence. (**b**) Grain yields of wild type (WT) and three different *SARK*::*IPT* lines (5T, 20T, and 8T) under well-watered and pre-anthesis (panicle elongation) or post-anthesis (2 weeks after flowering) water stress (6–10 days without watering). Although still reduced relative to the well-watered condition, the *SARK*::*IPT* plants have approximately double the grain yield following stress as the control plants. Letters a, b above the error bars indicate significant differences within each treatment. From Peleg and Blumwald (2011) and Peleg et al. (2011). Courtesy of Elsevier and Wiley, respectively

multiple biochemical capacities for synthesis. Processes must operate to allocate incoming assimilates to different reserve components in precise quantities and ratios; membrane transporters specific to different cell types, and to organelles within the cells, can determine the source materials for metabolism. Differential and coordinated expression of genes for enzymes for synthetic events can control where and when a particular anabolic pathway operates. Posttranslational regulation of enzyme activity, and cellular energy production (ATP) through respiration, also operate to control the flow of assimilates and intermediates into and within a metabolic pathway. An excellent example of this is illustrated in Fig. 3.6, which shows that there is a strong correlation between the presence of O_2, the production of ATP, and the synthesis of starch in specific regions of the developing barley grain. Even in regions where there is hypoxia, however, there are metabolic adjustments to ensure that pyruvate accumulation and fermentation do not occur.

During the development of seeds of some legumes, e.g., soybean, and of rapeseed, there is initially the synthesis of storage starch, followed by that of the major oil reserves. Synthesis of the former is less sensitive to cellular O_2 concentrations than the latter, which has a greater requirement for the production of ATP energy. The amount of O_2 present in the seed during early development is less than during the later stages; thus the change from starch to oil synthesis as development proceeds could be due a resultant increase in energy status of the cotyledon cells producing these storage reserves. The O_2 balance, in turn, may be controlled by nitric oxide (NO), the synthesis and stability of which increases in cells where there is low O_2, causing elevated NO:O_2 ratios. NO inhibits mitochondrial electron transport via the cytochrome pathway, thus lowering ATP production and the capacity to synthesize storage oils. The roles of O_2 and NO in respiration during germination are discussed in Sect. 4.4.4.

As a prelude to a consideration of the metabolic changes that occur in the seed as its reserve content is increasing, it is necessary to place these events into perspective with respect to the overall changes that occur during seed development (Fig. 3.1). The seed grows initially from a single fertilized egg (zygote) into a multicellular embryo by cell division and differentiation (Sect. 2.2). These events, collectively termed histodifferentiation, along with early cell expansion are marked by a rapid increase in whole-seed fresh weight and water content (Fig. 3.1). A period of rapid gain in dry weight then follows due to the synthesis and deposition of the stored reserves; cells expand to accommodate these reserves. The whole seed fresh weight remains relatively stable, although the seed loses water as this is displaced by the accumulating insoluble reserves within the cells of the storage tissues. The decline in water content slows as the seed approaches its maximum dry weight. Finally, the seed undergoes maturation drying and approaches the quiescent stage, when it may be shed from the parent plant, and there is a period of fresh weight loss accompanied by a rapid decline in whole seed water content. The duration of each of the major phases of development (Fig. 3.1) varies from several days to many months, depending upon species and prevailing environmental conditions.

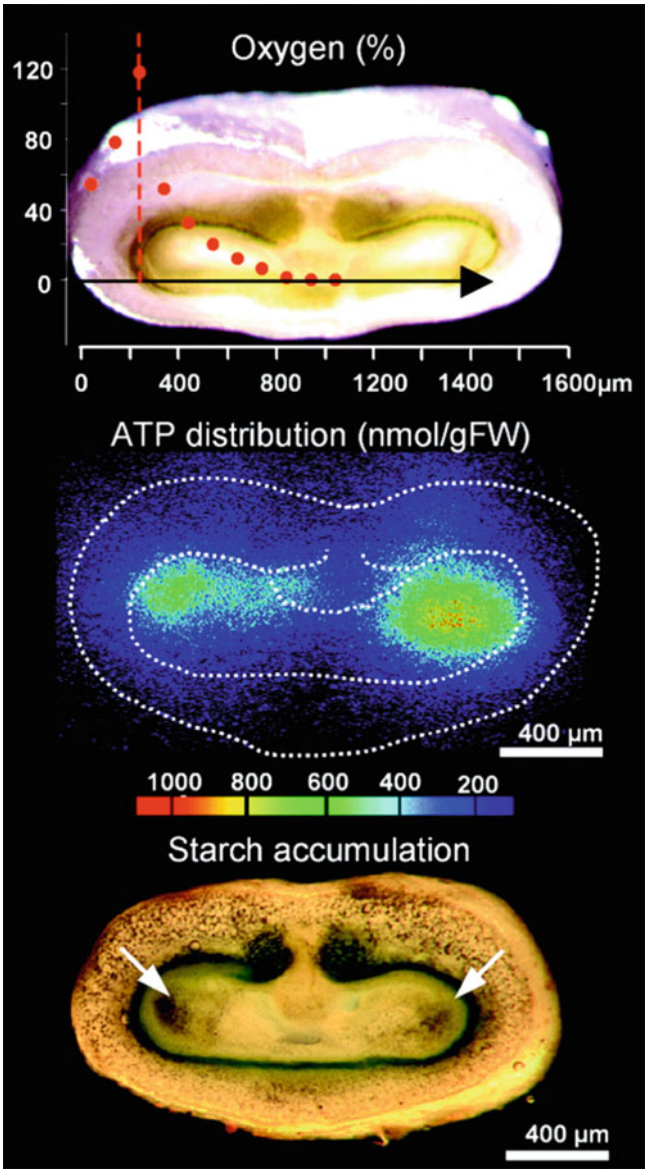

Fig. 3.6 Topographical comparison of the concentrations of O_2 and ATP with the synthesis of starch in the developing endosperm of barley. *Top picture*: the highest amount of O_2 is within the chlorophyll layer of the tissues (pericarp) surrounding the barley grain; it decreases from the outside to the inside of the endosperm. *Lower picture*: Synthesis of starch occurs in the lateral regions (*white arrows*) of the grain where O_2-dependent ATP production is the highest (*intense green* in the *middle figure*). From Rolletschek et al. (2004). With permission of Oxford Univ. Press

3.2.1 Starch Synthesis

Sucrose, the sugar translocated into the seed, is the substrate for starch formation, which is present in amyloplasts as the straight-chain amylose and the highly branched amylopectin (Fig. 1.2a). First it is converted in the cytoplasm (cytosol) of the starch-synthesizing cells to fructose (Fru) and UDPG1c (uridine diphosphoglucose) by sucrose synthase (sucrose-UDP glucosyl transferase) as follows:

$$\text{Sucrose} + \text{UDP} \leftrightarrow \text{Fru} + \text{UDPG1c}$$

The fructose is phosphorylated by a hexokinase to Fru-6-P, which is changed to Glc-6-P by hexose phosphate isomerase, and to G1c-1-P by phosphoglucomutase. The UDPG1c is also converted to Glc-1-P by UDPG1c pyrophosphorylase (UGPase).

$$\begin{array}{c} \text{UDPGlc} + \text{PPi} \leftrightarrow \text{Glc-1-P} + \text{UTP} \\ \updownarrow \\ \text{Fru} \rightarrow \text{Fru-6-P} \leftrightarrow \text{Glc-6-P} \end{array}$$

The first committed step in the synthesis of starch is the conversion of Glc-1-P to ADPG1c by ADPG1c pyrophosphorylase (AGPase) as follows:

$$\text{Glc-1-P} + \text{ATP} \leftrightarrow \text{ADPG1c} + \text{PPi}$$

This conversion to ADPGlc occurs in the amyloplasts of the storage cells of dicots (Fig. 3.7a), whereas this reaction takes place in both the cytosol and the plastids in the endosperm cells of cereal grains (Fig. 3.7b). The by-product of this reaction, inorganic phosphate (Pi, from pyrophosphate, PPi), inhibits the activity of ADPGlc, providing a negative feedback regulation of starch synthesis (a good example of product inhibition of an enzyme reaction), which may be important for the maternal plant to control filling of individual seeds. In mutants of corn that have a low AGPase activity (*shrunken-2*, *sh-2*) there is a large reduction in starch synthesis and an accumulation of unutilized sucrose in the endosperm, resulting in a "supersweet" type of sweet corn. Because these kernels have little starch and high water content, they collapse upon drying, resulting in the "shrunken" kernels. On the other hand, expression in the cytoplasm of maize and rice endosperm cells of a bacterial AGPase having higher activity than the plant enzyme results in increases of up to 25% in seed size without a reduction in seed number. This is consistent with studies suggesting that seed growth and crop yields in cereals may be sink limited rather than source limited.

The ADPG1c donates its glucose to the nonreducing end of a small primer (an $\alpha(1 \rightarrow 4)$-linked chain of Glc) thus increasing its chain length by one unit. The process is repeated until the amylose molecule is completed, the enzymes involved being a number of starch synthases (ADPG1c-starch glucosyltransferases), as follows:

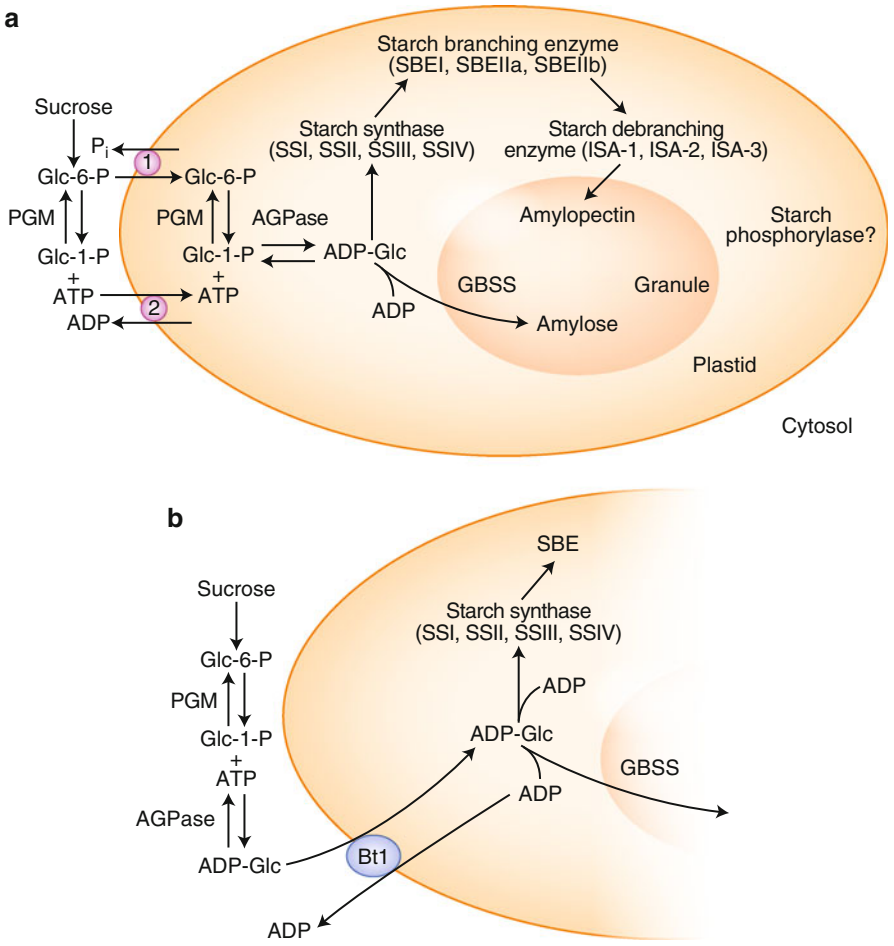

Fig. 3.7 (**a**) The pathway of starch biosynthesis in storage cells of dicot seeds, where AGPase is exclusively localized in the plastid (amyloplast). Hexose-phosphates and ATP are imported into the plastid from the cytosol via, respectively, the Glc-6-P/Pi antiporter (1) and the ATP/ADP transporter (2) located in the inner envelope membrane. Cytosolic and plastidial isoforms of phosphoglucomutase interconvert Glc-6-P and Glc-1-P. The antiport substrates for these transporters are generated from plastidial reactions involved in starch synthesis; Pi is generated from pyrophosphate produced by AGPase, and ADP is a by-product of the starch synthase reaction. ADP-Glc is utilized by the SSs, and the glucan chain branched and debranched by SBEs and debranching enzymes, respectively, to form amylopectin within the granule that is growing within the amyloplast. Granule-bound starch synthase also uses ADP-Glc, and is exclusively involved in amylose synthesis. (**b**) The initial pathway of starch synthesis in the endosperms of monocots such as cereals. These plants possess a cytosolic form of AGPase and import ADP-Glc from the cytosol via an ADP-Glc/ADP transporter termed Bt1. Monocot seeds also possess a plastidial form of AGPase (as in **a**), but much of the AGPase is present in the cytosol. The pathways after the synthesis of ADP-Glc are the same as in (**a**). Glc-6-P, glucose-6-phosphate; Pi, inorganic phosphate; Glc-1-P, glucose-1-phosphate; PGM, phosphoglucomutase; ADP-Glc, adenosine diphosphoglucose; AGPase, ADPG1c pyrophosphorylase; SS, starch synthase; SBE, starch branching enzyme; ISA-1 to 3, isoamylases; GBSS, granule-bound starch synthase. Based on Tetlow (2011). Reprinted with permission of Cambridge Univ. Press

$$\text{ADPG1c} + \text{Glc}_{(n)} \leftrightarrow \text{Glc}_{(n+1)} + \text{ADP}$$

These starch synthases are active within the stroma of the amyloplast (soluble starch synthase, SS) or within the starch granule itself (granule-bound starch synthase, GBSS) (Figs. 3.7–3.9). GBSS exclusively increases the length of the amylose chains; *waxy* mutants of cereals, e.g., maize, which lack the gene for GBSS fail to produce amylose. There are several starch synthase isoforms (SSI-SSIV, encoded by different genes) within the stroma of the amyloplast, and each plays a different role in amylopectin synthesis. SSI is responsible for the synthesis of short chains of 10 Glc residues or less (DP, degree of polymerization, <10), and extension of these glucan chains is achieved by the activities of SSII and SSIII, each of which acts on progressively longer chains. The *sugary-2* (*su-2*) mutant of maize, a sweet corn, lacks SSII and therefore produces low amounts of starch, more short chains with a DP of 6–10, and fewer chains that are 12–30 units in length; amylose content is elevated also, as is sucrose content. The *du1* mutants of rice and maize (lacking SSIII activity) have altered starch granule morphology and crystalline structure resulting from changes in amylopectin, and fewer glucan chains longer than DP 30, indicative of a role for this enzyme in their elongation. SSIV may play a role in the initiation (nucleation) of the granule, producing the first short glucan chains to which the other synthases attach the Glc units, although it likely contributes to this in conjunction with SSIII.

Synthesis of the branched form of starch, amylopectin, requires the presence of starch branching enzymes (SBEI, SBEIIa,b) (Figs. 3.7–3.9), which cleave internal $\alpha(1\rightarrow4)$ bonds on the amylose molecule to generate short chains that are then attached by $\alpha(1\rightarrow6)$-linkages to the same or adjacent amylose chains. These chains then have more Glc units added to the nonreducing end by the SS enzymes. SBEII enzymes transfer shorter chains and show a higher affinity for adding them to amylopectin than SBEI, which favors the branching of amylose. Mutations to the SBE genes lead to a variety of different endosperm phenotypes. The *amylose extender* (*ae*) mutation of SBEIIb of maize and rice results in fewer branch points in amylopectin, and a predominance of amylose; in wheat this occurs only when both SBEII genes are suppressed. The sweeter wrinkled pea seed mutant (*rugosus*) lacks a functional SBEI gene; consequently it contains less starch, and more sucrose and amylose. Because the cells of the endosperm or cotyledons are not packed with starch at maturity, the seed shrinks and becomes wrinkled following maturation drying. In the wrinkled pea, now known to be the recessive (*rr*) trait identified in Mendel's experiments, the failure to synthesize SBEI is due to an insertion of a transposon into its gene, which renders it transcriptionally nonfunctional.

The initial form of amylopectin that is formed is soluble and highly branched, as phytoglycogen (Fig. 3.8). This is "trimmed" by debranching enzymes (DBE), which are more usually termed isoamylases (ISA-1-ISA-3); these enzymes aggregate to form an active complex that removes the short branches. This results in amylopectin to which more Glc units and branches can be added. Mutations in the isoamylase genes (*isa1-3*) lead to the accumulation of the aberrantly branched phytoglycogen,

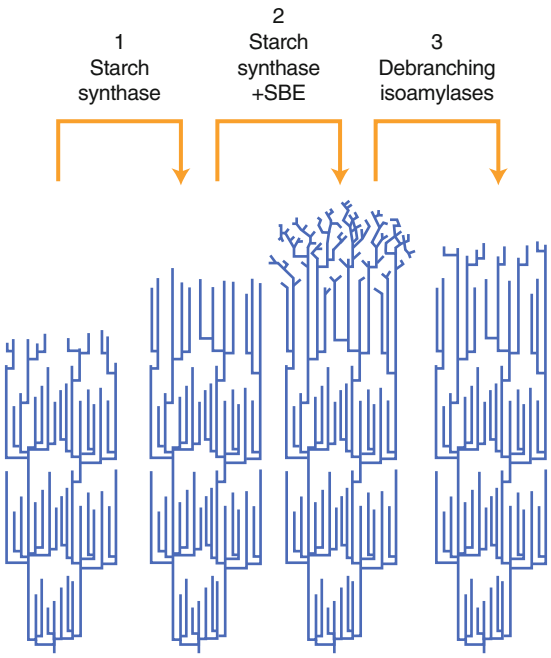

Fig. 3.8 The glucan trimming model illustrating how mature amylopectin is formed. Step 1. Short chains are elongated by starch synthases. Step 2. When chains reach sufficient length they become substrates for starch branching enzymes (SBE) and a highly branched preamylopectin (phytoglycogen) is formed. Step 3. Selective trimming of this structure by debranching isoamylases forms more short chains from which the next round of elongation and branching can occur. From Smith (1999). Courtesy of Elsevier

rather than starch, and a poorly defined granule structure. The *sugary-1* (*su-1*) mutant genotype of sweet corn lacks ISA-1.

Because of the coordinated activities of the stromal starch synthases and starch branching enzymes to produce amylopectin, it is not surprising that these form an active synthetic complex. Formation of one such complex occurs between SSI, SSII, and SBEII in the endosperms of wheat and maize, and requires that the SBEIIb component of this complex be initially phosphorylated (Fig. 3.9). Dephosphorylation causes dissociation of the complex and loss of synthetic activity. The debranching isoamylases do not appear to be associated with the complex.

An additional synthetic pathway involving starch phosphorylase(s) occurs in amyloplasts, as follows:

$$\text{Glc-1-P} + \text{Glc}_{(n)} \leftrightarrow \text{Glc}_{(n+1)} + \text{Pi}$$

While there are correlations between starch biosynthesis and the transcription of the gene for this enzyme and its activity, its importance in the overall synthetic

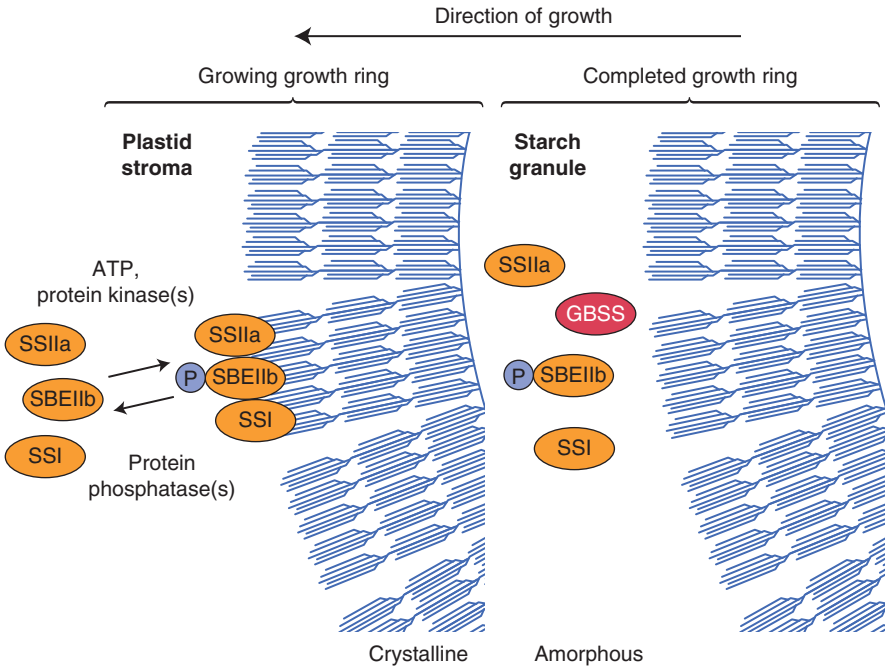

Fig. 3.9 Protein–protein interactions between amylopectin-synthesizing enzymes during starch granule formation in cereal grain endosperms. To the left, the assembly of the active protein complex (in wheat and maize) of SBEII, SSI, and SSIIa is dependent on protein phosphorylation. In maize, SBEIIb is phosphorylated (denoted as P in the diagram). In vitro dephosphorylation with alkaline phosphatase causes disassociation of the protein complex; in vivo this is presumably performed by a plastidal protein phosphatase. It is proposed that the SS/SBEII protein complexes work at the periphery of the nascent granule, being involved in the assembly of amylopectin chains that form the crystalline regions of the starch granules, and eventually the amorphous region within which the enzymes become entrapped. To the right, when the next growth ring is initiated, the enzymes that were initially present as complexes in the stroma are entrapped within the starch granule, as noncomplexed granule-associated proteins that are presumably inactive; this is also the location of the active GBSS. To see the rings of crystalline and amorphous starch, see Fig. 1.2b. SSI and IIa, starch synthases; SBEIIb, starch branching enzyme; GBSS, granule-bound starch synthase. Based on Tetlow (2011). Reprinted with permission of Cambridge Univ. Press

process is still unclear; its phosphorolytic action to recycle Glc-1-P during "trimming" of the growing starch molecule may be equally or more important.

The amyloplasts in the cotyledons of many legume species, including pea, develop from chloroplast-like (photoheterotrophic) plastids. Single starch granules arise between the stacks of thylakoids in the plastids at the time when reserve deposition commences, and they increase in size as more starch is deposited. The internal membranes are pushed against the outer membrane and lose their integrity during maturation drying. Cells located towards the inside of the cotyledons of pea contain larger starch granules; those towards the outside not only contain fewer and smaller ones but also have small chloroplasts in which no starch is deposited; these account for the pale (pea) green color of the mature seed.

3.2.1.1 Uses and Modifications of Starch

Starches have many food and industrial uses, depending upon how they are processed and upon their amylopectin to amylose ratio. Much of the transgenic work to modify starch quality has been aimed at potato tubers and cassava roots, rather than cereal grains. The demand for amylose-free starch from all sources is likely to increase since it is easily digested and can make clear pastes that do not retrograde (precipitate, crystallize) when cooked as microwave-ready food or when frozen in pies and puddings, for example. There also are nutritional and medical benefits to high-amylose starch, known as "Resistant starch." Unlike high-amylopectin starch, this resists digestion in the small intestine and passes through to the large intestine where it acts as a prebiotic dietary fiber. Here it encourages the growth of healthy bacteria, reduces intestinal pH, and helps maintain the health of the colon by producing short-chain fatty acids, particularly butyric acid, which has anti-inflammatory and anti-colorectal cancer properties. Resistant starches may also reduce the risk of type-2 diabetes. Hi-Maize is a commercial flour product made from the kernels of a conventionally bred cultivar developed in Australia; with an amylose content of 50–90% it is good source of resistant starch, although it is lower yielding than regular maize and hence the product is more expensive.

3.2.2 Synthesis of Polymeric Carbohydrates Other than Starch

Starch is not the only polymeric carbohydrate present in the storage tissues of cereals and legumes (Sect. 1.3.1). In cereals, the walls of the dead cells of the mature starchy endosperm are rich in glucans (barley, oats) or arabinoxylans (wheat, maize, sorghum), although they are present in relatively minor amounts, less than 10% compared to starch stored within the cells; hence they are not regarded as a storage form of carbohydrate. In rice endosperm cell walls, there are more or less equal amounts of glucan, arabinoxylan, and cellulose. The glucans are mixed linkage linear chains of Glc: $(1\rightarrow3,1\rightarrow4)$-β-glucans, with 3 or 4 $\beta(1\rightarrow4)$ linked Glc units interspersed between each $\beta(1\rightarrow3)$ Glc link; they are synthesized from UDP-Glc by a β-glucan synthase complex. Arabinoxylans are composed of a $\beta(1\rightarrow4)$ xylose (Xyl)-linked backbone with irregularly spaced $\alpha(1\rightarrow2)$ arabinose (Ara) sugars as side chains; their synthesis involves the activity of transferases that incorporate the sugars from UDP-Xyl and UDP-Ara into the growing polymer.

In some legumes, especially members of the lupin family, the amount of carbohydrate stored as cell wall hemicelluloses (as arabinogalactans, a pectin polymer) considerably exceeds (by up to 15-fold) that stored as starch, because of its presence in the extensively thickened cotyledon cell walls at maturity. The polymers consist of a $\beta(1\rightarrow4)$ galactose (Gal) backbone with branches of $\alpha(1\rightarrow5)$ Ara. Little is known about their synthesis in seeds, but UDP-Gal is the donor for galactosyl transferase, and there must be a transferase utilizing UDP-Ara; the genes for these have not been isolated. In seeds of the tropical legumes such as the kerosene tree, tamarind,

and jatobá, and the nonlegume *Nasturtium*, the abundant storage carbohydrate is cell wall xyloglucan (termed "amyloid" because, like amylose, it stains blue with iodine), with a backbone of β(1→4)-linked glucans and regular branches of single or short-chain α(1→6) Xyl, that may be branched further with β(1→2) Gal. UDP-Xyl and UDP-Glc, and presumably UDP-Gal, are the sources of the sugar units for incorporation by the appropriate transferases into the hemicellulose polymer.

In certain seeds, including some of the endospermic legumes, the major storage product is deposited in the cell walls as hemicellulose (Sect. 1.3.1). In species such as fenugreek, ivory nut, coffee, and date, the hemicelluloses—largely galactomannans, a polymer of mannose (Man) and Gal—may be deposited in such large quantities in the endosperm cells that the living contents are almost occluded by the inward thickening of the secondary cells wall; in fenugreek the endosperm storage cells are dead at maturity. Galactomannan synthesis in fenugreek commences in the endosperm cells adjacent to the embryo at an early stage of development, and then spreads towards the cells on the outer periphery of this tissue. No filling occurs in a single outer layer of cells, the aleurone layer (Fig. 5.12) which remains as the only living cells of the mature endosperm; it plays a vital role in galactomannan mobilization following germination (Sect. 5.6.2). The synthesis of galactomannan occurs in the Golgi apparatus, the polymer then being secreted through the plasma membrane and into the surrounding cell wall.

The source of carbohydrate for galactomannan synthesis is sucrose entering the endosperm from the maternal tissues, which is hydrolyzed to Glc and Fru by invertase. After several metabolic steps the hexoses from sucrose are converted to Man-1-P and then to the nucleotide sugar, GDP-Man (Fig. 3.10). A membrane (Golgi)-associated GDP-Man-dependent mannosyltransferase (β-mannan synthase) then transfers Man residues to a linear (1→4)-α-linked Man primer, to form the growing backbone chain of the galactomannan polymer. Simultaneously, another membrane-associated enzyme, UDP-Gal-dependent galactosyltransferase, transfers Gal residues as α(1→6)-linked unit side chains to the most recently added Man units on the growing chain, at intervals that are generally species specific. Gal cannot be transferred to preformed Man chains, and thus the activities of the mannosyltransferase and galactosyltransferase increase in parallel during galactomannan deposition. Usually, then, the ratio of Man:Gal in galactomannans is constant throughout seed development. Any deviations from this occur because of an increase in α-galactosidase activity at late stages of development, which removes some Gal side chains. In senna, for example, there is an increase in Man:Gal ratio from 2.3 to 3.3 over the last 5–10 days of endosperm development due to this post-synthesis "editing."

3.2.3 Oil (Triacylglycerol) Synthesis

There is considerable variation in the major fatty acid constituents of stored triacylglycerols (TAGs), also called fats, oils, or neutral lipids, in seeds. Some examples are presented in Table 1.3, although the diversity is much greater, for there are over

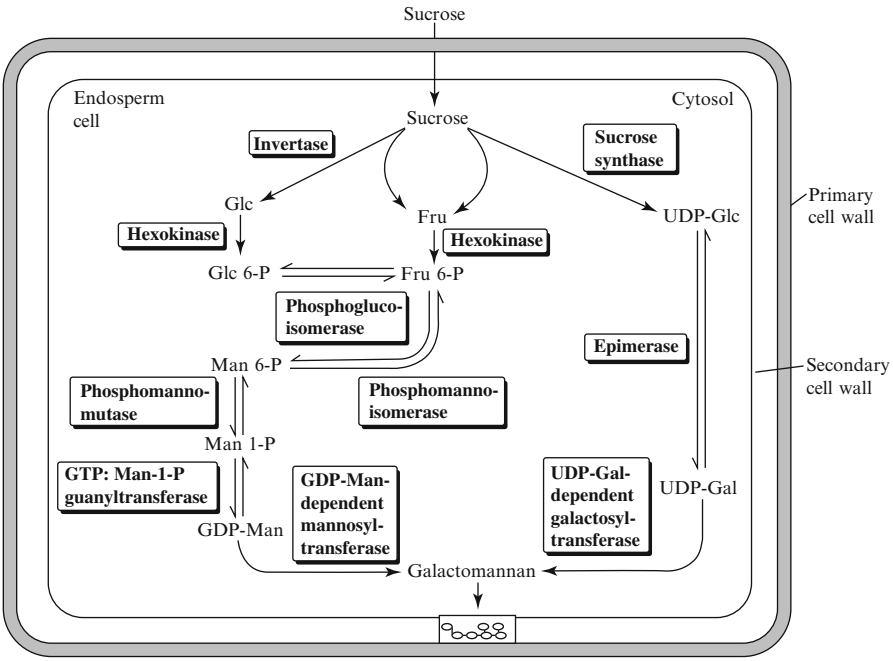

Fig. 3.10 Pathway for the synthesis of galactomannan in the endosperm of developing endospermic legumes. The galactomannan is synthesized in the Golgi apparatus and then transported to the inner side of the primary cell wall, to form the secondary wall. Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Man-6-P, mannose-6-phosphate; Man-1-P, mannose-1-phosphate; GDP-Man, guanosine diphosphomannose; UDP-Glc, uridine diphosphoglucose; UDP-Gal, uridine diphosphogalactose. From an article entitled Hemicellulose—Synthesis, by Bewley, J.D. in *The Encyclopedia of Seeds. Science, Technology and Uses*, Black et al. (2006), see reference in Advanced literature references in Chapter 1. Courtesy of CAB International

300 fatty acids present in plant oils, some unique to a particular family, or even to a species. For the sake of brevity and clarity only the synthesis of those fatty acids most commonly present within seed TAGs will be emphasized here.

Sucrose entering the developing oil seed is the source of C for TAG synthesis, which can be considered in three parts: (1) the production of the glycerol backbone; (2) the formation of fatty acids (or fatty acyl moieties); and (3) the esterification of glycerol with fatty acid components to give TAGs. It should be noted, however, that neither free glycerol nor free fatty acids are involved, but rather glycerol-3-phosphate and fatty acids linked to coenzyme A (fatty acyl-CoA) or an acyl carrier protein (ACP).

A simplified overview of TAG biosynthesis illustrating the main steps involved is shown in Fig. 3.11. The precursor of all fatty acids is acetyl-CoA, derived from sucrose. With the involvement of two major enzymes, a carboxylase and a synthase, and reducing power from NADPH, saturated long-chain fatty acids up to 18C in

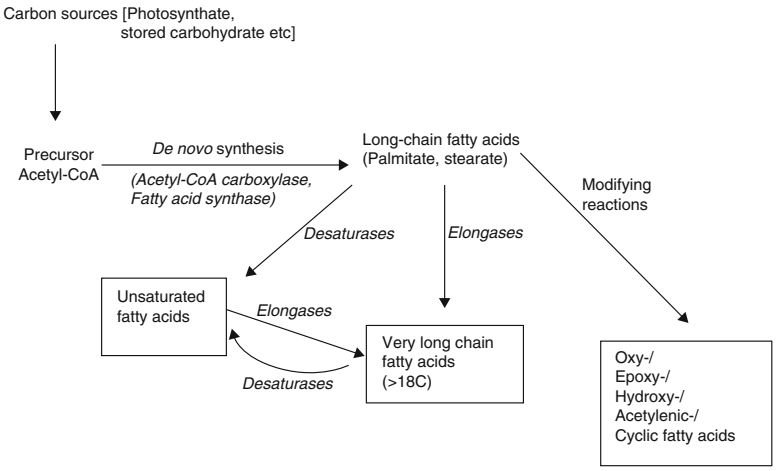

Fig. 3.11 A simplified schematic to demonstrate the major types of metabolic steps involved in the synthesis of fatty acids of various lengths and complexity. Courtesy of J.L. Harwood, Cardiff Univ

length, palmitic (16C) and stearic (18C) acids, are synthesized (denoted as palmitate and stearate because they are not present in the free acid form). These may then be elongated to very long-chain fatty acids by elongases, converted to unsaturated fatty acids by desaturases, or their structure modified in various ways (in addition to being subjected to elongation and/or desaturation).

In more detail (Fig. 3.12), the synthesis of TAGs involves several different compartments within a storage cell. Initially, sucrose is translocated into the developing seed and converted by the reactions of glycolysis in the plastid to hexose phosphate (Glc-6-P) and triose phosphates (triose-P). One of the latter, dihydroxyacetone phosphate, is reduced in the cytosol to yield glycerol-3-phosphate (G-3-P), which is later combined (esterified) with three fatty acids in the endoplasmic reticulum (ER) to form TAGs. The availability of G-3-P is proposed to be one factor restricting the overall rate of TAG accumulation.

Most commonly the Glc-6-P is imported directly into the plastid, but in vitro experiments suggest that in some species this occurs after conversion to other intermediates in the cytosol or mitochondria. The important initial product for fatty acid synthesis is acetyl-CoA, which provides 2C acyl units for incorporation into fatty acid chains. The first committed step in fatty acid biosynthesis is the carboxylation of this to malonyl-CoA by acetyl-CoA carboxylase (ACCase). This is then converted to malonyl-ACP by attachment to an ACP by malonyl transacylase. There is then a complex series of steps, involving several enzymes in a fatty acid synthase (FAS) complex, that increase the length of the fatty acid by 2C for each cycle of activity, due to the condensation and reduction of 2C units from malonyl-ACP into the growing

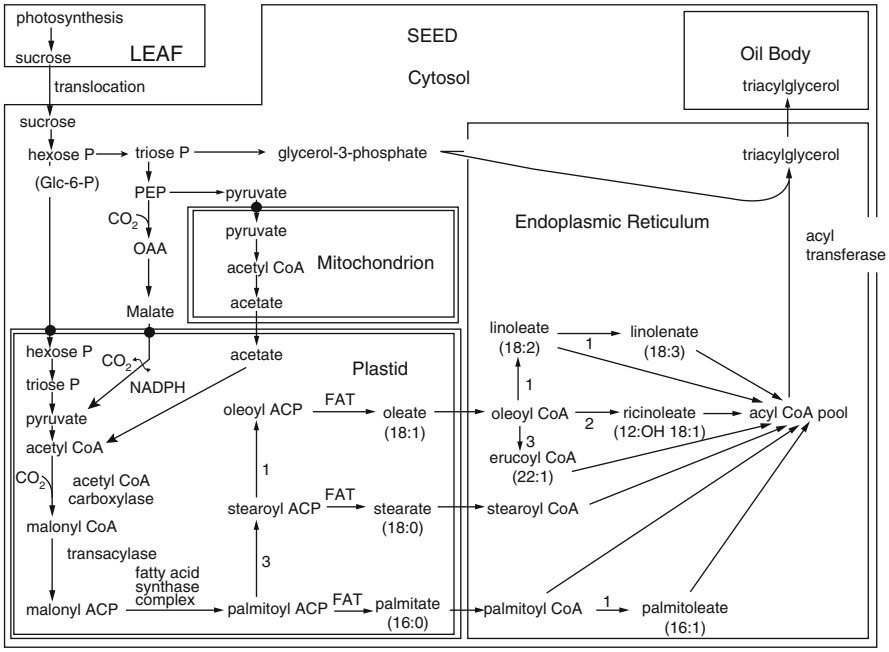

Fig. 3.12 The synthesis of fatty acids and oils in developing seeds involves the participation of enzymes in several cellular compartments, the cytosol, mitochondrion, plastid, and ER, the latter becoming modified to form the oil body. Numbered stages require the following enzymes: (1) FADs, (2) hydroxylase, (3) elongase complex. Other enzymes occur in the ER of some species to produce rarer fatty acids, e.g., by epoxidation, acetylenation, or methylation. Biochemical reactions in the ER are intimately associated with its membrane, as are the final stages of TAG production when fatty acids from the acyl-CoA pool are added sequentially by acyltransferases to glycerol-3-P. *ER*, endoplasmic reticulum; *FAD*, fatty acid desaturases; *Glc-6-P*, Glucose-6-phosphate; *PEP*, phosphoenol pyruvate; *OAA*, oxaloacetate; *ACP*, acyl carrier protein; *CoA*, coenzyme A; *FAT*, fatty acyl thioesterase

chain. This sequential addition of 2C units continues through 16C (16:0, palmitoyl-ACP) to reach 18C (18:0, stearoyl-ACP). Stearate or palmitate may be released from the synthase complex, or stearoyl-ACP may be aerobically desaturated (by a soluble fatty acid desaturase, FAD1) to yield oleoyl-ACP, from which free oleate (18:1) may be derived. The release of the fatty acids from the ACP complex is achieved by fatty acyl-ACP thioesterases (FAT). There are FAT enzymes that are specific for dissociating particular lengths of fatty acids from the complex, and in some species this results in the release of shorter chains, e.g., a lauroyl-ACP thioesterase from California bay laurel releases laurate, the 12C fatty acid, and in the coconut kernel there are FAT enzymes to release 8C (caprylate), 10C (caprate), laurate, 14C (myristate), and palmitate fatty acids (12C and 14C being the most predominant in TAGs). The relative specificity of the FAT enzymes is a factor in determining the ultimate fatty acid composition of the TAGs. Using this knowledge about the preferences of different FATs, the fatty acid composition of TAGs has been genetically modified so that fatty acids present in more exotic species can be synthesized in common crops. Introduction of

the appropriate FAT gene from the California bay laurel, for example, into rapeseed (canola) induces the formation of lauric acid (12:0) in the seeds of the transgenic plants, a fatty acid which is not typical of this species but has the properties of palm oil (see Sect. 3.2.3.1 for more applications).

The fatty acids leave the plastid after being linked with CoA in its outer membrane; they are thus imported into the ER (Fig. 3.12). The unsaturated acids, linoleic (18:2) and linolenic (18:3), are produced there by membrane-associated desaturases from the oleoyl residue of oleoyl-CoA after the acyl group has been transferred into a phospholipid, phosphatidyl choline. The unsaturated fatty acids are released from the phospholipid as their CoA derivatives, to enter the fatty acyl-CoA pool. Modifications to the fatty acids, such as hydroxylation to form ricinoleate (18:1 Δ9-OH) in the castor bean, or their elongation and desaturation to erucic acid (22:1) in rapeseed, also occur in association with the ER membrane. The acyl-CoA pool furnishes the fatty acid groups for their sequential condensation with G-3-P, eventually to form the TAGs, a process that involves acyl transferases.

Developing jojoba seeds synthesize unique liquid waxes, which are esters of unbranched, long-chain (C20, C22, C24) unsaturated fatty acids and alcohols: they do not produce TAGs. The waxes are produced due to the presence of two unique enzymes, a fatty acyl-CoA reductase that yields the fatty alcohol from fatty acyl-CoA and a wax synthase that esterifies the fatty alcohol to another acyl moiety of acyl-CoA. The waxes are of importance because they are difficult to synthesize commercially; the only other natural source is the endangered sperm whale. However, because of its slow growth and limited yield, low-cost, large-scale wax production from jojoba is a challenge; hence transfer of the wax synthetic capacity to more amenable crops by genetic engineering is an attractive proposition.

The characteristic fatty acid composition of plant TAGs is largely genetically determined through the different enzymes participating in fatty acid and oil synthesis, although environmental factors may result in modifications during seed development. Seed oils of plants grown in cool climates generally tend to be more unsaturated than in those grown in warm climates. The major influence is usually on the predominant fatty acid, but not all oil-rich species or lines are affected by growth temperature in this respect. The precise nature of a climatic influence on fatty acid composition in seeds is incompletely understood, although activity and synthesis of FAD2 (oleate desaturase which converts 18:1 [oleate] to 18:2 [linoleate] fatty acids) are increased at low temperatures in developing sunflower seeds, resulting in an increase in the linoleate:oleate ratio in the TAGs. In contrast, the ratio is decreased at higher temperatures due to a decline in thermal stability of FAD2 (although the influence of temperature on the availability of oxygen, a co-substrate in the desaturation reaction may also be important). Similarly, during seed development of oilseed rape the ratio of linolenate:oleate in storage oils is increased at low temperatures (e.g., 12°C), and lowered at high temperatures (e.g., 30°C). The influence of higher temperatures on the unsaturated fatty acid content of safflower seed oils is slight, however, perhaps because the thermal stability of FAD2 is higher than in sunflower.

The reserve TAGs within the cells of storage organs are confined to organelles, 0.5–2 μm in diameter, called oil (fat or lipid) bodies, and also oleosomes

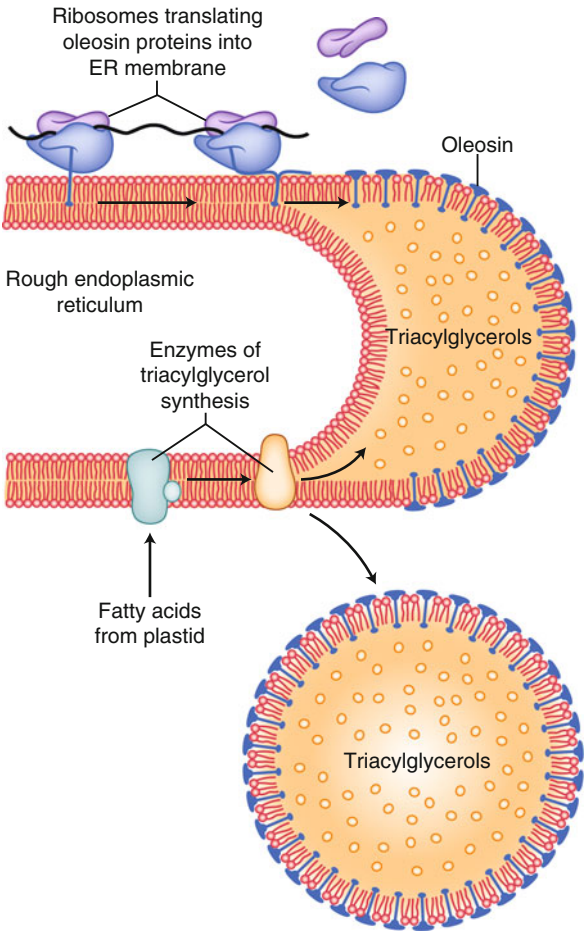

Fig. 3.13 An illustration of the formation of an oil body from the endoplasmic reticulum. Fatty acids and glycerol are combined in the phospholipid membrane of the ER and the resultant triacylglycerols are deposited within the bilayer membrane. Simultaneously there is the synthesis of oleosins on polysomes associated with the ER (rough endoplasmic reticulum) and these are incorporated into the outer layer of the membrane, which will surround the nascent oil body. When it reaches a certain size, the oil body may be budded off from the ER, although some may remain attached. Based on an article on Lipids by Somerville, C., Browse, J., Jaworski J.J., and Ohlrogge, J.B. in *Biochemistry and Molecular Biology of Plants*. 2000. Buchanan, B.B., Gruissem, W. and Jones, R.L. (eds.). Copyright American Society of Plant Biologists

(spherosomes). Unlike other cellular membranes, that of the oil body is a monolayer (half-unit membrane), and those in seeds contain unique proteins called oleosins. The origin of these bodies is the ER. Newly synthesized TAGs, being hydrophobic, accumulate between the two layers of the ER double membrane, leading to its swelling (Fig. 3.13). When the oil-filled vesicle reaches a critical size, it may bud off completely.

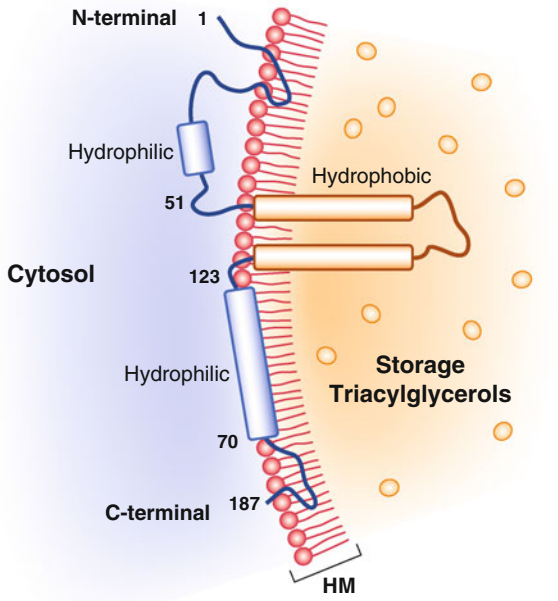

Fig. 3.14 Proposed model for the location of an 18 kDa oleosin protein associated with a maize scutellum oil body. The oleosin is composed of a hydrophobic region of amino acids (some of which are numbered) positioned in the oil (triacylglycerols) within the body, and hydrophilic regions present in the cytosol and on the surface to the body associated with the headgroups of phospholipid molecules of the single or half-unit membrane (HM). Modified from Qu and Huang (1990)

The oleosins, proteins of low molecular mass (15–26 kDa) are structurally incorporated into the membrane of the oil body during its ontogeny; they are synthesized on ER-bound polysomes as the TAGs are being deposited within the bodies (Fig. 3.13). In the maize scutellum and *Brassica* embryos, expression of the oleosin genes is increased by abscisic acid. Oleosins possess three structural domains (Fig. 3.14), an N-terminal hydrophilic portion, a central hydrophobic hairpin (interestingly, no other protein in any organism has such a long hydrophobic stretch), and a C-terminal hydrophilic portion. The difference in molecular mass of the various oleosins (isoforms), which vary between, and even within, species is due to extension of the C- and N-terminal amino acid domains. The oleosins serve a structural role in stabilizing the half-unit phospholipid membrane that surrounds the oil body. Without the oleosin coating, removal of water during drying would allow the unit membranes of adjacent oil bodies to merge and coalesce into large oil droplets. Oleosins provide the surface with a net negative charge, which by electrical repulsion prevents these organelles from coalescing and aggregating, especially during

maturation drying and subsequent imbibition during germination. Oleosins may also protect the phospholipids from hydrolysis by cytoplasmic phospholipases, and provide binding sites for lipases involved in post-germinative TAG mobilization. They are absent from fruit oil bodies, e.g., olive and avocado, which do not undergo dehydration, nor are they associated with oil bodies in the nonliving starchy endosperm cells of oat grains, resulting in the dispersal of oils between the starch granules and protein storage vacuoles as the grains mature.

The unique characteristics of oleosins are being utilized for the production of pharmaceutical proteins in seeds. DNA sequences coding for a protein of interest have been fused to the oleosin gene and transformed into a plant (e.g., safflower or canola) and expressed during seed development. The transgene was structured so that the expressed novel protein product lies on the cytoplasmic side of the oleosin protein that is incorporated into the oil body membrane. Since oil is lighter than water, it is simple to recover intact oil bodies by centrifugation of seed extracts. The modified oleosin protein can be isolated from the oil and the peptide of interest cleaved off enzymatically as a source of pharmaceuticals or other proteins of interest. Insulin produced in safflower seeds by this method is equivalent in biological action to that produced by current sources (recombinant bacteria and yeast) and is undergoing production and manufacturing trials.

3.2.3.1 Uses and Modifications of Fatty Acids

As described above, seeds produce oils with a wide array of fatty acid compositions, and this can be further modified by breeding and genetic engineering. As noted, genes for key enzymes of the synthetic machinery for commercially important oils, such as palm oil, have been transferred into seeds of more universally available crops, such as the Brassicas, for a more stable production system. The production of industrial oils for biofuels and biolubricants in seeds is also a topic of active research. Down-regulation of *FAD2-1* and *FatB* (encoding a palmitoyl thioesterase) in soybean seeds results in soybean oil with a reduced level of palmitic acid (<5%) and increased oleic acid content (>85%), which is an ideal composition for use as biodiesel.

Edible seed oils can also be modified for improved health and nutrition. Polyunsaturated maize or canola oils are chemically hydrogenated to make them more saturated and produce products with desired cooking, melting or eating properties, as in margarines. However, natural seed oils contain *cis*-bonds adjacent to their unsaturation sites, while chemical hydrogenation results in a mixture of *cis*- and *trans*-fats. Concerns have been raised about possible adverse health consequences of too much *trans*-fat in human diets, and food manufacturers are reformulating their products to reduce or eliminate them. Using both conventional breeding and transgenic methods, oilseeds have been produced that contain various compositions of (*cis*-) unsaturated fatty acids, such as high oleic sunflowers or soybeans, allowing them to be used singly or in combination to provide oils that meet diverse needs without requiring hydrogenation.

As outlined in Sect. 1.3.2 many seed oils contain essential fatty acids, ones that must be obtained in our diets, and genetic engineering approaches are being applied to modify and increase their synthesis. One of the more exciting prospects is the production of oils with very long chain polyunsaturated fatty acids (VLC-PUFAs), so-called "fish-oils." Omega-3 (ω-3) polyunsaturated fatty acids (~C20) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential for human health; increased content in the diet can improve brain and retinal health, and reduce the risks of coronary heart disease and type II diabetes. While these fatty acids can be supplied from seafood, this dwindling resource will not be sufficient to support the growing world population with the amounts recommended for optimum health. Producing fish oils in seeds has been very difficult to achieve due to the requirement for co-expression of the multiple genes that have to be reconstituted into the correct biosynthetic pathways in the transformed host plant. Researchers have overcome this problem by selecting specific genes from various origins and using appropriate promoters to express the genes in the appropriate cells at the correct time during seed development. Canola seeds have been engineered with three specific desaturases (Δ6, Δ12, and Δ15) to enhance the accumulation of ω-3 stearidonic acid (18:4), which is also present in fish oils and can be converted to EPA by the body. Soybeans have been engineered with six or more genes that enable them to produce oil with increased content of EPA + DPA. Choosing an appropriate host for modification can also be helpful. For example, some genetic lines of Ethiopian mustard lack erucic acid, which is produced through another branch of the VLC-PUFA pathway. Since the erucic acid and EPA synthesis pathways share the same upstream substrates, such "zero-erucic-acid" lines serve as an ideal genetic background for increased EPA synthesis. Two novel genes encoding 18C and 20C ω-3 desaturases from fungi, which catalyze the synthesis of EPA, have been introduced into zero-erucic-acid Ethiopian mustard lines, using a seed-specific promoter. This approach has increased EPA content to up to 29% of total fatty acids.

3.2.4 Storage Protein Synthesis

The final amount of stored protein present within a mature seed varies markedly among species, but usually there is a characteristic seed-specific major reserve protein (Tables 1.4, 1.5). The synthesis of a storage protein, or constituent polypeptide, involves the transcription of its gene (or genes, since some are encoded by small gene families, and some prolamins, e.g., α-zeins of maize, by multi-gene families) to produce the appropriate messenger RNA (mRNA), its translation and the insertion of the product into the ER lumen, posttranslational processing of the protein therein, targeting and sequestering within a storage compartment (the protein storage vacuole, PSV), and the eventual conjoining of constituent polypeptides to form the mature holoprotein. These steps are illustrated for the synthesis of the legumin (glycinin) storage protein in Fig. 3.15.

Examples of the quantitative and qualitative changes occurring during protein deposition within a cereal grain, a legume, and an oil-storing dicot are shown in Fig. 3.16a–d. In general, the syntheses of the various storage proteins in a particular seed are initiated at similar but not identical times during development, and proteins are accumulated at about the same rate. However, as noted for the α and β subunits of β-conglycinin (Fig. 3.16d), not all storage polypeptides are synthesized concurrently. Likewise in maize, different classes of the zein are synthesized and deposited within the PSVs as endosperm development proceeds (Fig. 3.16e). In the newly forming PSVs only β- and γ-zeins are present. Later, however, the interior of this vacuole fills with the major storage protein, α-zein, of which there are two forms, a 19 kDa one that is dispersed centrally within the PSV, and a more peripheral and less abundant 22 kDa form that is deposited at the interface between the core and the outer β-and γ-zein layer. Import into and the specific localization of the 22 kDa α-zein within the PSV are facilitated by the presence of a concurrently synthesized transmembrane protein (FL1). The β- and γ-zeins are displaced to form a continuous thin layer around the PSV periphery at maturity. A few minor patches of β- and γ-zeins occur embedded within the α-zein matrix in the interior regions of the mature PSV. Location of the β- and γ-zeins to the outside of the PSVs likely has importance to maintaining the structure of the endosperm as it undergoes maturation drying. The integrity of the bounding membrane of the PSVs is lost during drying, and interactions between the exposed outer hydrophobic zeins and neighboring starch grains may contribute to maintaining a stable vitreous endosperm structure in the mature seed.

Important points emerge with respect to these patterns of protein deposition. First, synthesis of each reserve protein occurs over a discrete period and not throughout the whole of development. The same is true in respect of the mRNAs for the component polypeptides and, therefore, for the expression of their genes. Second, reserve proteins are characteristically synthesized largely or only in certain parts of the seed, e.g., prolamins occur almost exclusively within the starchy endosperm of cereals, rather than in the surrounding aleurone layer. Therefore there is a strong temporal and spatial (tissue-specific) regulation of the expression of genes encoding reserve proteins and of their subsequent synthesis and deposition in PSVs.

3.2.4.1 Synthesis, Processing and Deposition of Storage Proteins

Between initial translation of the storage polypeptides and their final sequestration in PSVs they are subjected to several posttranslational events. A brief synopsis of the types of changes that can occur is as follows:

(1) *Cleavage of the signal peptide*. This hydrophobic region of amino acids at the N-terminus of the polypeptide is inserted through a signal recognition complex in the ER membrane, and is excised by a protease (signal peptidase) present on its inner (lumen) side (Fig. 3.17).

(2) *Glycosylation*. Many of the storage proteins in mature seeds are glycoproteins, having one or more oligosaccharide side chains covalently linked to specific

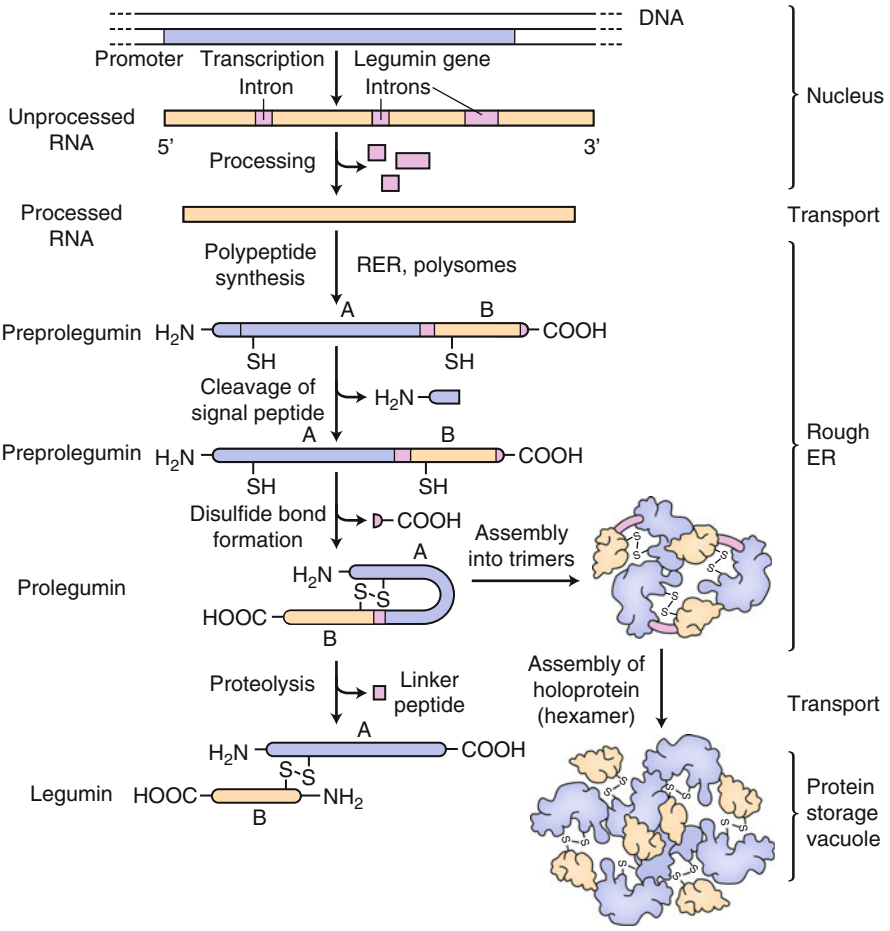

Fig. 3.15 A representation of the several steps involved in the synthesis of the legumin storage protein (glycinin) in soybean cotyledons. The gene for legumin possesses an upstream promoter sequence of several hundred nucleotides controlling its tissue-specific and temporal expression. The first transcriptional product is an unprocessed mRNA that contains untranslated intron regions of about 1,150 bases in total; these are spliced out to produce the mature legumin mRNA, which is 1,455 bases long. (The mRNA is also polyadenylated at the 3′ end [polyA tail]; not shown). This mRNA is transported from the nucleus to the endoplasmic reticulum (termed rough ER, RER, because of an association with ribosomes on the outer membrane surface) where it is translated on the polysomes. The mRNAs for the acidic (A) and basic (B) subunits are joined by codons for a 4-amino acid linker sequence in the primary translation product (preprolegumin). The mRNA also contains a code for the signal peptide at the amino-terminal ($–NH_2$) end and a pentapeptide at the carboxy terminal (–COOH) end. These are cleaved off and the primary product (prolegumin) is processed to yield the A and B subunits joined by the linker sequence and disulphide bonds (S–S); the signal peptide and the pentapeptide are cleaved off. Within the ER the prolegumin assembles into trimers, which enter the protein storage vacuole (PSV). In the final step of processing in the PSV the linker sequence is removed by proteolysis, leaving the A and B subunits joined only by the disulphide bonding. The mature hexameric legumin holoprotein is assembled from the trimers,

asparagine (Asn) residues in the constituent polypeptides. This occurs within the lumen of the ER as the polypeptide is still being translated (i.e., it is a co-translational event), and involves a lipid carrier molecule (dolichol phosphate) embedded in the ER membrane (Fig. 3.17). Examples of glycoproteins include the vicilins of legume seeds (e.g., β-conglycinin of soybean) and the lectins of *Phaseolus* bean (e.g., phytohemagluttinins); legumins are not glycosylated (Table 3.1). The oligosaccharide side chains are of two major types, and one or both types may be present on the same polypeptide: (a) simple or high-mannose (Man) oligosaccharides composed exclusively of Man and N-acetylglucosamine (GlcNAc), usually in a 5–9:2 ratio (Fig. 3.17); and (b) complex or modified oligosaccharides which are often rich in Man, but contain other residues in addition to GlcNAc, e.g., fucose (Fuc), xylose (Xyl) and galactose (Gal). Glycosylation may be important in enhancing the stability of proteins, and in the aiding their folding and assembly. It can also be involved in triggering allergic reactions when consumed by people with specific sensitivities.

(3) *Proteolytic processing*. This commences during transit of the protein to the PSV and is completed therein. Some polypeptides are cleaved into two or more smaller ones, and small linking peptides may be removed (Fig. 3.15) or the N- or C-terminus may be trimmed. These modifications, along with glycosylation, can result in a heterogeneous set of mature proteins emanating from a single polypeptide. For example, the 7S vicilins in pea, originally translated as an ~50 kDa polypeptide precursor, are cleaved and modified to produce up to eight proteins that range in mass from 12 to 35 kDa after processing. Of all the storage proteins, the 2S albumins are subjected to the most extensive posttranslational processing. Some examples of storage proteins that are subjected to different types of processing are in Table 3.1.

(4) *Folding and assembly*. The constituent polypeptides of a holoprotein begin to fold and assemble into their correct three-dimensional (tertiary and quaternary) structure within the ER lumen, an event that is required before the protein can be targeted and transported to its final destination. ER-resident proteins (molecular chaperones) facilitate this process, including a binding protein (BiP) which promotes correct folding of the storage polypeptides, and protein disulphide isomerase (PDI) which forges disulphide bonds within (intrachain) and/or between chains (interchain) using the -SH groups of the cysteine residues (Fig. 3.15). Misfolded proteins that fail to assume their proper conformation may be degraded in the ER lumen.

Fig. 3.15 (continued) linked by interchain hydrogen bonds. The mature A subunit contains 278 amino acids (approx. 40 kDa) and the B subunit 180 amino acids (approx. 20 kDa). Many polypeptides present in storage proteins are transcribed from individual, rather than linked genes and hence the step involving removal of the linker peptide is not required. To form a mature holoprotein, conjoining of the produced polypeptides can occur by disulphide bonding (interchain bonds). Modified from Krochko and Bewley (1988). Also see chapter on Reproductive Development by Bewley, J.D., Hempel, F.D., McCormick, S. and Zambryski, P. in *Biochemistry and Molecular Biology of Plants*. 2000. Buchanan, B.B., Gruissem, W. and Jones, R.L. (eds.). Copyright American Society of Plant Biologists

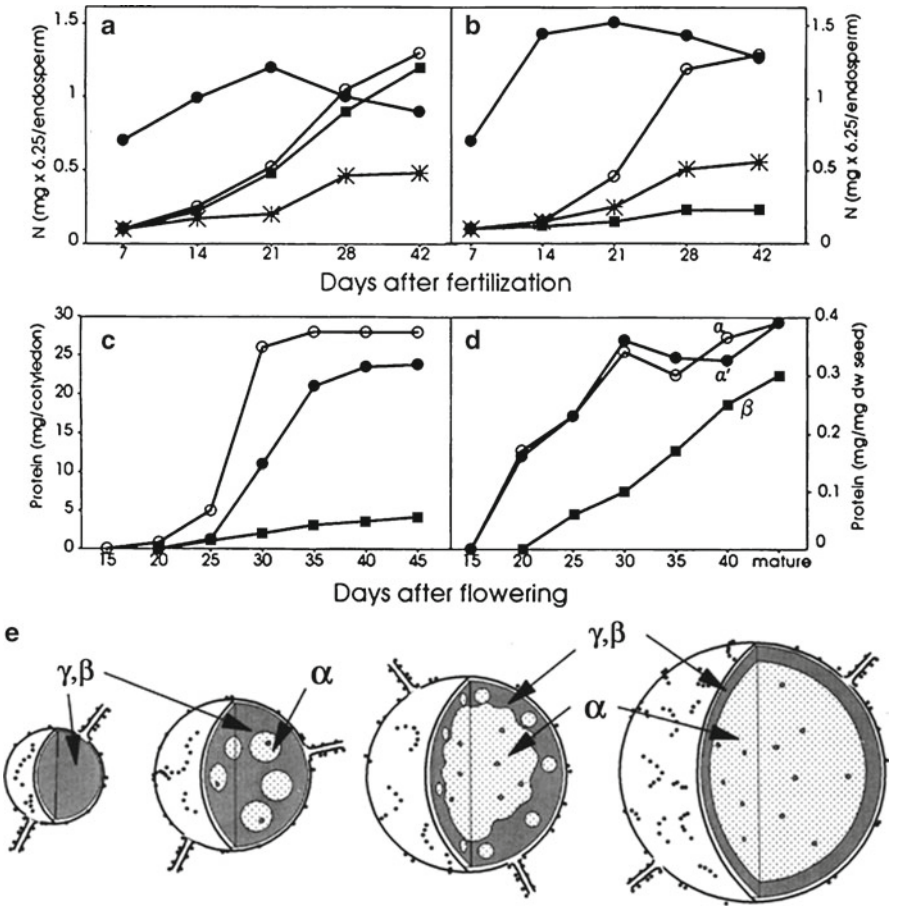

Fig. 3.16 (**a**, **b**) Changes in the endosperm protein fractions during grain development of (**a**) normal cv. Bomi barley and (**b**) the high-lysine barley mutant Risø 1508 Albumin (plus free amino acids (•); globulin (*); hordein (prolamin) (■); hordenin (prolamin) (o). (**c**) Accumulation of the legumins vicilin (o) and legumin (•), and of albumins (■) in developing cotyledons of broad bean. (**d**) Synthesis of α (o), α′ (•), and β subunits of the 7S storage protein β-conglycinin (■) in developing soybean seed cotyledons. While synthesis of the α subunits occurs simultaneously, that of the β subunit commences later. (**e**) Pattern of deposition of α-, β- and γ-zeins in a PSV of maize endosperm during development. Two α-zeins are present in the PSVs, the 22 kDa form being deposited in a ring between the γ-zein layer and the core, whereas the 19 kDa form is dispersed more generally throughout the storage body, but is almost excluded from the γ-zein-rich peripheral region. Dark dots on the protein storage vacuole and attached endoplasmic reticulum represent ribosomes. Not to scale. Data in (**a**, **b**) based on Brandt (1976), (**c**) on Manteuffel et al. (1976) and (**d**) on Gayler and Sykes (1981). (**e**) Lending and Larkins (1989). Copyright American Society of Plant Biologists

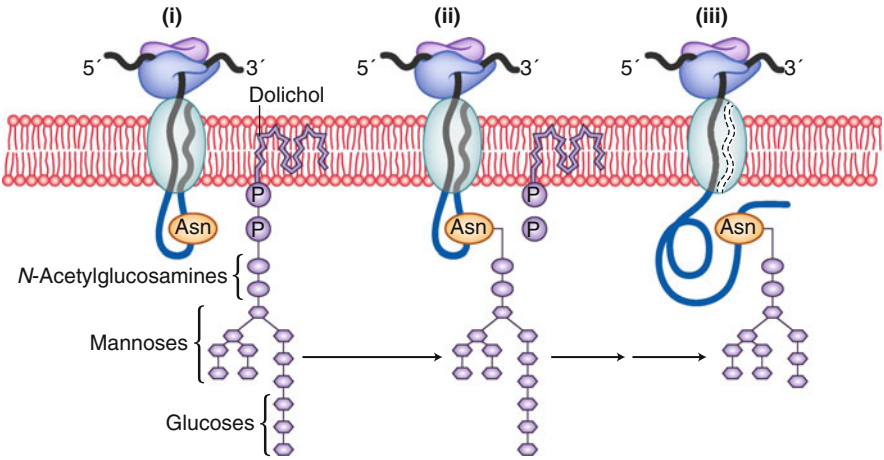

Fig. 3.17 Illustration of a growing polypeptide chain of a storage protein and its glycosylation while passing through the membrane of the rough endoplasmic reticulum (RER) as its mRNA is being translated by ribosomes. When approximately 70 amino acids have been translated, the growing polypeptide chain is long enough for the signal peptide at the N-terminus to enter a receptive complex in the RER (light green oval), which guides it through (Step i). The signal peptide is degraded enzymatically by a signal peptidase in the ER lumen as the chain continues to grow by translation on the cytosolic side of the membrane (Step iii). Glycosylation of the protein occurs as the polypeptide chain is growing, and requires the recognition of an appropriate Asn (in configuration Asn-X-Ser or Asn-X-Thr) to which the glycan chain can be transferred from dolichol phosphate located in the membrane (Step ii). Here a simple glycan is shown, with two glucosamines and nine mannoses $(Man)_9(GlcNAc)_2$, to which three glucoses are initially attached. These are removed by glucosidases as the protein begins to fold, aided by molecular chaperones (Step iii). Asn, asparagine; Ser, serine; Thr, threonine; X, any amino acid except proline. Based on figures in a chapter on Protein Sorting and Vesicle Traffic by Raikhel, N. and Chrispeels, M.J. in *Biochemistry and Molecular Biology of Plants*. 2000. See legend in Fig 3.15 for reference details. Copyright American Society of Plant Biologists

The ultimate destination for a storage polypeptide is a PSV, within which the final modifications occur to form the holoprotein. PSVs for prolamins in the cereal starchy endosperm are formed directly from the ER. Others, which contain globulin storage proteins, are formed from the large cell vacuoles present at the start of reserve synthesis; invagination of the tonoplast around concentrations of newly deposited storage polypeptides results in the formation of the many small discrete PSVs that are present in the mature seed. How newly synthesized polypeptides are transported through the secretory pathway from the lumen of the ER to enter the vacuole is still incompletely understood, but several potential routes are shown in Fig. 3.18. Taking them in numerical order:

1. This pathway is rare, but has been reported in developing pumpkin cotyledons for 2S and 11S storage proteins. Here the proteins form aggregates in the ER lumen at their sites of synthesis, and PAC (precursor-accumulating) vesicles containing them are budded off, move to the PSV and fuse directly with it, releasing

Table 3.1 Some examples of posttranslational proteolytic processing of storage polypeptides

No proteolytic processing[a]	**N-terminal domain removed**
Phytohemagglutinin[b] (lectin albumin, legumes)	Napin
Soybean agglutinin[b] (lectin albumin)	Brazil nut S-rich protein
β-subunit of β-conglycincin[b] (7S globulin, soybean)	Soybean α,α[1] β-conglycinin subunits[b]
Phaseolin[b] (7S globulin, *Phaseolus* bean)	Castor bean ricin[b] (lectin albumin)
Pea convicilin (7S globulin, pea)	
C-terminal domain removed	**Internal removal with or without loss of domain**
Soybean glycinin (11S globulin)	Napin
Wheat germ agglutinin[b] (lectin albumin)	Brazil nut S-rich protein
Barley lectin[b] (lectin albumin)	Castor bean ricin[b]
Napin (2S albumin, *Brassica* spp.)	11S globulins (legumins, legumes)
Brazil nut S-rich protein (2S albumin)	Pea vicilin[b] (7S globulin)
Cleavage and re-ligation	
Concanavalin A (lectin albumin, jack bean)	

See Sect. 1.3.3 for definitions of protein types
Based on information in a chapter on Synthesis, processing and deposition of seed proteins: The pathway of protein synthesis and deposition in the cell. Kermode, A.R. and Bewley, J.D. in *Seed Proteins*, Shewry, P.R. and Casey, R. (eds.). 1999. Kluwer Academic, Dordrecht
[a]Excluding removal of the N-terminal signal peptide
[b]Indicates proteins that are glycosylated at maturity

the proteins to the inside, thus bypassing the Golgi network. En route from the ER to the PVC, the PAC vesicles may fuse with vesicles released from the *trans*-Golgi, containing other storage proteins.

2. This is the most common and strongly supported pathway for all 7S and 11S globulins and some albumins, although details on the various intermediary steps and molecules involved remain to be confirmed. The storage polypeptides moving from the ER into the Golgi may possess specific target domains (variable linear sequences of amino acids that may be present at the C- or N-terminal ends, or located internally) that are recognized by undefined receptor proteins at the inner lumen surface and are transported laterally to the periphery of the *cis*-Golgi cisternae. The accumulation there of the storage polypeptides (also given the generic name, cargo proteins) leads to interactions and aggregations, increasing their density; they are eventually budded off from the *trans*-Golgi region within dense vesicles (DVs). The cisternal regions contain proteins (vacuolar sorting receptors, VSRs) that span the Golgi membrane, perhaps binding with a specific region (ligand) of the storage polypeptides on the inside, or another linking protein, while on the outside they possess amino acid sequences that recognize either the PSV membrane, or that of an intermediate body, the prevacuolar compartment (PVC, also termed multivesicular body, MVB). The DV or PVC, formed by the fusion of several DVs, eventually melds with the membrane of the PSV to release the contained polypeptides to the inside. In some instances,

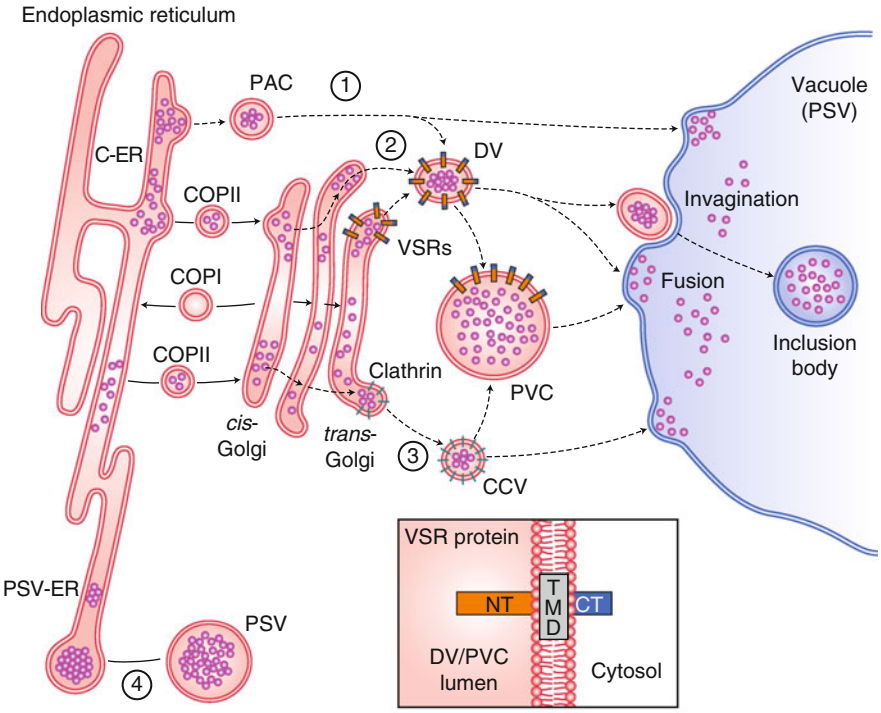

Fig. 3.18 Proposed pathways for the trafficking of storage polypeptides from their sites of synthesis on the rough endoplasmic reticulum to their destination in the protein storage vacuole (PSV). Initially the storage polypeptides are introduced into the Golgi network by COPII (coat-protein vesicle II, with the exception of Route 4); the emptied vesicles return as COPI to the ER. *Route 1*. The direct pathway using precursor-accumulating vesicles (PAC) which bypasses the Golgi network, but may have polypeptides added to them from the *trans*-Golgi *en route* to the PSV. *Route 2*. Polypeptides become assembled in the peripheral cisternal regions of the *trans*-Golgi and are released as dense vesicles (DV). These may become directly fused with, or ingested by, the vacuole, or form prevacuolar compartments (PVC) that likewise become associated with the vacuole. *Trans*-membrane proteins (elongated rectangles) guide the DVs or PVCs to the vacuole (see inset and legend below for more detail). *Route 3*. Clathrin-coated vesicles (CCV) containing the storage polypeptides are formed from the *trans*-Golgi. They are coated with the protein clathrin (*green lines*), and contain a vacuolar targeting signal of the BP-80 family. *Route 4*. Storage polypeptides aggregate within the lumen of the ER before budding off as discrete PSVs. The presence of polysomes on the surface of the cisternal ER (C-ER), which is the site of synthesis of polypeptides for Routes 1-3, and of protein storage vacuole-ER (PSV-ER) for Route 4 is not shown. The small circles denote the presence of storage polypeptides. The inset is a diagram of a membrane spanning VSR protein (vacuolar sorting receptor, which includes the BP-80 and RMR families of proteins). This contains an N-terminal domain (NT) responsible for binding to the cargo protein (storage polypeptide) in the ER cisternae and in the subsequent transport vesicle lumen, a trans-membrane domain (TMD) and a short cytoplasmic tail (CT) which recognizes the target PSV or PVC. Assembled from diagrams and information in Raikhel and Chrispeels (2000, see reference in legend to Fig. 3.17), Vitale and Hinz (2005), Robinson et al. (2005), Suen et al. (2010)

the whole of the PVC or DV may be invaginated into the PSV, resulting in localized regions of membrane-bound storage proteins (inclusion bodies) within the larger storage vesicle.

3. This somewhat controversial route (because it is normally associated with trafficking of enzymes to lytic vesicles, in which the contents are hydrolyzed, rather than to storage vesicles), is purported to be the one for many 2S albumins, e.g., ricin from castor bean and the 2S protein from Brazil nut. Here the storage polypeptides, in association with a VSR of the BP-80 family, are packed into clathrin-coated vesicles (CCVs), instead of DVs, in the *trans*-Golgi and transported to the PSV for release; alternatively they may fuse with the PVC. There is also a contention that CCVs only transport VSRs like BP-80 and proteases that process the storage polypeptides, not the storage polypeptides themselves.
4. This pathway is unique to cereal endosperm prolamins, and involves neither the Golgi complex nor secretion into the cell vacuole to form PSVs. Whereas the storage globulins are synthesized in association with cisternal-ER (C-ER), a region composed of stacks of rough endoplasmic reticulum, before passage to the vacuole (route 2), the prolamins are synthesized on protein storage vacuole ER (PSV-ER), which has a less ordered structure. The prolamins are inserted into the PSV-ER lumen, where they aggregate, causing localized swelling and the eventual budding off of the storage body when maximum distension is reached. Proteins that are ER-resident frequently contain a C-terminal tetrapeptide sequence of amino acids: histidine (or lysine)-aspartic acid-glutamic acid-leucine (HDEL or KDEL), which is the signal for retention in the ER. However, this sequence is not present in cereal prolamins, and their retention may be because they aggregate densely in the ER lumen. In the wheat endosperm, as the endosperm cells reach maturity, the two populations of PSVs, one derived directly from the ER (containing prolamins) and the other from the vacuole (containing globulins), coalesce to form a single protein-storing organelle, with the globulins occurring as inclusions within the prolamin matrix. In oats the prolamins are inclusions within the globin matrix. In contrast to this mode of prolamin (zein) deposition into the PSV-ER in the starchy endosperm of maize, in the cells of the aleurone layer, where they are less abundant, synthesis and deposition into PSVs essentially follows pathway 2.

There are a number of proteins in the mature seed that are insufficient in amount to be categorized as storage proteins, but nevertheless are important, especially with respect to tolerance of maturation drying. These include the LEA (late embryogenesis abundant) proteins Sect. 2.5.2.2.

3.2.4.2 Uses and Modifications of Storage Proteins

Prolamin storage proteins in cereals are nutritionally deficient in the essential amino acids lysine, threonine and tryptophan, whereas legume globulins have insufficient S-containing amino acids (Sect. 1.3.3). Attempts have been made to

improve seed protein quality by identification of appropriate mutants, selective breeding and genetic engineering.

With respect to improving the lysine content of storage proteins in maize, a defect in the transcription factor Opaque2 has been identified in the *opaque-2* (*o2*) mutant of this species. This results in lower transcription of zein genes, allowing the synthesis of more of the proteins that contain adequate amounts of lysine; proteins that contain 70% more of this amino acid than normal maize are produced in the mutant. However, the initial *o2* mutants exhibited undesirable traits such as soft and starchy kernels, reduced yield, and increased disease susceptibility that precluded their commercialization. More recently breeders have found phenotype modifier genes, called *o2* modifiers, and using them have produced QPM (quality protein maize) lines in which the negative characteristics of the mutation are reduced while maintaining a higher lysine content in the protein. Other approaches used to improve cereal grain quality include: (1) increasing lysine production in the endosperm cells (not incorporated into storage proteins) by over-expressing lysine biosynthesis genes that are resistant to the feedback regulation by this amino acid end product; (2) decreasing lysine degradation by suppressing genes for its catabolism; (3) increasing the proportion of lysine-containing proteins by suppressing zein genes devoid of lysine; or (4) combinations of these. One of the best high-lysine maize lines, which has been approved but not commercially released, contains 5.8 mg/g dry weight of this amino acid in the kernel, which is a 225% increase over that in normal maize. Down-regulation of genes encoding the low-lysine containing C-hordein, a major storage protein in barley grains, has resulted in an increase in other proteins containing more lysine, as well as threonine and methionine. Expression of bacterial lysine biosynthesis genes in canola using the seed-specific β-phaseolin (*Phaseolus* bean vicilin) promoter has been used to double the total seed lysine content. Similar approaches in soybean have increased seed lysine content fivefold.

A major target for increasing the nutritional quality of legume seeds has been to introduce genes for proteins that are rich in the S-containing amino acids cysteine and methionine. This has been achieved successfully several times by over-expressing genes for certain S-rich 2S albumins in a legume seed, such as those from Brazil nut or sunflower. However, in the case of Brazil nut proteins, preliminary studies quickly showed that persons with sensitive allergic responses to the nuts would also react to soybean seeds expressing the nut protein, so further development was stopped. Due to the severity of reaction of some people to certain seed storage proteins (e.g., those of peanuts, Sect. 1.3.3), work is now in progress to eliminate these from seeds and thus reduce their allergenicity.

3.2.4.3 Regulation of Storage Protein Synthesis

Obviously, the genes for storage proteins are present in all cells of a plant, yet the proteins themselves are synthesized only in the seed; they may be restricted to

certain tissues therein, and limited to being produced during discrete periods of seed development. This temporal and spatial regulation of storage protein synthesis is orchestrated by precise mechanisms at the level of gene expression. This is the result of information contained in the genomic DNA encoding the storage proteins, and the cell-type-specific regulatory proteins that mediate the utilization of the genomic information. That there is similarity among species in their regulatory mechanisms of storage protein synthesis is evident from transgenic experiments, where the promoters and genes from one species can direct expression in seeds of a different one, even those of a different family, e.g., legume proteins in tobacco (Solanaceae) or Arabidopsis (Brassicaceae).

The DNA sequence upstream of the protein-coding region of a gene, the promoter region, is not expressed but rather regulates the transcription of the associated coding region. Analyses of the promoter regions of a number of seed storage proteins have identified DNA sequences common to many or related groups of promoters; these are termed DNA motifs. For example, the promoters of cereal prolamins contain the specific nucleotide sequence TGTAAAG (prolamin box; Fig. 3.19a), approximately 300-base-pairs upstream of the point where transcription starts, the transcription initiation site. Another common motif that contains ACGT is found close to the prolamin box (Fig. 3.19a). These DNA motifs in the promoters are called *cis*-elements and are recognized by *trans*-acting proteins, termed transcription factors. The prolamin box-binding factor (PBF) and OPAQUE2 (O2) protein, which physically interact with each other, bind to the prolamin box and ACGT core, respectively (Fig. 3.19a), and activate the expression of prolamin genes.

Regulation of the expression of legume 11S globulin genes involves an upstream legumin box that contains a seed-specific motif CATGCA(TG), the so-called RY motif (named for the alternating R [purine] and Y [pyrimidine] bases) (Fig. 3.19b). This *cis*-acting element is present in the promoter regions of many seed-expressed genes in both monocots and dicots. B3 transcription factors (named after the protein domain "B3") bind to the RY motif and activate globulin expression (Fig. 3.19b). The RY motif is frequently found together with GACACGC (coupling element: CE) and ACGTGG/TC (ABA-responsive element: ABRE) (Fig. 3.19c). As its name suggests, the ABRE motif is typically found in the promoter regions of genes induced by ABA, which include many of those encoding storage protein genes. ABA plays several roles during seed development, including the promotion of reserve accumulation (Sect. 2.3).

In Arabidopsis, *LEC2*, an embryo identity gene (Sects. 2.3.2, 2.3.4) and *FUSCA3* (*FUS3*), a seed maturation-associated gene (Sect. 2.3.4), both encode B3 transcription factors that directly bind to the RY motifs in the promoter region of the Arabidopsis 2S storage protein 3 (*At2S3*) gene (Fig. 3.19d). Another well-known B3 transcription factor, ABA INSENSITIVE 3 (ABI3), does not directly bind to the promoter but is associated with the activation of the same gene, which is mediated by another transcription factor called the basic leucine zipper protein (bZIP). LEC2, FUS3, and ABI3 are all seed-specific proteins.

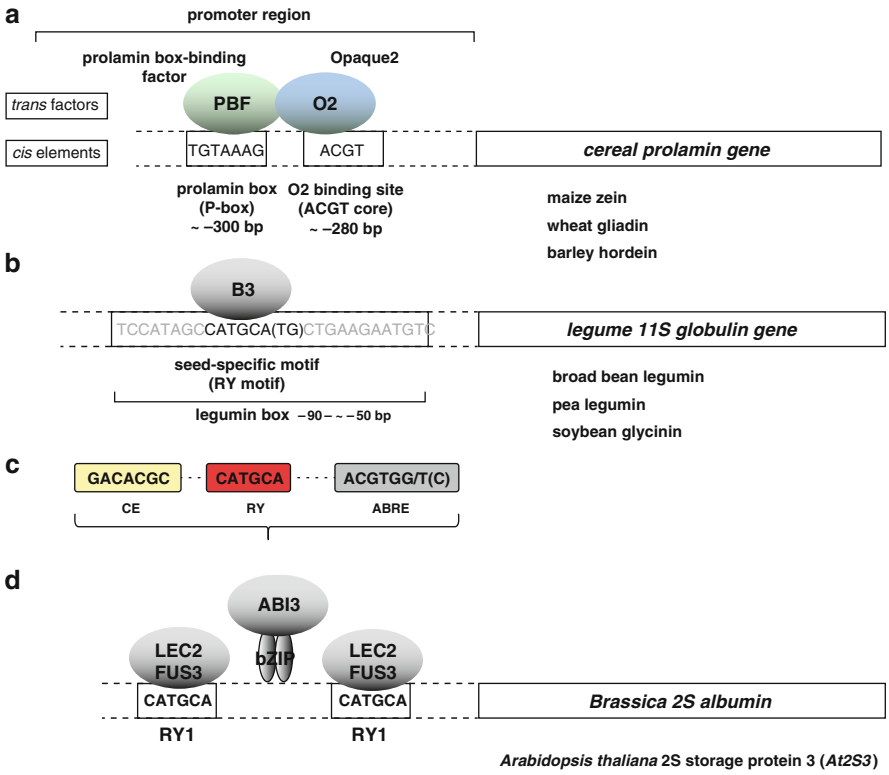

Fig. 3.19 Schematic representation of *cis*-acting elements (specific DNA sequences in the promoter region called motifs) and *trans*-acting factors (DNA-binding proteins called transcription factors), and their interactions in the induction of seed storage protein genes. (**a**) Cereal prolamin genes contain a prolamin box (TGTAAAG), to which is bound a prolamin box-binding factor (PBF), and a second motif, the ACGT core, that is bound to by Opaque 2 (O2). PBF and O2 physically interact with each other. (**b**) Legume 11S globulin genes contain a seed-specific RY motif (CATGCA) that binds to the B3 domain-containing transcription factor. (**c**) Typical structure of an RY seed motif together with the coupling element (CE: GACACGC) and the ABA-responsive element (ABRE: ACGTGG/T[C]). (**d**) Brassica (e.g., Arabidopsis) 2S albumin genes contain RY motifs that are directly bound by LEC2 and FUS3, both B3 transcription factors. ABI3, another B3 transcription factor, interacts with the promoter region of the same gene indirectly through a basic leucine zipper (bZIP) transcription factor. (**a**–**c**) based, respectivly, on information in Vicente-Carbajosa, J. et al., 1997. *Proc. Natl. Acad. Sci. USA* 94, 7685–7690; Bäumlein, H. et al., 1992. *Plant J.* 2, 233–239; Nakabayashi, K. et al., 2005. *Plant J.* 41, 697–709. (**d**) modified from Kroj et al., 2003. *Development* 130, 6065–6073

3.2.5 *Phytin Synthesis*

Phytin is an important reserve compound as a source of phosphate and mineral ions in seeds (Sect. 1.3.4). Synthesis of phytin occurs concurrently with that of storage proteins, and both are sequestered within PSVs.

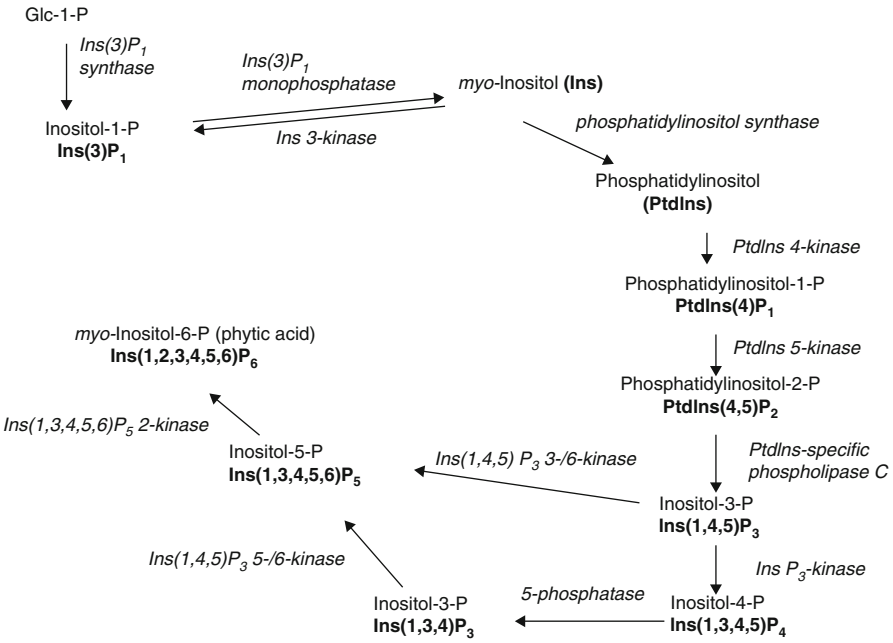

Fig. 3.20 Possible modes of synthesis of phytic acid in seeds, based on evidence from limited studies of metabolic pathways and mutants thereof. The abbreviated name of each intermediate is given, as well as (in bold) the D-numbering convention of the International Union and Biochemistry and Molecular Biology; e.g., PtdIns(4,5)P_2 is phosphatidylinositol-2-P, with phosphates in positions 4 and 5 of the ring. Ins: inositol; PtdIns: phosphatidyl inositol; $P_{1\text{-}6}$: number of phosphates associated with *myo*-inositol. For convenience the prefix *myo*- has been omitted from inositol, other than at the beginning and end of the pathway. Based on information in Raboy and Bowen (2006)

Two independent synthetic pathways for phytin have been proposed in plants, but only the so-called "lipid-dependent" or phospholipase C (PLC) pathway appears to operate in developing seeds, although in a divergent manner (Fig. 3.20). Glc-6-P derived from sucrose is converted to *myo*-inositol (Ins) via Glc-1-P and inositol-3-P [Ins(3)P_1]; in turn this is attached to a phosphatidyl moiety (a membrane lipid component, perhaps associated with either the inside or outside of the ER membrane) to form phosphatidyl inositol (PtdIns). To this there is the sequential addition of three phosphates (from ATP, the P source for all phosphorylation reactions) by specific kinases, and liberation from the phosphatidyl group, to yield Ins(1,4,5)P_3, with phosphates in the 1,4 and 5 positions of the inositol ring. There is some debate as to what happens next. One option (Fig. 3.20) is for the further addition of another P to position 3 on the ring, followed by removal of that from position 5, the resultant Ins(1,3,4)P_3 then being simultaneously phosphorylated twice by a single kinase, and the last P added by another kinase to yield phytic acid [Ins(1,2,3,4,5,6) P_6].

Another option is for the Ins(1,4,5)P_3 to be doubly phosphorylated by a single kinase to Ins(1,3,4,5,6) P_5, and thence on to phytic acid as previously. It is also possible that both of the optional pathways (or other variations) operate in the same or different tissues within the developing seed. The association of ions with IP_6 presumably occurs randomly by the attraction of the metallic cations to the strong negative charges on the exposed phosphate groups. This may occur before or after inclusion within the PSV; in the latter case, specific ion transporters need to be present in the membrane for import into this body.

Studies on the developing castor bean endosperm using electron microscopy are suggestive that, following its synthesis, phytin is trafficked similarly to Route 2 for proteins (Sect. 3.2.4.1, Fig. 3.18). That is, phytic acid is synthesized within the lumen of the cisternal ER (C-ER) prior to being packaged into transport vesicles (in which they are associated with ions); these migrate to the vacuolar (PSV) membrane (perhaps via the Golgi complex) with which they fuse. The phytin particles are released into the PSVs and condense therein to form an electron-dense globoid; an alternative suggestion is that the transport vesicles containing phytin are invaginated intact into the PSVs, coalesce therein, and thus form a large, distinct membrane-bound phytin body. Or, PSVs may contain several nonfused smaller phytin bodies, since not all species contain distinct globoids at seed maturity.

During the early development of rice embryos the gene for Ins(3)P_1 synthase (*RINO1*) is expressed in the scutellum and aleurone layer, and phytin globoids appear coincidentally with the increase in transcripts.

As mentioned in Sect. 1.3.4, the negatively charged nature of the phytin molecule allows it to bind to essential dietary minerals such as zinc, calcium and iron, and hinders their absorption by animals. Also, the excreted high phosphorus content in animal waste can cause pollution of waterways by enhancing algal growth. Attempts have been made to lower the phytin content of seeds through genetic engineering. In maize, the *IPK1* gene encodes Ins(1,3,4,5,6)P_5 2-kinase, the enzyme that catalyzes the final step of phytic acid biosynthesis (Fig. 3.20). The expression of this gene has been eliminated by a precise genome modification using zinc-finger nucleases (ZFNs), which are composed of specific DNA-binding domains linked to a DNA-cleaving domain, essentially creating genomic scissors. Unlike in traditional genetic engineering, this method allows for targeted genome modification through DNA sequence-specific recognition of the target gene by zinc-finger motifs and the action of endonucleases to cause a double-stranded break in the targeted DNA region. Using this method, a gene can be suppressed, activated or modified. When maize *IPK1* was disrupted in this manner, this resulted in a significant reduction in phytate in the mature kernels. As well as disrupting the gene, an herbicide-resistant gene was also inserted, resulting in transgenic plants delivering two phenotypes: low phytate seeds and herbicide-resistant plants. In an alternative strategy, silencing of a gene encoding a transporter protein that moves phytate into PSVs (*Ipa1-1*) reduces phytate content in maize and soybean seeds by about 80% and increases inorganic phosphate content. Yet another approach has been to express late in maize seed development a fungal enzyme (phytase) that breaks down phytate. This potentially

improves phytate digestibility and reduces phosphate excretion by farm animals; the bioavailability of iron is also increased. This phytase-expressing maize is undergoing final regulatory trials before commercial release in China.

3.2.6 *Modifications of Non-storage Compounds to Improve Nutritional Quality*

In addition to changes to the major storage reserves noted in the above sections that are being attempted or accomplished by selective breeding or genetic modification, seeds have also been the target of research to add or improve minor components, and thus increase their nutritional value. The synthesis and/or accumulation of a number of vitamins, secondary metabolites (flavonoids, resveratrol) and minerals (iron, zinc) have been enhanced in seeds. Only two examples will be noted here.

About half of the world's population depends upon rice grains as its staple food. But these are a poor source of essential micronutrients and minerals, especially the polished grain (i.e., with the aleurone layer removed to enhance grain storage capacity). In South East Asia some 40% of children under five suffer from impaired vision and disease susceptibility because of vitamin A deficiency, with up to 500,000 children going blind each year. Genetic engineering has been used to produce rice grains containing β-carotene, which the body can convert into vitamin A. To achieve this, genes were introduced to take an intermediate product of an existing rice biosynthetic pathway (geranyl–geranyl diphosphate [GGPP]) and convert it to β-carotene in the endosperm, which gives the grains a yellow or golden color (Fig. 3.21a). In the most recent version ("*Golden Rice 2*"), only two introduced genes are required, one from maize (phytoene synthase, *Psy*) and one from a bacterium (carotene desaturase, *ctrl*). The amount of vitamin A produced by the modified rice could supply over 50% of the recommended dietary allowance (RDA) of vitamin A from a very modest amount (e.g., 50 g) of rice, if consumed daily. This amount is well within the consumption habits of most young children and their mothers in rice-eating regions (130–200 g rice per day). In addition, the conversion of β-carotene from *Golden Rice 2* into vitamin A in the body is even more efficient than from other food sources, such as carrots or green leafy vegetables. The genes responsible for *Golden Rice 2* are being transferred into appropriate rice varieties for Asian countries at the International Rice Research Institute in the Philippines, but a release date remains uncertain due to regulatory issues and vocal opposition to genetic engineering in some countries.

This metabolic engineering approach has been extended to maize (Fig. 3.21b–d), where genes encoding enzymes in biochemical pathways to produce not only β-carotene but also vitamin C and folate (vitamin B) were introduced and expressed in the endosperm. This resulted in a 169-fold increase in β-carotene, a sixfold increase in vitamin C and a doubling of folate contents in the grains. As for *Golden Rice*, this multivitamin maize could nutritionally benefit the world's poorest people, but it remains to be seen whether it will be allowed to be commercialized in the countries where it is most needed.

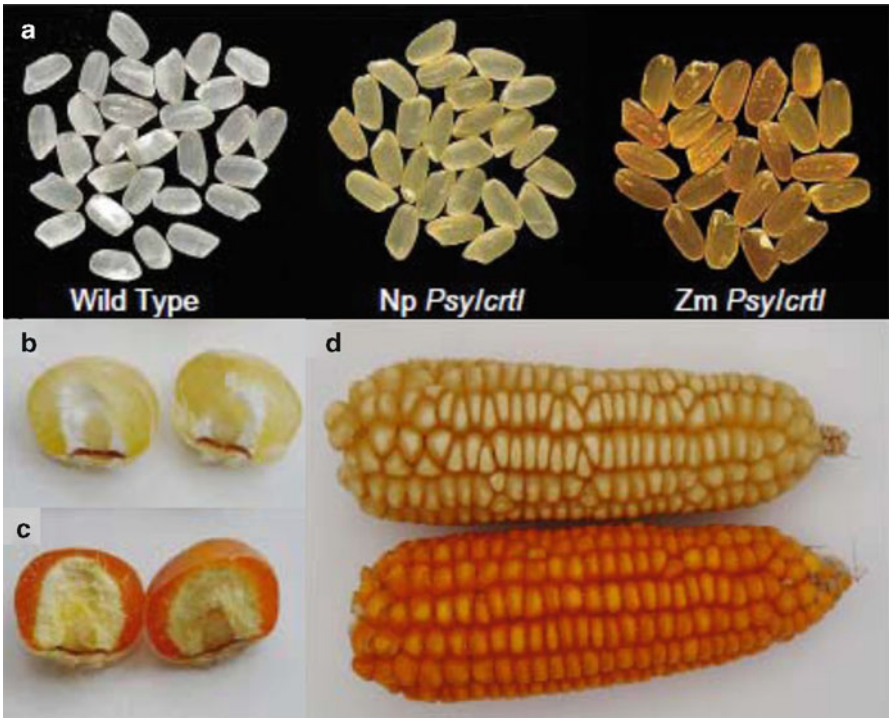

Fig. 3.21 Genetically modified rice and maize grains that synthesize and accumulate vitamins in the endosperm. (**a**) Polished grains of wild-type rice and grains transgenically expressing phytoene synthase (*Psy*) from either daffodil (Np) or maize (Zm) in combination with a carotene desaturase from the bacterium *Erwinia uredovora* (*ctrl*). The maize *Psy* gene greatly increases the β-carotene content, as is evident in the darker golden color (i.e., *Golden Rice 2*) compared to the daffodil *Psy* gene used in the initial version. (**b–d**) Kernels of wild-type (**b**) and multivitamin (**c**) kernels and cobs (**d**) of maize. The multivitamin kernels were engineered to synthesize and accumulate β-carotene, vitamin C, and folate (vitamin B). As in rice, the presence of β-carotene results in the golden color. (**a**) From Paine et al. (2005), (**b–d**) from Naqvi et al. (2009). Courtesy of Nature Publishing Group and the National Academy of Sciences, USA, respectively

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Chapter 4
Germination

Abstract Seed germination refers to the physiological process culminating in the emergence of the embryo from its enclosing coverings, which can include the endosperm, perisperm, testa, or pericarp. The absorption of water by the seed (imbibition) activates metabolic processes that subsequently lead to expansion of the embryo and penetration of the radicle (or other organ) through the surrounding tissues. Respiration to supply metabolic energy for these processes is activated immediately following imbibition. Transcripts (mRNA) synthesized in seeds during development are present in dry seeds, but most are degraded soon after imbibition. The transcription of germination-related genes and their translation into proteins begins within the first few hours following hydration. Expansion of the embryonic tissues is opposed by the restraint of the tissues enclosing them; an increase in embryo growth potential and/or a decrease in the strength of the covering tissues allow germination to be completed. Cell division generally only begins following the completion of germination. Partially hydrating seeds for a period of time followed by dehydration (seed priming) can accelerate their germination when they are subsequently planted, a practice that is utilized commercially to enhance the speed and uniformity of crop establishment.

Keywords Germination • Imbibition • Respiration • Transcription • Transcriptome • Translation • Proteome • Growth • Cell division • Priming

4.1 Seed Germination: Definition and General Features

In the scientific literature the term *germination* is often used loosely and sometimes incorrectly, and so it is important to clarify its meaning. *Germination begins with water uptake by the seed (imbibition) and ends with the emergence of the embryonic axis, usually the radicle, through the structures surrounding it.* This latter event is sometimes referred to as "visible germination," at which point the seed has

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completed germination (or germinated). However, for brevity, the word germination is often used to indicate its completion, e.g., terms like "50% germination" indicate that 50% of a seed population has completed germination, and a "germination time course" actually reflects the % of seeds that have completed germination at particular points in time.

Germination *sensu stricto* does not include seedling growth; this commences after germination is completed. The sometimes-used term "germinating seedling" is obviously erroneous. Seed analysts often refer to germination as emergence from the soil because their interests lie in monitoring the establishment of a growing plant of agronomic value. Although such use of the term germination is not encouraged by physiologists, its widespread use by seed technologists is acknowledged. A better term, however, would be "*seedling emergence*" or "*seedling establishment*." Processes occurring in the nascent seedling, such as the extensive mobilization of the major storage reserves, are also not part of germination: they are post-germination events. Unfortunately, there is an abundant literature on physiological, cellular, and molecular aspects in which germinated seeds have been used to incorrectly draw conclusions about germination; the information drawn from such studies should be treated with caution.

A seed that is mature and dry is said to be *quiescent*. Quiescent seeds are resting organs, generally having low moisture contents (5–15%) with metabolic activity almost at a standstill. A remarkable property of seeds is that they are able to survive in this state, often for many years (Chap. 8), and subsequently resume a normal, high level of metabolism. For germination to occur, many quiescent seeds need only to be hydrated under conditions that support metabolism, e.g., a suitable temperature and the presence of oxygen. Quiescence should not be confused with dormancy (Chap. 6), which relates to the inability of imbibed and metabolically active seeds to complete germination under favorable conditions.

When dry, viable seeds imbibe water, a chain of events is initiated which ultimately results in the emergence of the embryo, usually the radicle, signifying that germination has been successfully completed. The cellular changes that occur during germination are complex (Fig. 4.1), for upon imbibition metabolism must be initiated to permit the recovery from structural damage caused by maturation drying and oxidation while dry, basal cellular activities must be reestablished, in some species dormancy must be alleviated, and the embryo must prepare for emergence and subsequent early seedling growth. Thus it has been difficult to distinguish between events that are directly linked to the completion of germination itself, and those necessary for the other changes that are taking place. There are several reasons for this, some related to the nature of the seed, and others to the research approaches employed. Utilizing whole seeds for germination studies can pose problems, because the seed is a multicellular organism, with several different organs and tissue types, as detailed in Chaps. 1 and 2, yet it is only a part of the axis region that elongates to complete germination. In many seeds, the major cell mass is the storage tissue, which does not play a major role in germination per se; its importance comes later with the requirement for a supply of nutrients to the growing seedling (Chap. 5).

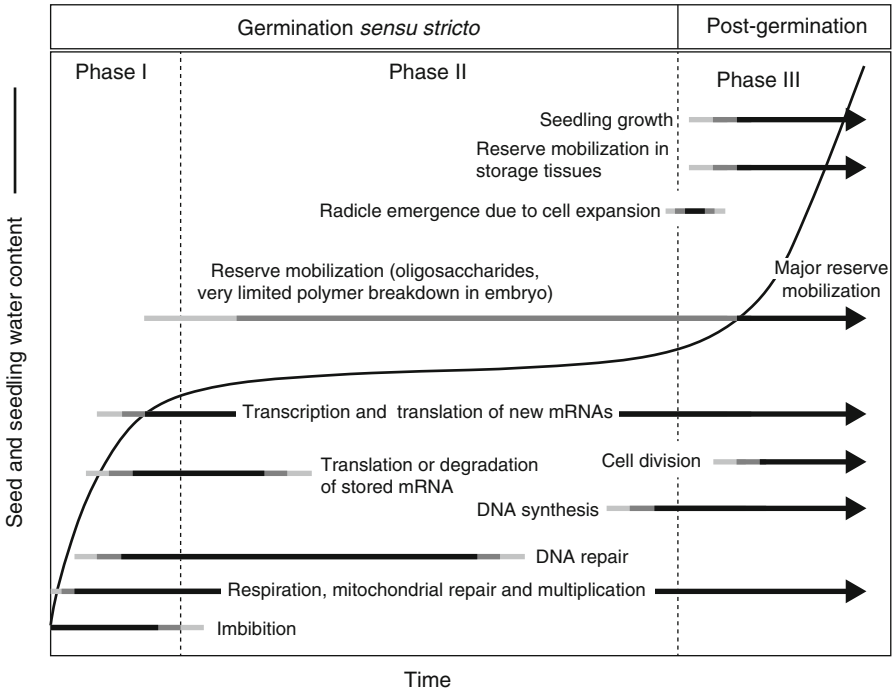

Fig. 4.1 Time course of water uptake and some important changes associated with germination and early seedling growth. Initial absorption of water, imbibition in Phase I, is primarily a physical process; physiological activities may commence within minutes of a cell becoming hydrated, well before all seed tissues become fully imbibed. During Phase II seed water content is fairly constant and metabolic activities increase with substantial transcription of new genes. Radicle emergence through the surrounding structures at the end of this Phase marks the completion of germination, and in Phase III there is further uptake of water as the young seedling becomes established, utilizing the major stored reserves. The curve is a stylized time course for water uptake. The time taken for these events to be completed varies among species and the germination conditions to which the seed is subjected. After Nonogaki et al. (2010). Courtesy of Elsevier

Yet its metabolism is de facto included in analyses of intact germinating seeds. Removal of the axis from larger seeds (e.g., legumes and cereals) has been carried out for some experiments, but even this region contains many cells (of different types), relative to the few that become engaged in elongation to effect radicle emergence (Sect. 4.6.1), thus potentially masking important changes that are integral to this final act of germination. Cellular events associated with germination may occur in a seed that does not achieve radicle emergence. Even when conditions are apparently favorable for germination, so that imbibition, respiration, synthesis of nucleic acids and proteins, and a host of other metabolic events all proceed, cell elongation resulting in emergence of the embryo does not occur, for reasons that are still poorly understood: such a seed exhibits dormancy (Chap. 6).

4.2 Measurement of Germination

As described above, the visible indication that germination *sensu stricto* has been completed is when the radicle (or other embryonic tissue) emerges from the tissues enclosing it. This point marks a clear end to germination and the start of seedling growth. Prior to radicle emergence, it is difficult or impossible to predict how far a given seed has progressed toward completing germination, such as by measuring water uptake or respiration. On the other hand, it is not germination of a single seed that is of interest, but rather the germination properties of a large number or population of seeds, e.g., all the seeds produced by one plant or inflorescence, or all those collected in a soil sample, or all those subjected to a certain experimental treatment. Thus, a fundamental measure of the germination capacity of a seed population is the *percentage* of seeds that have completed germination at a given time. To determine the total potential viability of a seed population, this time may be quite long to give all nondormant seeds the opportunity to complete germination. Seldom, if ever, do all seeds in a population complete germination simultaneously, and therefore observing radicle emergence repeatedly over time, and plotting the percentage of the seed population that has completed germination at each time point, results in characteristic seed germination time courses (Fig. 4.2). Time course curves like these are commonly produced in seed germination tests and provide considerable information about the speed and uniformity of germination.

Seed populations tend to germinate in a characteristic pattern over time, resulting in a sigmoid curve when the percentage of seeds that have completed germination is plotted versus time (Fig. 4.2). This reflects the fact that like many biological properties, germination times are approximately normally distributed, or exhibit a bell-shaped distribution. Some seeds in a population will complete germination very quickly, most will do so around the average time, and some will be much slower than the average, resulting in the overall sigmoid shape of cumulative germination time courses. In fact, this curve is generally somewhat skewed to longer germination times, meaning the time required for the first half of the population to complete germination is shorter than for the second half (Fig. 4.2). This skewed pattern is well described by a population model that is based upon a normal distribution of threshold values among seeds (Sect. 7.2.1).

Germination time courses reveal information about the timing, uniformity and extent of germination in seed populations. For example, curve (a) in Fig. 4.2 illustrates a time course for a seed population that completes germination rapidly, uniformly, and completely. The corresponding probability distribution of germination (curve [a] in Fig. 4.2b) is narrow and somewhat right-skewed, but approximately symmetrical. The width of the distribution indicates the spread in germination times (the inverse of uniformity), which is also proportional to the slope of the germination time course curve near its midpoint. That is, steep slopes of germination curves indicate high uniformity or a narrow distribution in time to completion of germination. [Note that the slope of the germination time course curve is an indication of the *uniformity* of germination, not the *rate* of germination; see discussion of germination

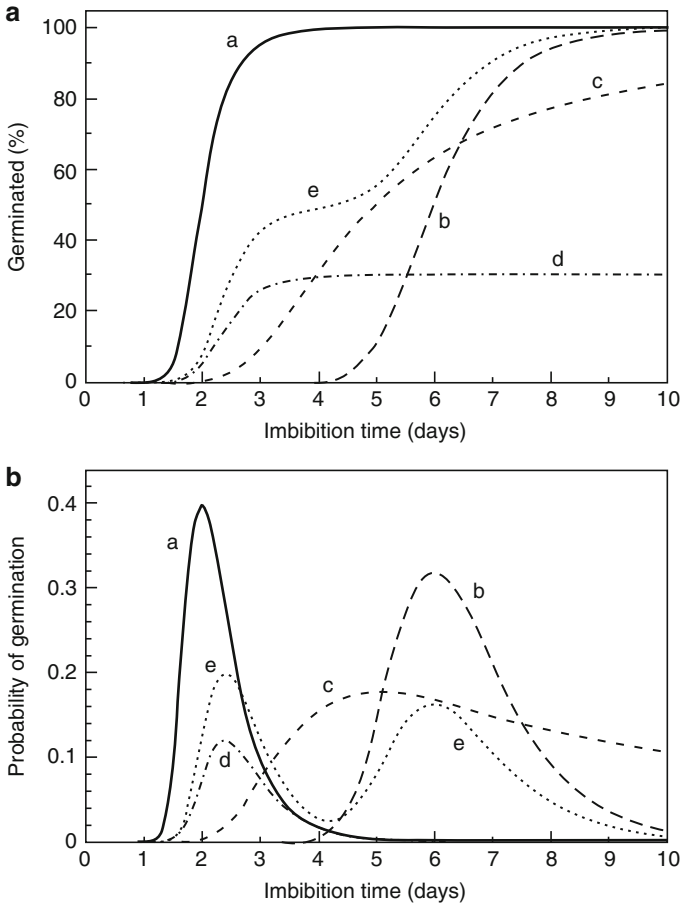

Fig. 4.2 Some characteristic generalized time courses (a–e) and distributions of germination. (**a**) Time course curves illustrate some of the different patterns often encountered with germination of seed populations. (**b**) Distributions or probabilities of germination occurring across time based on the corresponding time courses in (**a**)

rates below]. Uniformity of germination is also sometimes indicated by the time between two germination percentiles, such as the time between 10 and 90% or between 25 and 75% germination; smaller values indicate greater uniformity. [Statistically, it would be better to use 16 and 84% as the range in this method, as this represents one standard deviation on each side of the mean of a normal distribution.] Curve (b) in Fig. 4.2a illustrates a seed population with a longer Phase II of imbibition and less uniform germination, but which still achieves 100% germination. The corresponding distribution (curve [b] in Fig. 4.2b) is shifted to longer times and is broader than for curve (a). Curve (c) in Fig. 4.2a illustrates an intermediate

case where the seed population is quite nonuniform, with some seeds completing germination relatively early and others requiring much longer times. Extending the test for longer than the 10 days illustrated would be required to determine the total percentage of viable seeds in this population. Wild (non-crop) seed populations often exhibit germination time courses such as this, indicating a wide range of germination potentials among seeds within the population. This is also the type of germination time course that occurs when water availability is reduced, i.e., completion of germination is delayed and uniformity is reduced.

When only a fraction of the population is capable of germinating under the conditions of imbibition, as in the case of a partially dormant or nonviable seed lot, a time course such as curve (d) in Fig. 4.2a can result. The nondormant viable seeds will complete germination, but the dormant or nonviable seeds do not, resulting in a time course that reaches a final percentage less than 100%. Seed germination time courses can also exhibit more complex patterns, as illustrated by curve (e) in Fig. 4.2a. This type of curve reveals that the total seed population is composed of two subpopulations with different germination characteristics. In this case, 50% of the seeds have germination properties like the nondormant seeds in curve (d), while the other 50% have properties like those in curve (b). The germination of the total population reflects the sum of the behavior of the two subpopulations, and the germination distribution shows two peaks (Fig. 4.2b). This type of germination pattern can be observed for some plants that characteristically produce seeds with different depths of dormancy or germination requirements, or when seed lots with different germination characteristics have been blended together. It is also possible that seeds that are initially dormant at the start of imbibition can lose dormancy under the temperature and light conditions present, such that they will gain germination capacity during the germination test. This can result in time courses like curve (e) or even more complex patterns. See Chaps. 6 and 7 for more information on how dormancy and environmental conditions influence germination of seed populations.

Germination time courses also reveal information about the *rate* of germination. This can be defined as the reciprocal of the time taken for germination to be completed, starting from the time of sowing, and is expressed in units of hours^{-1} or days^{-1}. Although the term "rate" is often used in the seed literature to refer to germination percentage (e.g., "the germination rate was 90%"), this is clearly incorrect. *Percentage* always refers to the fraction of individuals in the seed population that have or have not completed germination, while *rate* should be used only in connection with the inverse of the time required for a specific percentage of the population to complete germination. It is important to note that as defined here, germination is a quantal parameter, meaning that a given seed either has or has not completed germination at a given time. Thus, any given seed has as a unique rate of germination, which is the reciprocal of the time from the start of imbibition until radicle emergence. Since all seeds in a population do not complete germination simultaneously (Fig. 4.2), the rate must be specified for a given percentage, often the median or 50% percentile, but the actual percentile used is arbitrary. The time (t) to 50% germination would be t_{50}, and the germination rate for the 50th percentile would be $1/t_{50}$.

A common method of calculating germination rate is to determine the time at which 50% of the germinating seeds completed germination. In this method, the mean time to germination is equal to $\Sigma(t \cdot n)/\Sigma n$, where t is the time in days, starting from day 0 (the day of sowing) and n is the number of seeds completing germination on day t. The mean germination rate (GR) therefore equals $\Sigma n/\Sigma(t \cdot n)$. However, if the total germination percentages of two populations differ, this means that different percentiles of the total population would be compared, depending on the final germination percentage, which can underestimate the differences in germination rates. This is illustrated in Fig. 4.3, where the same sets of germination time courses are analyzed for germination rates by different methods. In Fig. 4.3a, the times to 50% germination are based on the total seed population. This value increases sharply as germination is delayed, and becomes infinite when final germination does not reach 50% (Fig. 4.3c). However, when the time to 50% germination is based on the final germination within a given period, the value for t_{50} increases more slowly, and is based on different percentages of the total populations. For example, seed populations 1 and 2 in Fig. 4.3 reached 100% germination, so both would be compared at 50% of the total population. However, population 5 only reached 40% germination at the end of the experiment, so the t_{50} would be based on the 20th percentile of the total population. Thus, when the final germination percentage differs widely, this method results in the comparison of different percentiles in the different populations, which is inherently misleading. Instead, if some populations do not achieve 50% germination, all populations could be compared at a lower percentage. For example, all of the populations could be compared at 20% (Fig. 4.3b), which would result in relative germination rates showing a similar relationship to the ones shown for the total populations in Fig. 4.3c.

In many cases, both the rate and the final percentage germination provide useful information about a seed population. A number of indices have been proposed that attempt to combine both of these into a single parameter. For example, one index uses the following equation, $\Sigma(n_i \cdot t_{(x-i)})/\Sigma n_i$, where n_i is the number of seeds germinated on day i, and x is the total number of days of the test. This equation gives rapid germination higher weight than slower germination. However, it is still possible for this index to give similar values to two time courses with very different overall patterns. Due to the wide diversity of possible germination patterns (e.g., Fig. 4.2), it has proven difficult to develop a single index that satisfactorily incorporates both percentage and rate. In general, it is best to examine complete germination time courses and use separate measures of percentage, uniformity, and rate when quantifying or comparing the germination properties of seed populations. As germination is a quantal parameter based on percentages, probit analysis can be of great value in handling germination data. A number of different equations and distributions have also been used to fit cumulative germination time courses, such as the logistic, the Richards, the Weibull, and the Gompertz functions. These equations are flexible and in many cases can empirically fit germination time courses very well, allowing the derived parameters in the equations to be used as indices of different aspects of germination kinetics. It is beyond the scope of this book to deal further with these mathematical methods, but further discussion can be found in Chap. 7 and in references listed for this Section.

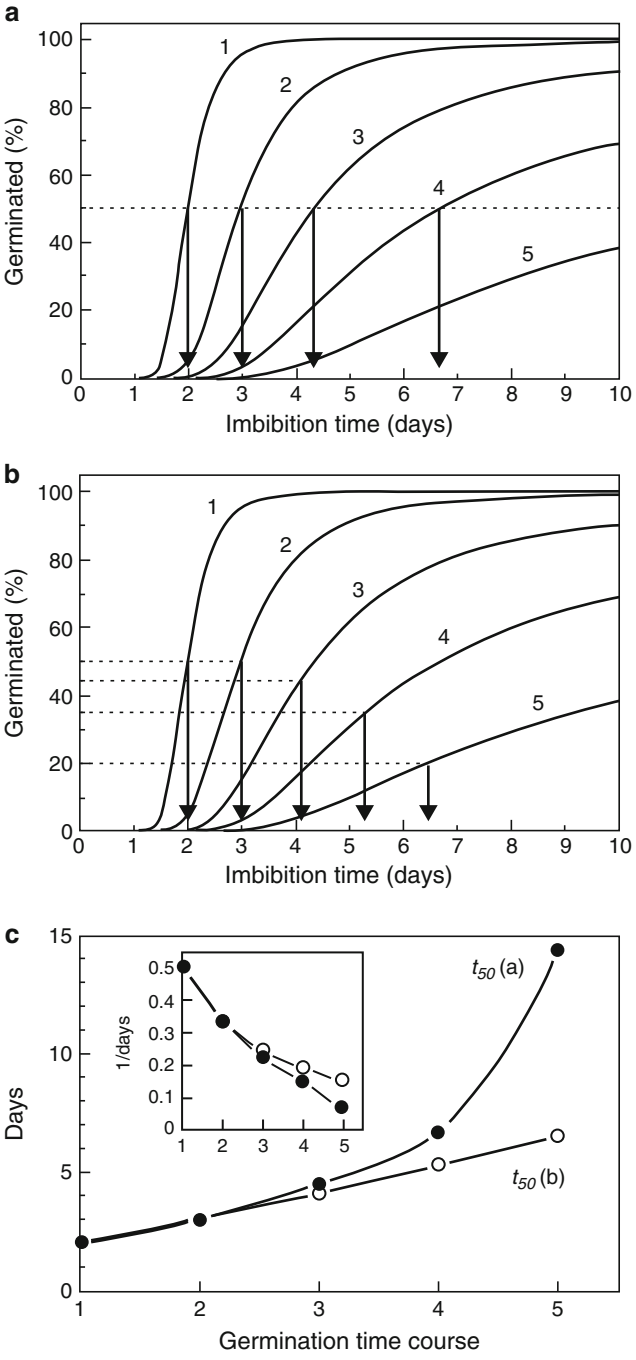

Fig. 4.3 Germination time courses and measurements of median germination time. (**a**) A series of germination time courses (1–5) showing the times to 50% germination (t_{50} *total population*) of the entire seed population. This method compares the same fraction of the population (50th percentile)

4.3 Imbibition

For seeds that are dry at maturity, absorption of water, or imbibition, is required for the resumption of metabolism and initiation of cellular events leading to radicle emergence. Described here are the physical and structural changes associated with the absorption of water by seeds and how they can affect germination.

4.3.1 Uptake of Water from the Soil

The uptake of water by seeds is an essential, initial step in germination. Air-dried seeds generally have moisture contents (water content as a percentage of seed dry weight) in the range of 5–15%, depending upon the seed oil content and the ambient air relative humidity (Sect. 2.6.3), while fully imbibed seeds may have moisture contents of 75–100%. These values correspond to approximately 5–13% and 40–50% on a fresh weight basis (water content as a percentage of the total seed weight) (Fig. 2.29). Both the initial rate of hydration (Phase I) and the final plateau moisture content attained (Phase II) (Fig. 4.1) can influence seed germination.

Several factors govern the movement of water from the soil into the seed, but particularly important are the water relations of the seed and of the soil. Water potential (ψ) is an expression of the free energy status of water, and in plant physiology is generally expressed in terms of pressure units. The bar was the unit most frequently used in the past (1 bar = 10^3 dynes cm^{-2}, 10^2 J kg^{-1}, or 0.987 atm), but the term megaPascal (MPa) is now preferred (-1 MPa = -10 bar). Pure water has the highest potential, and by convention it is assigned a zero value. Other potentials, therefore, have positive (i.e., >0) or negative (i.e., <0) values. Net movement of water occurs down an energy gradient from high to low ψ. The total water potential of the cells in a seed (ψ_{cell}) comprises three components as follows:

$$\psi_{cell} = \psi_s + \psi_p + \psi_m \tag{4.1}$$

The solute or osmotic potential (ψ_s) accounts for the effect of the concentration of dissolved solutes in the cell on the energy status of water. The greater the concentration of solutes of all types (i.e., both nonionic such as sugars and ionic such as

Fig. 4.3 (continued) in each case. Note that population 5 does not reach 50% germination until over 14 days, beyond the time period shown. (**b**) The same germination time courses as in (**a**), but showing the times to 50% of the final germination percentage (t_{50},*final germination*) after 10 days. This method compares the times to germination of different fractions of the total population as the final germination percentage is reduced. (**c**) Comparison of the values for t_{50} calculated as illustrated in (**a**) or (**b**) above for the different time courses (1–5). For a given percentile of the population, the t_{50} increases exponentially (t_{50} (**a**)), but as a fraction of the final germination percentage, it increases linearly (t_{50} (**b**)). The corresponding germination rates ($1/t_{50}$) are shown in the inset

potassium), the lower (more negative) is the ψ_s and hence the ψ_{cell}. Thus, the presence of solutes in the cell can create a ψ gradient for water uptake if the ψ of the water outside the cell is higher. The pressure or turgor potential (ψ_p) occurs because as water enters a cell, the relatively rigid cell walls resist expansion. Since water is essentially incompressible, the movement of water into the cell results in an increase in the internal pressure that raises the energy status of water. Values of ψ_p are therefore positive and increase ψ_{cell}. In essence, the negative ψ_s due to the presence of solutes attracts water into the cell until the buildup of pressure due to the water influx increases ψ_p sufficiently to balance it. Thus, in a nongrowing cell in equilibrium with an excess of pure water ($\psi = 0$ MPa), ψ_s and ψ_p would have equal but opposite values (e.g., −1 MPa vs. 1 MPa), and net movement of water would cease. The matric component (ψ_m) accounts for the effects of the tight association of water to tiny capillaries (e.g., among the polymers comprising the cell walls) and the surfaces of macromolecules such as starch and proteins. This binding lowers the energy content of water, so ψ_m values are negative. ψ_m is an important component of ψ in dry seeds and is responsible for the ψ gradient that initially drives water uptake. Matric forces are often minimal or ignored in hydrated plant tissues, as once there is sufficient water available for free liquid water to occur, ψ_m falls to negligible values. An exception is in the xylem and apoplastic system, where the evaporation of water in the leaves creates tension in the water columns in the xylem vessels that pulls water through the vascular system. However, this tension is usually considered as a negative pressure (ψ_p) rather than as a component of ψ_m.

The soil also has its own water potential (ψ_{soil}), which is the sum of its ψ_s, ψ_p, ψ_m components, and gravitational effects are sometimes included as well. However, the pressure component is zero in soils open to the atmosphere, and as the concentration of solutes in soil water is generally low (saline soils being an exception), ψ_s is also negligible in soils. Thus, ψ_{soil} is primarily due to the capillarity of soil particles and water binding to surfaces, or to ψ_m. The difference in ψ between the soil and the seed is one of the factors that determines availability and rate of flow of water to the seed. Air-dry seeds have ψ values (ψ_{seed}) of −50 to −350 MPa due to the large negative ψ_m component, while a soil at field capacity (i.e., drained only due to the action of gravity) has a ψ_{soil} of approximately −0.03 MPa. Thus, there is a very large initial ψ gradient of up to 5 orders of magnitude for water uptake into the seed from moist soil. As the seed moisture content increases during imbibition and the matrices become hydrated, ψ_{seed} increases (i.e., becomes less negative) and ψ_{soil} decreases as water is withdrawn. Continued availability of water to the seed depends on the ψ of the zones of soil immediately surrounding the seed and on the rate at which water moves through the soil to replenish it, i.e., the hydraulic conductivity of the soil. In a moist soil at field capacity, the small amount of water required to hydrate a seed would generally be readily replenished by water movement in the soil. However, soil hydraulic conductivity decreases exponentially as soils dry, and can become a limiting factor in drier soils. The degree of contact of the seed with the surrounding soil can be important for imbibition, both with respect to the surface area of the seed that is in contact with soil moisture (i.e., seed–soil contact) and the ability of water to move into the seed. Seed–soil contact is more critical for large seeds than for

small seeds, which can come into closer contact with smaller soil pores. Crop planting equipment generally has a press wheel or some similar mechanism for firming the soil around the seed to assure good seed–soil contact. Seeds also swell during imbibition, which can improve seed–soil contact. Some seeds release mucilage from their seed coats, which creates a continuous connection with the surrounding soil water and enhances water absorption. However, factors such as soil hydraulic conductivity and seed–soil contact primarily affect the rate of imbibition and not the final seed moisture content achieved, which will be determined at equilibrium by ψ_{soil}. As will be discussed subsequently (Sect. 4.3.5), germination is generally inhibited at ψ values much higher than those that markedly affect soil hydraulic conductivity, so the water conductivity of a soil does not limit germination per se.

Water can also move to the seed through the vapor phase, which is not limited by soil conductivity to liquid water but rather by gas diffusion rates through the soil air spaces. It is difficult in a soil situation to experimentally separate seed–soil contact from vapor phase movement, and temperature cycles in the soil can result in condensation of liquid water that could be absorbed by the seed. Nonetheless, water movement through the vapor phase may be more important than is often appreciated, as even at ψ_{soil} of −1.5 MPa, the equilibrium relative humidity is 98.9%. However, as germination rates and percentages of many seeds are sensitive to reductions in ψ of even less than this (Sect. 4.3.5), imbibition through the vapor phase in relatively dry soils will increase seed moisture content but cannot raise the ψ_{seed} above ψ_{soil}, which remains the determining factor for germination.

Another factor that can influence seed water uptake is the permeability of the seed coat or other tissues enclosing the embryo. Many plants produce seeds that once they are dry, have seed coverings that are impermeable to water uptake. These so-called "hard seeds" exhibit a form of physical dormancy, because if a dry seed cannot imbibe water, it cannot germinate. This type of dormancy is discussed in Sect. 6.3.2.

4.3.2 Phase I, Imbibition and Imbibitional Damage

Under optimal conditions of supply, the uptake of water by seeds is triphasic (Fig. 4.1), reflecting different physical and metabolic processes that drive water movement in each phase. As noted above, when a dry seed is brought into contact with water there is a very large ψ gradient for the movement of water into the seed. Assuming that the seed coat is permeable (at least in some places; see below), water will enter the seed rapidly in response to the low ψ_m of the dry seed. Phase I, or imbibition per se, is largely a consequence of these matric forces, and water uptake occurs regardless of whether the seed is dormant or nondormant, viable or nonviable. In fact, nonviable seeds often imbibe more water than viable seeds, as the cells in viable seeds eventually develop turgor pressure and the resulting ψ_p counteracts further water uptake. In nonviable seeds, the cellular membranes are not intact, ψ_p remains zero and solutes are released into the apoplast, allowing for continued water uptake.

In some seeds, initial water uptake occurs in specific locations or through inherent structural features in the surrounding tissues. For example, water enters initially through the micropylar region of tobacco and maize seeds. In certain legumes (e.g., *Vicia* and *Phaseolus* spp.), water uptake occurs primarily through the micropyle, and in some hard-coated seeds of the Papilionaceae (e.g., white sweet clover and Arabian fenugreek) a plug covering a special opening—the strophiolar cleft—must be loosened or removed before water can enter, and then only through that region. In some species of *Geranium*, a specific hinged valve of palisade cells adjacent to the micropyle controls water entry into the seed. In seeds of western white pine, the micropyle is actually impermeable to water, which enters the seed through the surrounding testa. High-resolution magnetic resonance imaging (MRI) of imbibing western white pine seeds shows that water enters through the testa and megagameophyte and then accumulates in the embryos, starting first from the cotyledon end and then hydrating the entire embryo (Fig. 4.4).

Soon after seeds start to take up water, there is often leakage of solutes such as sugars, organic acids, ions, amino acids, and proteins into the surrounding medium. A wetting front is formed as water permeates the seed, and there is an abrupt boundary of water content between wetted cells and those about to be wetted. Particularly in large seeds such as beans or peas, physical tension can be established between the outer tissues that are hydrating and therefore swelling, and the inner tissues that are still dry. When imbibition is too rapid, as when the testa is removed or damaged, these internal tensions can result in disruption of cell walls, blistering of the cotyledon surface and extrusion of cellular contents (Fig. 4.5). In addition to this direct extrusion, cellular membranes may also leak ions and solutes during hydration. The leakage of electrolytes from both sources can be easily quantified by measuring the conductivity of water in which seeds have been soaked, and higher conductivity (greater leakage) is associated with poorer quality seed lots. The conductivity test has been developed into a standard seed-quality test for peas, for example, but is not applicable to all seeds due to differences in the permeability properties of tissues surrounding the embryo. Studies with tracers having different physiochemical properties (e.g., lipophilicity, electrical charge) show that some covering tissues are highly permeable to solutes (e.g., *Phaseolus* bean seed coats), others are selectively permeable to some solutes (tomato, pepper, and onion), while those of some seeds (cucumber and lettuce) are impermeable to solutes. In the latter seeds, nonviable embryos release solutes that are retained within the endosperm/perisperm envelope, resulting in osmotic water uptake and swelling of the outer envelope, termed "osmotic distension." In the case of muskmelon seeds, the presence of callose in the outer cell walls of the perisperm envelope enclosing the embryo is apparently responsible for the selective permeability of this layer (i.e., permeable to water but not to solutes), which does not depend upon intact or viable phospholipid membranes. These enveloping tissues that prevent the release of solutes from the embryo until they are penetrated by the growing radicle may serve to retain solutes for reabsorption by the embryo following hydration and also prevent their loss to the soil, where they could stimulate the growth of fungi and bacteria that might invade the seed and lead to its deterioration.

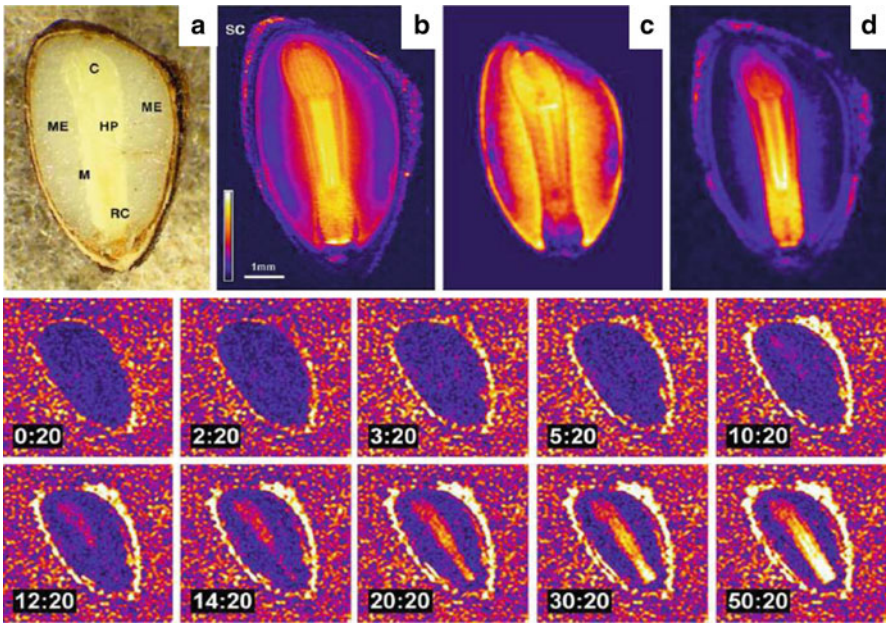

Fig. 4.4 (**a**) Low-resolution digital photograph of a cut imbibed western white pine seed. (**b**) Magnetic resonance (MR) image of an intact imbibed seed showing signals for both oil and water. Intensity (concentration) scale is from low (*blue*) to high (*white*). (**c**) Chemical shift selective MR image of oil in the same seed as in (**b**). (**d**) Chemical shift selective MR image of water into the same seed as in (**b**). Lower panels show a time series of imbibition for the water signal (h:min), with initial hydration of the seed coat, followed by water accumulation first in the cotyledon end of the embryo and eventual saturation of all the embryonic tissues. Labels in (**a**): ME, megagametophyte; C, cotyledons; HP, hypocotyl; M, meristematic region; RC, root cap; and in (**b**): SC, seed coat. From Terskikh et al. (2005) which includes time-lapse movies of imbibition

Seeds that are hydrated wholly or partially via the vapor phase in atmospheres of high relative humidity before introduction to liquid water, and seeds that do not undergo drying during their final stages of maturation, do not leak solutes when placed in water. This indicates that imbibition damage and solute leakage are associated with rapid liquid water uptake into seeds that are initially at low water content. In addition, the temperature of imbibition also affects the severity of imbibitional damage, being more severe when initial water uptake occurs at low temperatures (Fig. 4.6). This imbibitional chilling injury is a major problem in some warm season crops such as maize and soybean when dry seeds are planted in cold soils. The "cold test," in which seeds are first imbibed and held for seven days at 10°C then moved to 25°C for four additional days before scoring the seedlings, is often used as a vigor test to evaluate the susceptibility of a seed lot to imbibitional chilling injury. The low and high temperatures and the days at each temperature are adjusted for application to various species.

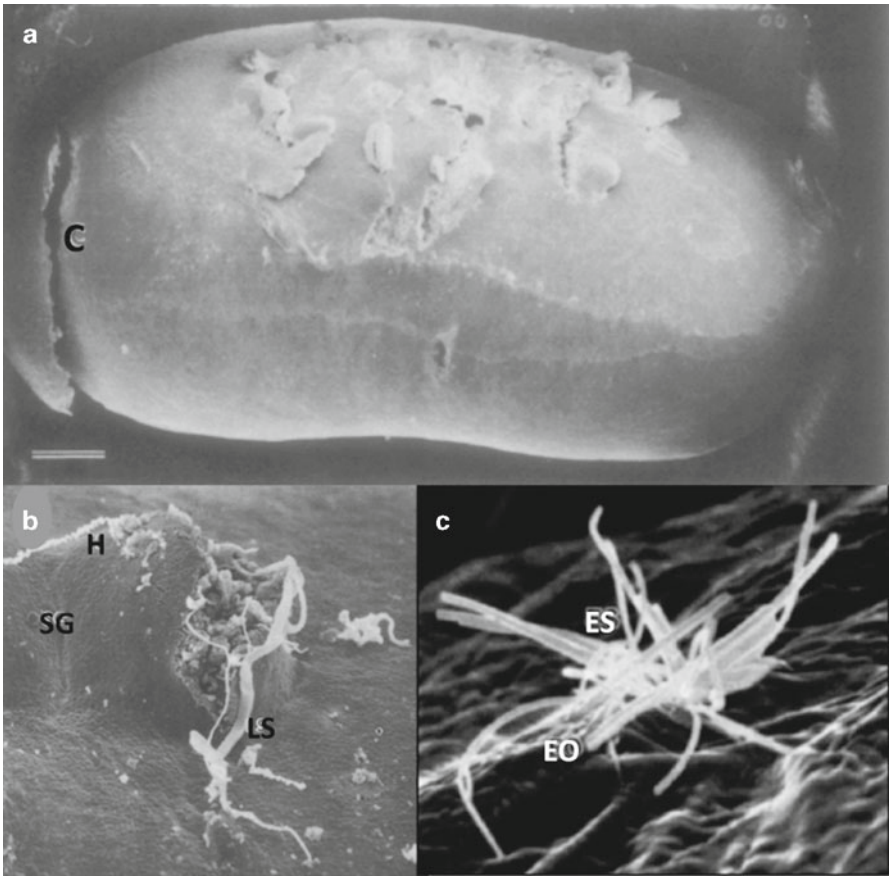

Fig. 4.5 (**a**) Blisters on the surface of a *Phaseolus* bean cotyledon after 45 min of imbibition. Rapid imbibition can create tension between outer hydrated tissues and inner dry tissues that result in pressure-driven extrusion of cellular contents. C, cracked cotyledon. (**b**) Close-up of a surface blister showing stream or ribbon of extruded cellular protoplasmic contents. SG, starch granule; H and LS, small helical and large longitudinal striations of the extrusion stream. (**c**) Extrusion streams on the surface of an imbibed pea cotyledon. EO, extrusion orifice; ES, extrusion streams. (**a**) and (**b**) from Spaeth (1987). Copyright American Society of Plant Biologists. (**c**) from Spaeth (1989). Courtesy of the American Society of Agronomy-Crop Science Society of America-Soil Science Society of America

Research on cell membrane structure in relation to water content and temperature has provided an explanation for the imbibitional chilling phenomenon. Cellular membranes are composed of a bilayer of phospholipids. The hydrophilic head groups of the molecules face outward, while the hydrophobic lipid chains associate in the inner part of the membrane (Sect. 2.5.2.1, Fig. 2.21a). This structure is dependent on the presence of water to maintain the hydrophobic/hydrophilic orientation. As water is removed during dehydration, the membrane changes from the more

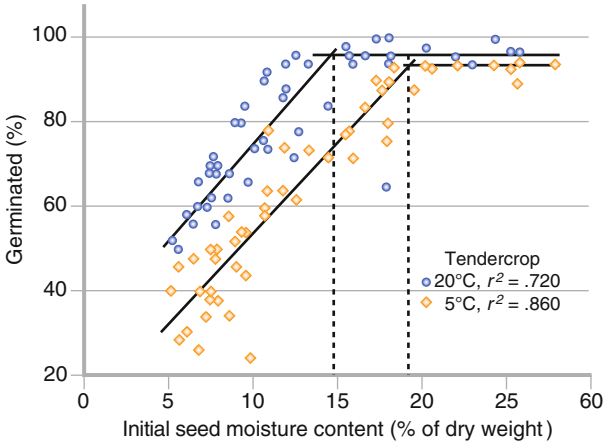

Fig. 4.6 Effect of initial moisture content and temperature on imbibitional damage of *Phaseolus* bean seeds cv. Tendercrop. Seeds were germinated at 20°C continually or at 5°C for 24 h and then moved to 20°C. Below threshold moisture contents (*vertical dashed lines*) seed viability was reduced linearly with further reductions in the initial seed moisture content. This threshold moisture content was higher (19%) at (◇,orange) lower temperature (5°C) than it was (15%) at (○, blue) higher temperature (20°C). Coefficients of determination (r^2) are for the slopes below the thresholds. From Wolk et al. (1989). Copyright American Society of Plant Biologists

fluid or liquid crystalline state to the less fluid gel state due to closer packing of the molecules, which restricts their motion. Thus, membranes of a dry seed will be primarily in the gel state, which is not a good barrier to the leakage of cellular contents. If the seeds are then rapidly exposed to water during imbibition, water enters before the membranes can revert to the liquid crystalline state, and leakage and cellular damage occur (Fig. 4.7a). The transition between the liquid crystalline and gel states is also dependent upon temperature. If the dry membranes are warmed, they can "melt" into the liquid crystalline state, and when water is then introduced, little leakage or damage occurs (Fig. 4.7b). Humidification through the vapor phase also allows the membrane state transition to occur before liquid water is introduced, even at cooler temperatures (Fig. 4.7c). This explains why imbibitional damage is greater at lower temperatures, and why pre-humidification to increase the moisture content before imbibition at low temperatures can also ameliorate damage (Fig. 4.8). At warmer temperatures and/or higher moisture contents, the membranes are already in the liquid crystalline state, and thus can tolerate rapid water influx. Sugars such as trehalose (in animals and yeast) or sucrose (in plants) (Sect. 2.5.2.1) can substitute for water during dehydration by interpolating between the polar head groups of the phospholipids (Fig. 2.21b), maintaining the bilayer structure and liquid crystalline state at lower moisture contents and temperatures. The large amount of sucrose present in most mature seeds is likely to be involved in desiccation tolerance and in prevention of imbibitional damage by maintaining the liquid crystalline membrane structure at lower temperatures.

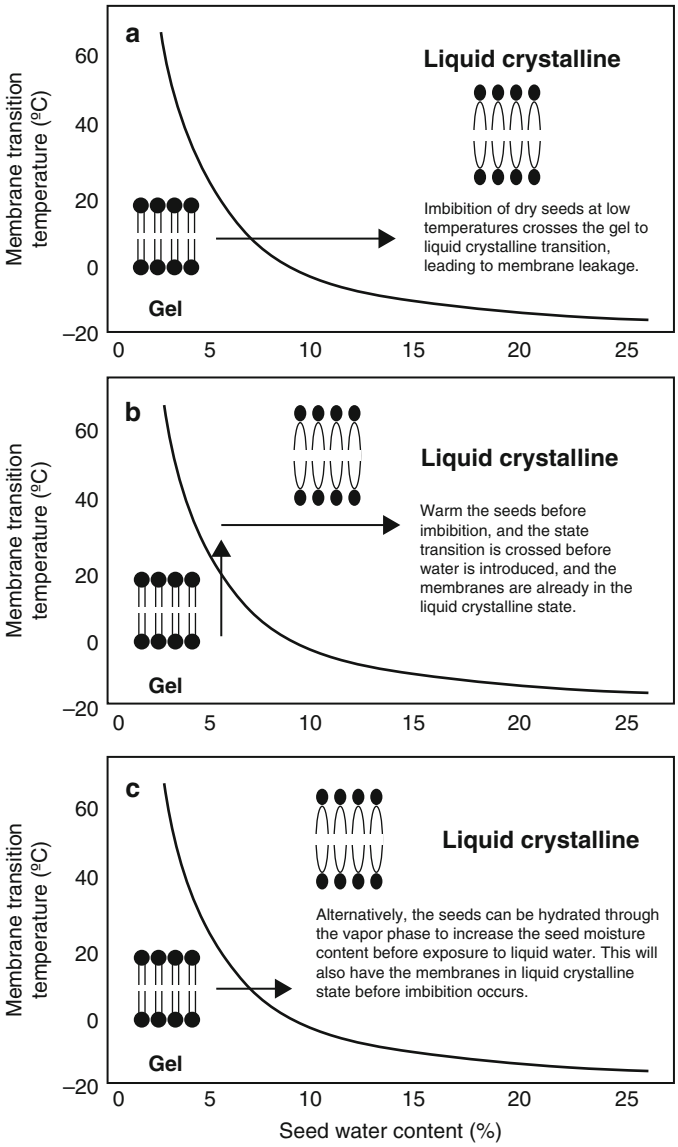

Fig. 4.7 Membrane state transitions during seed imbibition. The curves show the boundary between the gel and liquid crystalline states of the cell membranes at different combinations of temperature (*y*-axis) and seed water content (*x*-axis). (**a**) Rapid imbibition at low temperature causes abrupt transition from gel to liquid crystalline state as water is absorbed, resulting in leakage and cellular damage. (**b**) Rapid imbibition at warmer temperatures is not damaging even at low water contents because membranes are already in the liquid crystalline state. (**c**) Even at lower temperature, seeds can be hydrated through the vapor phase, causing the membrane state change before liquid water is introduced

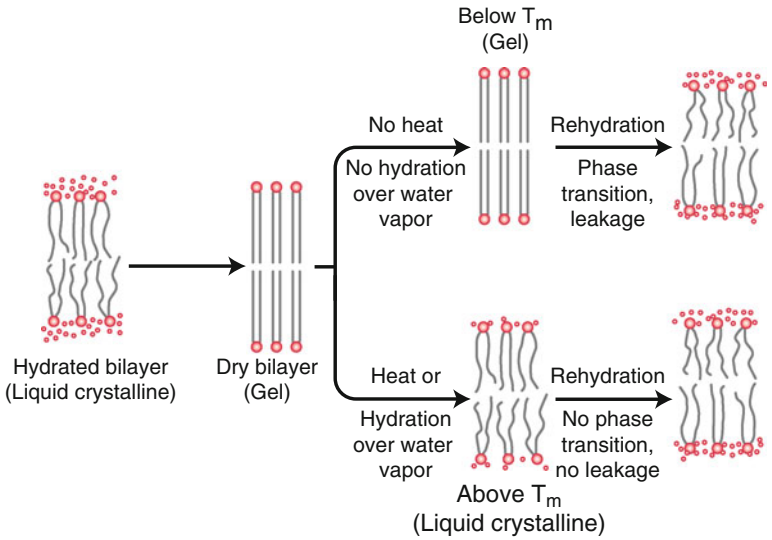

Fig. 4.8 Diagrammatic representation of a mechanism for imbibitional leakage. In hydrated viable cells, cell membranes are present as a hydrated bilayer with polar head groups of phospholipids oriented toward the outside (water molecules are represented by small, open circles) and hydrophobic fatty acid chains in the center. Upon drying, the more mobile liquid crystalline phase changes to the gel phase, depending on the temperature. Direct hydration of membranes in the gel phase with liquid water results in a phase transition back to the liquid crystalline phase, and leakage occurs during this phase transition (*upper pathway*). If a gel phase membrane is heated to above its transition temperature (T_m) before it is returned to water, it will undergo a phase transition before rehydration and will not leak when exposed to liquid water (*lower pathway*). Hydration through the water vapor will also result in the transition to liquid crystalline phase and prevent rehydration damage. From Crowe et al. (1989)

4.3.3 Phase II, The Lag Phase

As water is absorbed into the seed, the value of the matric component (ψ_m) becomes less negative as the cellular components and cell walls become hydrated and the ψ gradient for water uptake decreases. This slows the rate of water uptake, which approaches a plateau or period of only slowly increasing water content. The total ψ_{seed} will come into near equilibrium with that of the external water source, as indicated by little net movement of water into the seed. Internally, the ψ_s of the seed cells is balanced by turgor pressure (ψ_p) as the cells approach full hydration. Further water uptake during Phase II must be due to lowering of ψ_s by the limited mobilization of stored polymeric reserves within the cells that will expand and their conversion to more osmotically active building blocks, such as of starch to sugars or proteins to amino acids. The breakdown of phytin can also release ions that are important osmolytes in cells. This, plus the swelling of cellular constituents,

particularly proteins, upon hydration, causes an increase in seed volume that can result in cracking of the testa. In some seeds, such as those of the Brassicaceae and Solanaceae, cracking of the testa can be distinguished from the emergence of the radicle through the underlying endosperm envelope. Interestingly, the testae of dormant seeds do not crack upon imbibition, while abscisic acid (ABA), a germination inhibitor, can prevent radicle emergence but not testa cracking. This suggests that at least some embryo expansion in addition to simple hydration of the existing tissues is required for cracking of the testa, and that ABA may act primarily by inhibiting the cellular growth associated with radicle emergence (Sect. 4.6).

During Phase II, even though net water uptake is minimal, major metabolic events take place in both dormant and nondormant seeds (Fig. 4.1). In dormant seeds, these are limited to the restoration of cellular integrity, mitochondrial repair, initiation of respiration, and DNA repair, but with little breakdown of storage reserves (unless the seed remains in a hydrated dormant state for a prolonged period) or synthesis of germination-associated mRNAs or proteins. In nondormant seeds, additional events occur in preparation for radicle emergence (Sects. 4.4, 4.5). Some of the processes that are completed during Phase II are the reformation of the cellular cytoskeleton and the repair of damage to DNA accumulated during dry storage. In dry seeds, the microtubules that form the cytoskeleton that is important for many cellular processes, including cytoplasmic streaming, organellar movement, and cell wall formation, depolymerize and form discrete granular bodies within the cells (Fig. 4.9a). Within 8 h of imbibition, these tubulin protein subunits become organized into the cytoskeleton that is clearly associated with the plasma membrane adjacent to the cell walls (Fig. 4.9b). Similarly, incorporation of nucleotides into DNA prior to DNA duplication or cell division indicates that DNA repair is an early event occurring during Phase II (Fig. 4.9c, d).

4.3.4 Phase III, Completion of Germination

Although dormant seeds may achieve Phase II, only seeds that complete germination enter Phase III of imbibition, which occurs due to the cellular expansion associated with radicle protrusion. Thus, water uptake during Phase III is not properly imbibition per se, but rather the initial consequence of the completion of germination. As plant cells expand by absorbing water and stretching their cell walls, the increase in water uptake during Phase III indicates the initiation of embryo growth into a seedling as germination is completed (Sect. 4.6). Water uptake is driven by a decrease in ψ_s due to production of osmotically active substances that create a gradient for water movement relative to the ψ of the water source, and also by relaxation of the cell walls of the embryo and of any enclosing tissues, reducing ψ_p. Endosperms and nonpersistent (hypogeal) cotyledons do not expand and hence do not show a Phase III of water uptake: eventually their water contents decline as degeneration (programmed cell death) occurs.

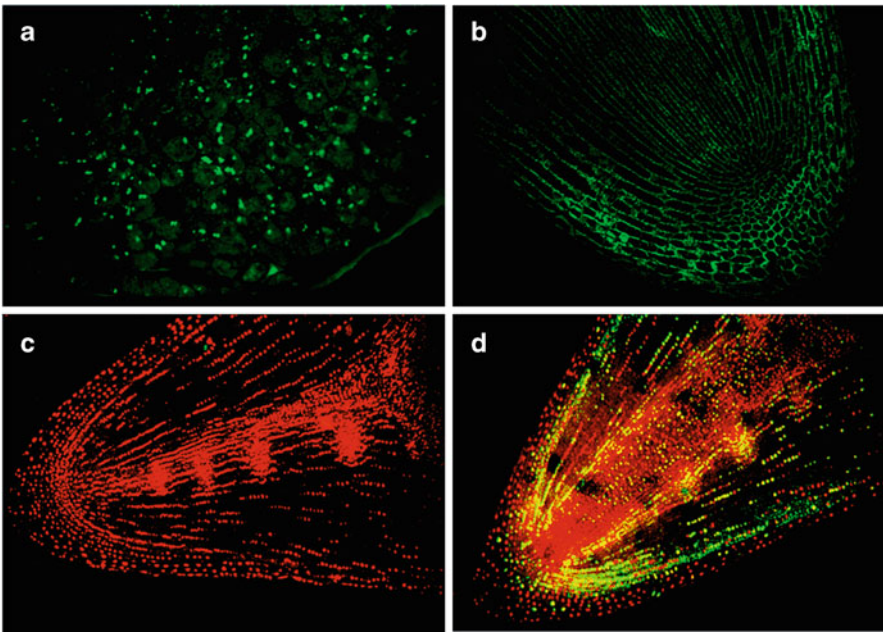

Fig. 4.9 Changes in the cytoskeleton and nuclei in the tomato radicle and lower hypocotyl following imbibition. (**a**) Granules of tubulin (detected using fluorescent antibodies for β-tubulin) present in radicle tip cells of dry seeds. (**b**) Following 8 h of imbibition, these tubulin granules are no longer present and tubulin is now detected in association with the cytoskeleton adjacent to the cell walls. (**c**) Cross section of a dry radicle tip stained with propidium iodide (PI, *red*) to show nuclei containing DNA. (**d**) Radicle tip after 9 h of imbibition stained with PI to show nuclei and with bromodeoxyuridine (BrdU; *green*/*yellow*) to highlight nuclei in which DNA synthesis has occurred. Because no DNA duplication or cell division has occurred at this time, BrdU incorporation is an indication of DNA repair. Images courtesy of R. Bino, Wageningen Univ.; see de Castro et al. (2000)

4.3.5 Kinetics of Imbibition

The duration of each of these phases of imbibition depends on certain inherent properties of the seed (e.g., hydratable substrate content, seed coat permeability, seed size) and on the prevailing conditions during hydration (e.g., temperature, initial moisture content, water and oxygen availability). Different parts of a seed may pass through these phases at different rates; e.g., an embryo or axis located near the surface of a large seed may commence elongation (i.e., enter Phase III of water uptake) even before its associated bulky storage tissue has become fully imbibed (i.e., completes Phase I). As an example, when the water content of whole dent corn grains reaches 75% on a dry weight basis, the water content of the embryo is 261%, but that of the remainder of the grain is only 50%. Hydration of the endosperm is slower because water has to penetrate the surrounding pericarp which is not structurally

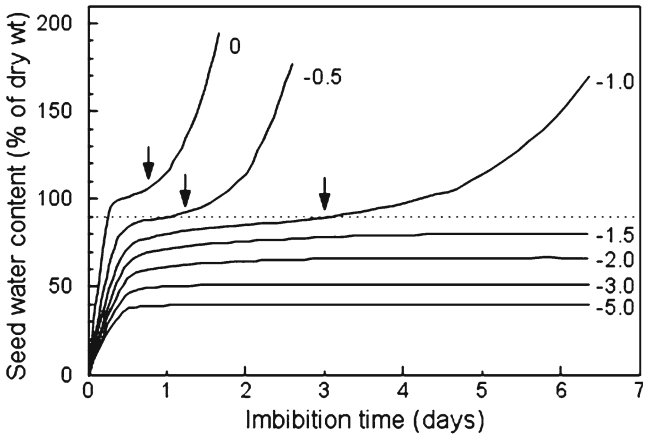

Fig. 4.10 Water uptake time courses for lettuce seeds imbibed in solutions of different ψ (MPa values indicated by each curve). At high ψ, all three phases of imbibition are evident, while only Phases I and II occur at lower ψ. The arrows indicate the time of radicle emergence (completion of germination) in each solution. The dashed line indicates the approximate minimum water content required for radicle emergence to occur. Based on Bradford (1986)

modified to permit its rapid uptake. The water contents of seeds and individual tissues within the seed are also dependent upon the composition of storage materials, as oil-storing tissues containing abundant oil bodies that exclude water will have lower water content at any ψ than will tissues with lower oil content.

The duration of the phases of imbibition is markedly affected by temperature and by the ψ of the water sources for the seed, particularly the duration of Phase II and whether Phase III occurs. Both reductions in temperature and ψ have relatively less effect on Phase I, as the ψ gradient for water uptake is initially very high. However, the duration of Phase II is extended as temperature or ψ decreases, and the final moisture content achieved during Phase II is dependent upon the ψ of the environment. Reductions in both temperature and ψ also slow respiration rates, extending the time required during Phase II to prepare for growth. As shown in Fig. 4.10, relatively slight reductions in ψ lower the plateau moisture content during Phase II, with reductions below about −1.0 to −1.5 MPa often completely preventing progress into Phase III, although some seeds can germinate at as low as −2.5 MPa. Apparently, there is a limit to the extent that seeds can lower their ψ_s to absorb sufficient water to drive the cell expansion required for completion of germination, resulting in a minimum water content below which the embryo cannot expand, and Phase II is extended indefinitely. This phenomenon is utilized in a technique called seed priming, which allows imbibition to the extent required for germination-related metabolism (Phase II), but prevents radicle emergence (Phase III; Sect. 4.7). Further discussion of the effects of temperature and of ψ on germination is in Sect. 7.2.

In concluding this section on imbibition, it is evident that the kinetics of water uptake into seeds is influenced by the properties of the seed, as well as by the environment in which it is situated. A ψ gradient between the seed and its surroundings

(the germination substrate or the soil matrix) is the driving force for water uptake, but the permeability of the seed to water determines the rate at which it can be absorbed. Seed permeability is influenced by morphology, structure, composition, initial moisture content and temperature of imbibition. Water uptake rate in the absence of permeability barriers is initially rapid, but then declines to very low values as the seed nears ψ equilibrium with its surroundings. Further water uptake is dependent primarily upon embryo growth associated with completion of germination and subsequent seedling growth.

4.4 Respiration: Oxygen Consumption and Mitochondrial Development

4.4.1 Pathways and Products

Three respiratory pathways are assumed to be active in the imbibed seed, namely glycolysis, the pentose phosphate pathway, and the citric acid (Krebs or tricarboxylic acid) cycle. Glycolysis operates under aerobic and anaerobic conditions to produce pyruvate, but in the absence of sufficient O_2 this is reduced further to ethanol plus CO_2, or to lactic acid if no decarboxylation occurs. Anaerobic respiration, also called fermentation, produces only two ATP molecules per molecule of glucose respired, in contrast to six ATPs produced during pyruvate formation under aerobic conditions. In the presence of O_2, further utilization of pyruvate occurs within mitochondria: oxidative decarboxylation of pyruvate produces acetyl CoA, which is completely oxidized to CO_2 and water via the citric acid cycle to yield up to a further 30 ATP molecules per glucose molecule respired. The generation of ATP occurs during oxidative phosphorylation when electrons are transferred to molecular O_2 along an electron transport (redox) chain via a series of electron carriers (cytochromes) located on the inner membrane of the mitochondrion. An alternative pathway for electron transport, which does not involve cytochromes, may also operate in mitochondria. The pentose phosphate pathway is an important source of NADPH, which serves as a hydrogen and electron donor in reductive biosynthesis, especially of fatty acids. Intermediates in this pathway are starting compounds for various biosynthetic processes, e.g., synthesis of amino acids, polyphenols, nucleotides, and nucleic acids. Moreover, complete oxidation of hexose via the pentose phosphate pathway and the citric acid cycle can yield up to 29 ATPs.

4.4.2 Respiration During Imbibition and Germination

Respiration by mature dry seeds (usual moisture content <15%) is extremely low when compared with developing or germinating seeds, and often measurements are confounded by the presence of a contaminating microflora. When dry seeds are

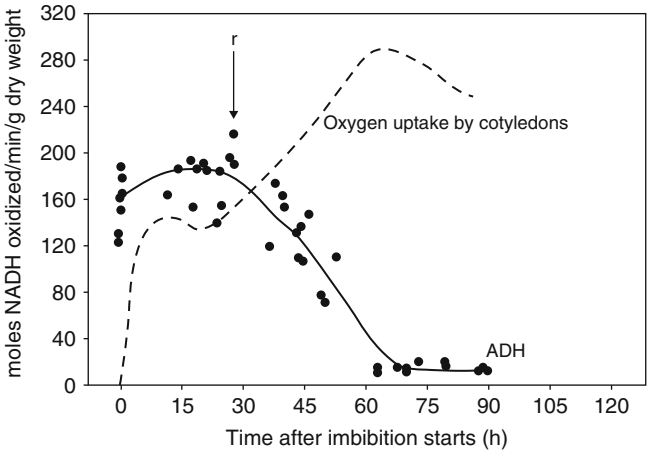

Fig. 4.11 Alcohol dehydrogenase (ADH) activity (*solid line*) in, and O_2 uptake (*dashed line*) by, cotyledons of dark-germinated intact pea seeds cv. Rondo. *Arrow* (r) marks the time of penetration of the testa by the radicle. After Kollöffel (1968)

introduced to water, there is an immediate release of gas. This so-called "wetting burst," which may last for several minutes, is not related to respiration, but is the gas that is released from colloidal adsorption to the dry seed matrices as water is imbibed. This gas is released also when dead seeds or their contents, e.g., starch, are imbibed.

The onset of respiration per se occurs within minutes of the start of imbibition. Initially there is a sharp increase in O_2 consumption, which can be attributed in part to the activation and hydration of mitochondrial enzymes involved in the citric acid cycle and electron transport chain. Respiration increases linearly as more cells within the seed become hydrated. Upon completion of imbibition there is then a lag in respiration as O_2 uptake is stabilized (Fig. 4.11) or increases only slowly. Hydration of the seed parts is now completed and all preexisting enzymes are activated. Presumably there is little further increase in respiratory enzymes or in the number of mitochondria during this phase. The lag phase in some seeds occurs in part because the coats or other surrounding structures limit O_2 supply to the imbibed embryo or storage tissues, leading temporarily to partially anaerobic conditions. Removal of the testa from imbibed pea seeds, for example, appreciably diminishes the lag in respiration. Another possible reason for this lag is that the activation of the glycolytic pathway during germination is more rapid than the development of efficient mitochondria. This could lead to an accumulation of pyruvate due to deficiencies in the citric acid cycle or oxidative phosphorylation (electron transport chain); hence, some pyruvate would be diverted temporarily to the fermentation pathway, which is not O_2 requiring.

Following penetration of the surrounding structures by the embryo there is a second respiratory burst (Fig. 4.11). In the embryo, this can be attributed to an

increase in activity of newly synthesized mitochondria and respiratory enzymes in the proliferating cells of the growing axis. The number of mitochondria in storage tissues also increases, often in association with the mobilization of reserves. Another contributory factor to the rise in respiration in both seed parts could be an increased O_2 supply through the now punctured testa (or other surrounding structures).

A supply of substrate for respiration must be available during germination. This may be provided to a small extent by hydrolysis of the major reserves, e.g., triacylglycerols, which are present in almost all parts of the embryo, including the radicle and hypocotyl, although their greatest concentration is in storage tissues. However, extensive mobilization of these reserves is a post-germinative event (Chap. 5). Most mature seeds contain sucrose, and many contain one or more of the RFOs: raffinose (galactosyl sucrose), stachyose (digalactosyl sucrose), and verbascose (trigalactosyl sucrose), although they usually are present only as a minor component (Sects. 1.3.1, 5.3). The distribution and amounts of these sugars within seeds are very variable, even between different cultivars of the same species. During germination of seeds of several species, e.g., legumes, Brassicas, cereals, and in dormant and germinating white spruce, sucrose and the RFOs are hydrolyzed, and the activity of α-galactosidase, which cleaves the galactose units from the sucrose, increases as raffinose and stachyose decline. However, there is conflicting but limited evidence as to whether the utilization of RFOs is essential during germination. Inhibition of their hydrolysis in pea seeds markedly delays the completion of germination, the implication being that other substrates are utilized as an energy source, but this takes longer to be effected. However, soybean lines with low RFO contents germinate as efficiently as those with normal amounts. To what extent these two species represent the requirement for RFOs during germination of other legume- or nonlegume species remains to be determined.

4.4.3 Mitochondrial Development and Oxidative Phosphorylation

Mitochondria in dry and freshly imbibed seeds are functionally and structurally deficient, being poorly differentiated internally. In seeds of some species, ATP may be produced initially and inefficiently by glycolysis, whereas in others its production throughout germination is by mitochondrial oxidative phosphorylation using the electron-transfer (-transport) chain. Hence, from the start of imbibition, ATP synthesis and O_2 consumption are sensitive to cyanide, an inhibitor of cytochrome c oxidase, a vital intermediate in the transfer of electrons from NADH to oxygen. Regardless of the initial mode of respiration, a general feature of germination is that mitochondrial efficiency increases with time from the start of imbibition. This may be due to increased proficiency of existing mitochondria and/or an increase in their numbers. It appears that there are two distinct patterns of mitochondrial development in imbibed seeds: (1) repair and activation of organelles already existing within the mature dry seed, as typified by the pea cotyledon and the storage tissues of some

Table 4.1 Seeds exhibiting repair or biogenesis of mitochondria in their storage tissues following imbibition, as related to their major nonprotein reserve

Repair and activation (starch-storing)	Biogenesis (oil-, TAG-storing)
Cowpea	Okra
Mung bean	Pumpkin
Black gram	Cucumber
Egyptian kidney bean	Castor bean
Soybean	Peanut
Garden pea	
Maize[a]	

[a]Embryo. Although the major storage product in the kernel is starch in the endosperm, this is absent from the embryo where oil is the major stored reserve, in the scutellum
TAG triacylglycerol
Based on Morohashi (1986) and references therein

other starch-storing seeds; and (2) production of new mitochondria, as typified by the peanut cotyledon and the storage tissues of other oil-storing seeds (Table 4.1). The pattern of mitochondrial development has not been followed so extensively in the axes of these or other dicot species during germination, although it would appear to be similar. In maize embryos two subpopulations of mitochondria, light and heavy based on their density, exist in the dry seed. The former, which may be residual from development, gradually deteriorate following embryo imbibition, but the latter undergo considerable changes, including improvement in internal structure, first during and then following germination. In the dry seed, the incomplete organelles, termed pro-mitochondria, lack a clear inner structure (Fig. 4.12a), but by 24 HAI, during germination, redifferentiation to the more typical mitochondrial structure has occurred in many, along with the biosynthesis of new organelles, both being completed by 48 HAI, after the completion of germination (Fig. 4.12b, c). The reassembly of the pro-mitochondria requires expression of the organellar and nuclear genomes leading to the synthesis of new structural proteins and enzymes. Even as early as 6 HAI the heavy mitochondria contain the components necessary for biogenesis, plus an active electron-transfer chain and the capacity to synthesize ATP (Fig. 4.12 legend). In Arabidopsis seeds, early during germination there is mitochondrial DNA replication and transcription, followed later by an increase in proteins important for the bioenergetic functions of this organelle. In imbibing rice embryos, the increased organization and function as pro-mitochondria mature is accompanied by an elevation in mitochondrial transcripts and in nuclear transcripts for proteins that are imported into this organelle, the import mechanism being operational within 2 HAI. The abundance of rice mitochondrial proteins is considerably different between grains germinating in anaerobic and aerobic conditions; fewer of these proteins are present under anaerobic conditions, but this is not necessarily reflected in their transcript abundance. This could indicate that there is posttranscriptional regulation of these proteins under anaerobic conditions due to alterations in transcript degradation, activity of transcription factors, protein synthesis capacity, or protein degradation.

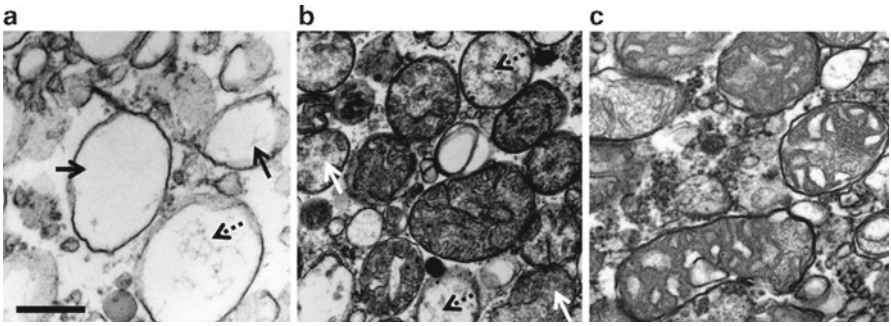

Fig. 4.12 Transmission electron micrographs of the heavy mitochondrial population obtained by subcellular fractionation of (**a**) dry maize embryos, (**b**) 24 h after the start of imbibition (HAI) embryos, still germinating, and (**c**) germinated embryos at 48 HAI. With time the internal structure of the mitochondria becomes more defined. *Arrows* indicate examples of poorly differentiated mitochondria; *dotted arrows* point to poorly developed internal cristae, the site of oxidative phosphorylation. Bar: 500 nm. Below is an indication of the activity of some mitochondrial enzymes before, during, and following germination

Activities of mitochondrial enzymes (expressed as nmol min^{-1} mg^{-1} protein)

Enzyme	Dry seed	6 HAI (germinating)	48 HAI (germinated)
Cytochrome c oxidase	200 ± 7	188 ± 5	186 ± 8
Citrate synthase	15 ± 4	56 ± 6	139 ± 13
Pyruvate dehydrogenase complex	2 ± 1	12 ± 1	19 ± 4
α-Ketoglutarate dehydrogenase complex	2 ± 1	8 ± 1	14 ± 3

Cytochrome c oxidase is associated with the electron transport chain for the synthesis of ATP. Citrate synthase and α-ketoglutarate dehydrogenase are enzymes of the citric acid cycle, and pyruvate dehydrogenase yields acetyl-CoA from pyruvate, which then enters the cycle. Values are from three separate experiments ± standard error. From Logan et al. (2001). Copyright American Society of Plant Biologists. Also see Howell, K.A. et al. (2006) *Plant Mol. Biol.* 60, 201–223 for comparable structural and metabolic changes in mitochondria of germinating rice embryos

It has been suggested that in some seeds an alternative oxidase (cyanide-insensitive) pathway operates in the mitochondria early after imbibition, until the electron-transfer pathway becomes actively engaged in ATP synthesis. This alternative pathway cannot produce ATP, and electrons from NADH oxidation are passed directly to oxygen. Its importance in seeds is unknown, although it may help to prevent the buildup of damaging reactive oxygen species (ROS) (Sect. 8.4.1) until O_2 can be used more effectively in ATP production.

4.4.4 Respiration Under Low Oxygen Conditions

As outlined above, many seeds experience conditions of temporary low O_2 availability (hypoxia) during early imbibition. Consequently, both ethanol and lactic

acid, fermentative products of anaerobic respiration, accumulate within the seed; they occur in different proportions in different species. Upon penetration of the enclosing structures by the radicle, these anaerobic products decline as they are metabolized under conditions of increasing aerobiosis. Lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) are responsible for both the synthesis and the removal of lactate and ethanol, respectively. Under aerobic conditions, the former enzyme converts lactate back to pyruvate, which can then be utilized by the citric acid cycle, and the latter converts ethanol to acetaldehyde, which is oxidized to acetate by acetaldehyde dehydrogenase. The acetate is activated to acetyl-CoA, which can be used in many metabolic processes. Not surprisingly, ADH and LDH tend to be present in seeds during germination, and often they are even present in the dry seed, e.g., ADH in pea cotyledons (Fig. 4.11). In seeds of some species the activities of ADH and LDH increase appreciably (tenfold or more) during germination. After germination has been completed, and when conditions are more aerobic, ADH (Fig. 4.11) and LDH become negligible: loss of both ethanol and lactate from the seed parallels this decline.

In conditions of low O_2 availability, as can occur during development of a seed in its surrounding fruit structures, as well as during germination (e.g., in nature when soils become waterlogged), a low-O_2 sensing and balancing mechanism operates to control energy production and utilization. In seeds of soybean and pea, an increase in nitric oxide (NO) occurs under hypoxic conditions; this, in turn, reduces O_2 consumption, generating localized decreases in both ATP availability and biosynthetic activity. Increasing the supply of O_2 results in a decline in NO, which is metabolized via hemoglobin, thereby reversing the inhibition. A proposed model for the role of NO in low-O_2 sensing is shown in Fig. 4.13. Essentially, the availability of O_2 controls the endogenous concentration of NO, which in turn regulates the rate of O_2 consumption through its availability. NO also inhibits mitochondrial cytochrome oxidase (and thus ATP production), and hence, the activity of this enzyme is indirectly dependent on O_2 concentration. For a consideration of the relationship between NO and the breaking of dormancy, see Sect. 6.6.7.2.

The period of natural hypoxia in seeds during germination can last from a few hours to several days. Sowing seeds under water can prolong these conditions. Seeds of many species will actually complete germination when submerged in water, although subsequent growth of the radicle is stunted, and if the germinated seeds remain waterlogged they will die. Seeds of certain aquatic species geminate better under conditions of reduced O_2 availability, e.g., bulrush, and some, e.g., common or soft rush, will germinate readily even after submergence for up to 7 years. Grains of aquatic species such as rice and barnyard grass, a noxious weed in rice fields, germinate and grow under water and show some interesting adaptations to these conditions of reduced oxygen availability. Both rice (Fig. 4.14a, b) and barnyard grass grains will germinate in a totally O_2-free environment (anoxia), although only the coleoptile elongates; root growth is inhibited, as is emergence of the leaves from the coleoptile. The growth rate of barnyard grass under anoxic conditions is about 25% of that in air. The seedlings of both species produce considerable quantities of ethanol, much of which (up to 95% in rice) diffuses into the surrounding water. Even so, under anoxic

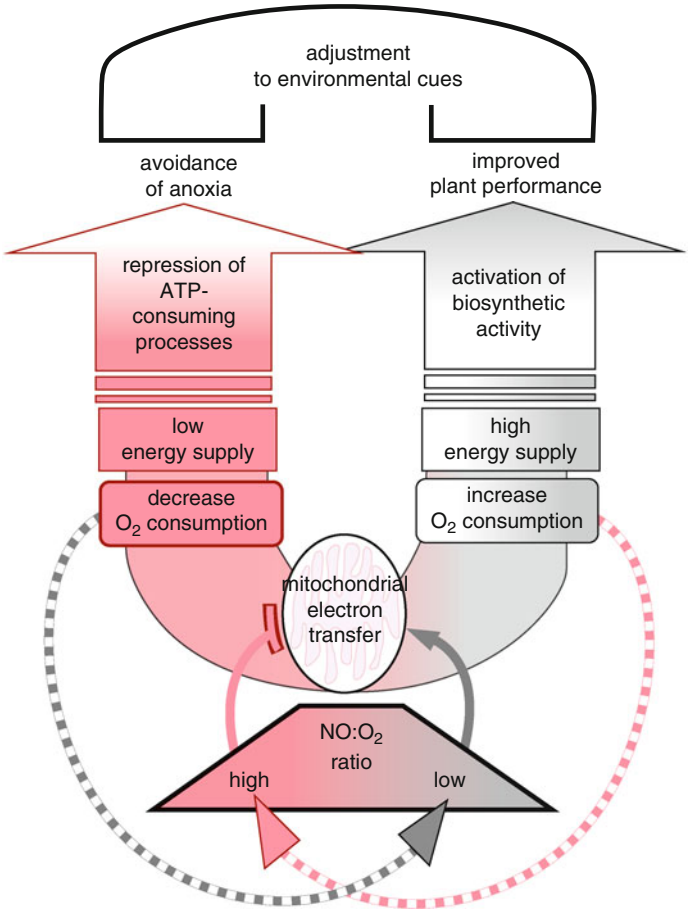

Fig. 4.13 A model to explain the role of nitric oxide (NO) in low-oxygen sensing and balancing, and in the control of energy (ATP) production and utilization for synthetic events in seeds. Under O_2-deficit conditions both the stability and synthesis of NO increase, causing elevated NO:O_2 ratios. NO inhibits the occurrence of mitochondrial electron transport via the cytochrome pathway. The efficiency of inhibition depends upon the internal O_2 concentration; thus the NO:O_2 ratio triggers O_2 balancing in an auto-regulatory manner, avoiding seed anoxia. At high ratios the inhibition of mitochondrial activity conserves O_2 and tends to increase its availability. Concomitantly, the decrease in ATP availability represses metabolic events that are dependent upon this energy source. Increasing O_2 availability decreases the NO:O_2 ratio, suspending mitochondrial inhibition and increasing O_2 consumption and ATP synthesis, eventually promoting seed biosynthetic activity. The arrows indicate that increased O_2 consumption results in a higher ratio of NO to O_2, whereas decreased consumption causes the reverse. After Borisjuk et al. (2007). Courtesy of Wiley. For details on the mitochondrial pathways that function during anaerobiosis see Igamberdiev and Hill (2009)

conditions the seedlings may contain close to 100 times more ethanol than the aerated controls. In rice, mitochondrial ADH activity rises along with ethanol production owing to induction of *ADH* genes and de novo synthesis of the enzyme. Increases in lactate occur also, but they are very small in comparison to ethanol.

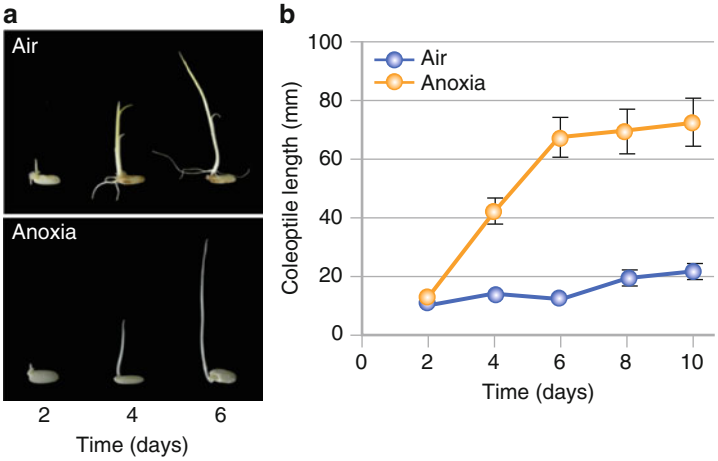

Fig. 4.14 Aerobic and anaerobic germination and seedling growth in rice. (**a**) Seedlings 2, 4, and 6 days after sowing. Under aerobic conditions the roots, coleoptile, and primary leaf are produced, while anoxic seedlings lack the primary leaf and roots and only the coleoptile grows. (**b**) Coleoptile lengths under aerobic (Air) and anaerobic (Anoxia) conditions. Under anoxia, coleoptile length is, on average, three times longer than under aerobic conditions 10 d after sowing. From Magneschi and Perata (2009). With permission of Oxford Univ. Press

In rice and barnyard grass, synthesis of ATP commences in the absence of O_2 very soon after imbibition, probably by operation of the glycolytic pathway, terminating in the conversion of pyruvate to ethanol. Mitochondrial development in seedlings of both species under anoxic conditions is not very different from that in fully aerated seedlings. Both exhibit substantial increases in many of the citric acid cycle enzymes under anoxia, for example. Although oxidative phosphorylation obviously does not occur in grains imbibed in the absence of O_2, the mitochondria develop and maintain their capacity for ATP production because oxidative phosphorylation commences almost immediately when seedlings are returned to aerobic conditions.

In closing, it should be noted that there are considerable changes in metabolic and transcriptional responses of rice grains to low oxygen availability other than those directly related to mitochondrial activity. For example, a substantial number of changes in transcripts for proteins associated with carbon and nitrogen metabolism, and some for lipid metabolism, occur during germination in anaerobic conditions, compared to aerobic. Some of these changes, however, are related to the need to offset losses in aerobic ATP production by increasing fluxes through glycolytic and fermentative pathways through up-regulation of the enzymes involved therein.

4.5 RNA and Protein Synthesis

The transition from metabolic quiescence and arrested growth in the dry seed to the resumption of metabolism during germination requires a number of biochemical changes that are catalyzed by enzymes and other functional proteins. Intensive cellular

activity during this period also requires changes in structural proteins. Therefore, de novo RNA and protein synthesis are critical processes leading to seed germination. Here, the changes in the seed transcriptome and proteome in imbibed seeds are considered with respect to their transition from quiescence to germination.

4.5.1 Transcriptomes of Dry and Germinating Seeds

Dry seeds of Arabidopsis, barley, rice, and lettuce contain the transcripts of more than 10,000 genes; these are called stored or residual mRNAs, which are likely associated as messenger ribonucleoprotein complexes (mRNPs). As would be expected, they largely reflect gene expression activity during the late stages of seed development, with a high abundance of mRNAs coding for seed storage reserves, heat shock proteins, LEAs (Sects. 2.5, 3.2), and biosynthetic enzymes for storage reserves. The majority of these residual mRNAs that were encoded by genes specific for seed maturation are quickly degraded following imbibition and are not replaced. Notably, the ABA-responsive element (ABRE), a DNA motif typically present in the promoter regions of genes that are expressed during late embryogenesis (Sect. 2.4.2), is overrepresented in the genes for residual mRNAs, reflecting the key role of ABA in regulating their expression during seed maturation. Some residual mRNAs present as mRNPs in dry seeds may be translated into proteins following imbibition, probably to support basic "housekeeping" functions until replaced by de novo synthesized transcripts, but there is no experimental evidence for them having essential roles in the completion of germination.

Global transcriptome analyses (profiling the presence and abundance of transcripts) of several species using high throughput approaches such as microarrays (hybridization-based assays for all or part of the genes in a genome) indicate that transcriptional activity is initiated in seeds during the first few hours after the start of imbibition (HAI). Examples of the changes that occur to selected transcripts in germinating tomato seeds are shown in Fig. 4.15. Expression of residual transcripts for proteins associated with development and maturation decline sharply over the first 24 HAI and are virtually absent by 48 HAI (completion of germination is first visible at 36 HAI, and all seeds are germinated by 60 HAI). Hence, they are not replaced during germination. In contrast, expression of genes encoding proteins associated with imbibition and germination increases steadily during germination and its completion, as do those for enzymes that become involved mostly in post-germinative mobilization of stored reserves.

Transcriptome analyses of imbibed rice grains indicate that more than 1,000 genes are up- or down-regulated, respectively, between 1 and 3 HAI, while only a limited number (<50) of changes in mRNAs occur in the first HAI. There is also a rapid increase in new transcripts during 1-3 HAI in Arabidopsis seeds. One group of the genes up-regulated by 3 HAI is also high at 6 HAI but declines by 12 HAI; therefore, the proteins they encode may have unique roles during this specific period of imbibition.

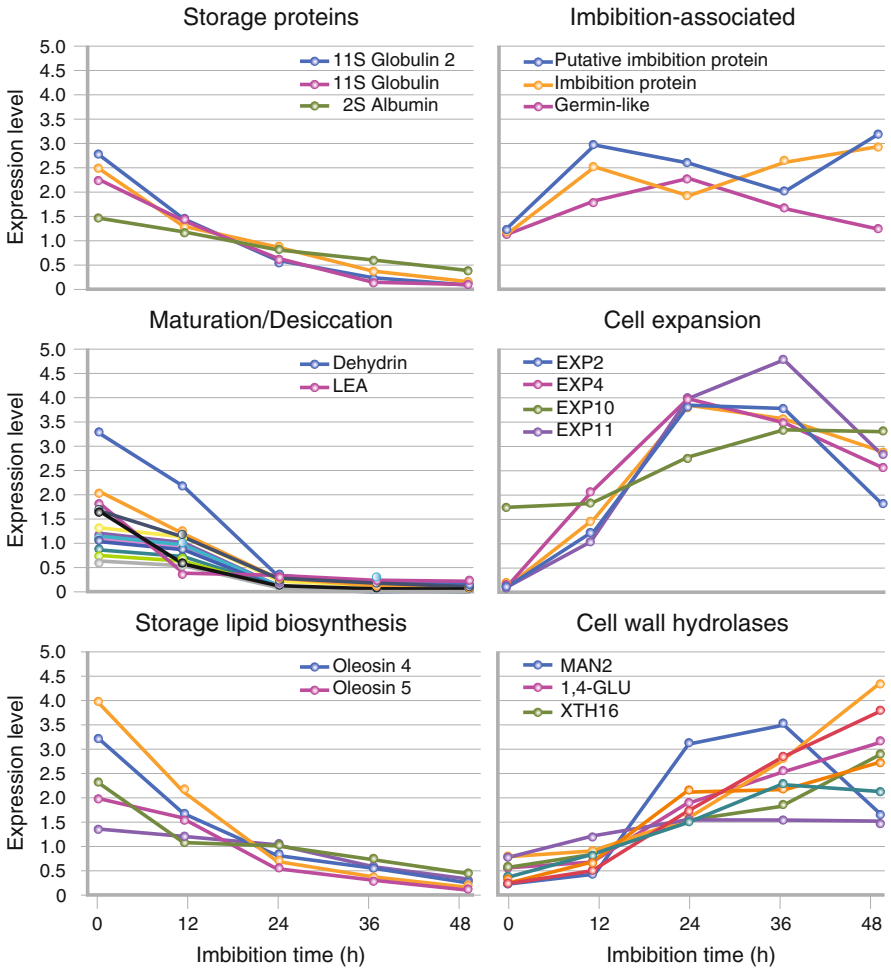

Fig. 4.15 Temporal expression patterns of differents groups of genes in tomato seeds following imbibition. Although the genes shown here represent only a subset of all those expressed during imbibition, germination, and early seedling growth, they represent examples of the normal range of expression patterns. Transcripts for developmental-related synthesis decline in abundance following imbibition (e.g., those for storage proteins, maturation/desiccation and storage lipid biosynthesis), while others increase in expression during germination (which is completed between 36 and 60 HAI) and during late germination and early seedling growth (e.g., imbibition-associated, cell expansion and cell wall hydrolases). *LEA*, late embryogenesis abundant protein; *EXP*, expansin; *MAN2*, endo-β-mannanase; *1,4 GLU*, endo-β-1,4-glucanase; *XTH16*, xyloglucan endotransglycosylase/hydrolase. See also Fig. 2.24 for changes in transcripts after imbibition. Previously unpublished data from the PhD thesis of C. F. Machado, Univ. Guelph (2007)

Most of the genes up-regulated around 3 HAI in Arabidopsis seeds are those associated with primary metabolism, including those for the pentose phosphate pathway (Sect. 4.4.1), as the resumption of respiration is among the first cellular

activities to be initiated during early imbibition (e.g., within 30 min). Metabolomic analyses (profiling of extant metabolites) of rice grains indicate that glycolysis and the TCA cycle (Sect. 4.4.1) are activated during early imbibition to support energy-demanding processes. The up-regulation of genes associated with respiration occurs in dormant and nondormant seeds, and thus resumption of respiration is not specifically associated with germination per se, but rather with the essential reestablishment of basal metabolism. This also confirms that seed dormancy is not a condition of suspension of general metabolic activities (Chap. 6).

While it is clear that large-scale de novo transcription occurs soon after imbibition, there is contention as to whether this is required for the completion of germination. It is possible that at least the early stages of germination can be supported by residual mRNA only. While there are reports that germination of Arabidopsis seeds can occur in the presence of inhibitors of RNA synthesis, some experimental issues remain, and conclusive supportive evidence is lacking (Sect. 4.6.1). Experiments in the 1960s with massively γ-irradiated seeds showed that radicle protrusion due to cell expansion could be accomplished even with sufficient damage to DNA to completely suppress cell division, resulting in stunted "γ-plantlets." However, RNA synthesis was not prevented in these irradiated seeds. On the other hand, the loss of stored transcripts associated with germination repression, either during dry after-ripening (Sect. 8.5) or following imbibition, may be a prerequisite for germination.

In general, transcriptomic analyses comparing dry and imbibed seeds indicate that the majority of residual mRNAs decline in abundance while those associated with germination and growth appear and increase in abundance following imbibition. Moreover, protein synthesis becomes increasingly dependent on the latter with time of imbibition prior to completion of germination.

During Phase II, after initial water uptake, many genes associated with germination are expressed, including those encoding transcription factors, hormone metabolism enzymes and signaling proteins (Sect. 6.6.1), and cell wall modification enzymes (Sect. 4.6.1). Global correlation analyses of genes expressed in germinating and non-germinating (dormant) Arabidopsis seeds reveal characteristic groups of genes that are associated with germination or with dormancy (Fig. 4.16). There is little overlap between the transcripts present in each of these groups. Identified in Fig. 4.16 (dark region) also are genes that are uniquely up-regulated in dormant (group 1) or germinating (group 3) seeds, and although their expression is connected to that of many other genes in the same group (surrounding grey region), they are not connected to those of the other unique group. Interestingly, the connecting region (group 2) includes some genes known to be associated with other major developmental transitions, such as the initiation of flowering, which recently have been reported to be associated with germination also. These networks are conserved with respect to the homologous genes in other species, including wheat and lettuce, indicating that they can serve as a useful guide to identify common genes and pathways involved in germination across species. These correlated gene expression networks can be accessed at www.vseed.nottingham.ac.uk, and a valuable public resource for retrieving and viewing seed transcriptome data is at www.bar.utoronto.ca.

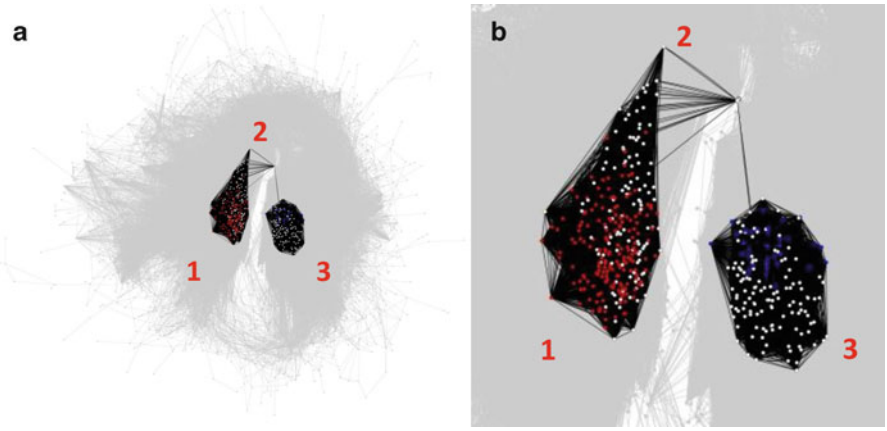

Fig. 4.16 Correlation networks among transcripts expressed in imbibed seeds. (**a**) All genes detected in microarrays of Arabidopsis seed transcripts were analyzed, and their expression patterns correlated with each other (*grey* network in the background; each dot represents a gene, the *grey* lines are connections ["edges"] between them indicating co-expression). Three groups of genes were identified: (1) those expressed in dormant seeds; (2) those expressed in seeds transitioning toward germination; and (3) those expressed in germinating seeds. The relatively small overlap is noteworthy, as indicated by the very few grey lines directly connecting groups 1 and 3 without going through region 2, and indicates the distinct transcriptional networks of dry/dormant and germinating seeds. (**b**) Genes that are specifically up-regulated in either dormant (*red dots*) or germinating (*blue dots*) seeds and connected to at least 500 other genes are shown in the black regions superimposed on the general expression correlation network, among other genes that are also highly connected (*white dots*). These genes are presumably master regulators. The single highly connected gene in the transition network is that encoding an actin, *ACT7*, which is involved in cellular-mechanical functions related to the cytoskeleton. Adapted from Bassel et al. (2011)

4.5.2 Proteomes of Germinating Seeds

Cell-free protein-synthesizing systems can be constituted from dry embryos (e.g., wheat germ) and used to translate mRNAs in vitro, indicating that all of the functional components necessary to reform the translational apparatus are present therein. Thus, ribosomes are present in the dry seed and soon after imbibition they combine with mRNAs to form into polysomes capable of protein synthesis (polysomes are absent from dry seeds). Transcripts encoding ribosomal proteins increase during early imbibition, particularly in nondormant seeds; thus an increase in ribosomal biogenesis is an early germination-associated event. Proteomic analyses (profiling of extant proteins in a tissue at a given time) indicate temporal changes in the protein complements of imbibed seeds, which reflect both de novo synthesis and turnover of proteins (Fig. 4.17). By separating extracted proteins using two-dimensional gel electrophoresis, cutting out the individual protein spots, determining their amino acid sequences by mass spectrometry, and comparing these to protein

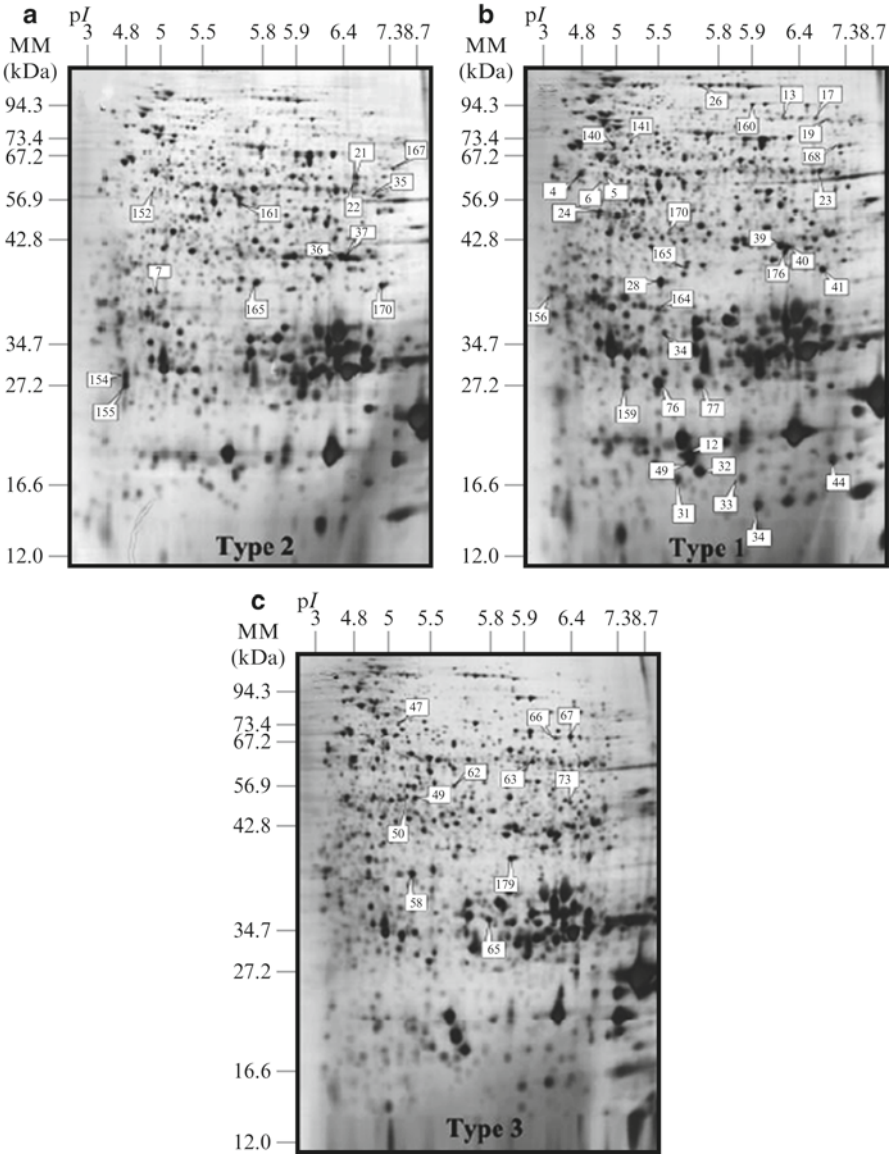

Fig. 4.17 Proteins extracted from germinating Arabidopsis seeds and stained following their separation by two-dimensional gel electrophoresis. Proteins from (**a**) dry, (**b**) 1-day-imbibed, and (**c**) 2-day-imbibed seeds. Numbers indicate identified proteins whose abundance either (in **a**) decreased (Type 2) or (in **b**) increased (Type 1) during germination prior to radicle protrusion, or (in **c**) increased after radicle protrusion (Type 3). MM, molecular mass. From Gallardo et al. (2002). Copyright American Society of Plant Biologists

sequence databases, up to 1,500 proteins have been detected in germinating Arabidopsis seeds, of which nearly 500 have been identified. Several increase prior to radicle emergence, a number of which are associated with the limited mobilization of storage proteins (e.g., subunits that are solubilized as the storage proteins are degraded) (Sect. 5.8.1). Tubulin, a protein component of cellular microtubules, also increases in abundance in imbibed seeds. Other proteins specifically associated with embryo expansion and radicle protrusion are discussed in Sect. 4.6. In addition, posttranslational modifications of proteins such as protein phosphorylation and dephosphorylation, or ubiquitination that affects their stability, play critical roles in germination. The resumption of protein synthesis, utilizing extant or de-novo-synthesized mRNAs, is essential for the completion of germination because inhibitors of translation such as cycloheximide effectively block embryo expansion. Information concerning the composition of the proteome of germinating seeds can be obtained from: http://www.seed-proteome.com.

4.6 The Completion of Germination

As noted in Sect. 4.1, germination *sensu stricto* is completed by the emergence of the embryo from its surrounding tissues; in most species the radicle is the first structure to appear. This has to physically break through the tissues that enclose it, such as the endosperm and testa. Therefore, a balance between the expansive force that the embryo generates during and following imbibition and the physical restraint of the surrounding tissues, which also changes following imbibition, determines if and when germination is completed. Considered here are the biophysical, biochemical and molecular changes in the embryo and the surrounding tissues, and their interactions, that are associated with the completion of germination.

4.6.1 Embryo Growth Potential Versus Enclosing Tissue Constraints in Radicle Emergence

For the radicle to emerge from a seed, the balance of embryo growth potential and the mechanical resistance of the tissues that surround it must change. The change could occur through the increase of embryo growth potential, the decrease of the mechanical resistance of the surrounding tissues, or both simultaneously (Fig. 4.18). Imbibition of water alone into seed tissues causes cell expansion that could contribute to the generation of the initial growth potential of the embryo. However, this process is passive, and the initial force of this swelling is generally not sufficient to cause rupture of the surrounding tissues. Additional expansion of the embryo is required to create the force that eventually ruptures them. In physiologically dormant seeds, repression of embryo expansion, which is probably imposed by ABA, prevents the completion of germination (Fig. 4.18).

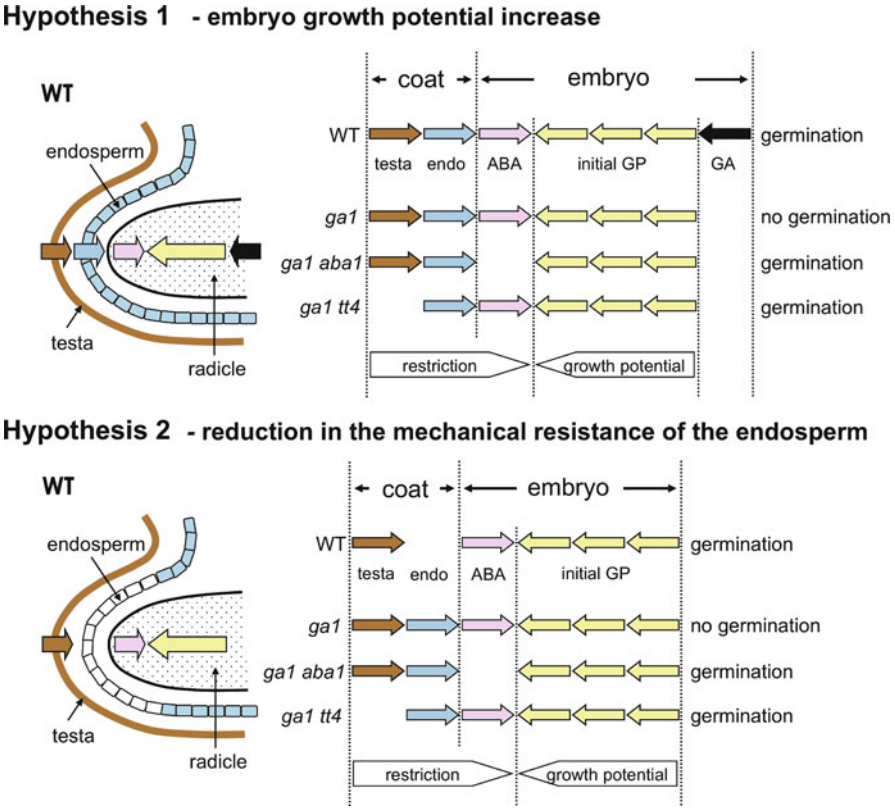

Fig. 4.18 Two hypotheses to explain the relationship between the embryo radicle and the adjacent micropylar endosperm and testa during seed germination. Radicle emergence is determined by a balance between the mechanical resistance of the testa and endosperm (coat) and the expansion or growth potential of the embryo. In hypothesis 1, the growth potential of the wild type (WT) embryo is increased by GA during germination. The two restrictive factors in the covering tissues, the mechanical resistance of the testa (*brown arrow*) and micropylar endosperm (*blue arrow*), and inhibition of embryo expansion imposed by ABA (*pink arrow*) are balanced against the initial growth potential of the embryo (three units of *yellow arrows*). An additional increase in embryo growth potential (*black arrow*) is induced by the action of GA synthesized in this tissue, which changes the balance of the forces inside the embryo (and between it and the coat) to induce radicle protrusion. GA-deficient mutant *ga1* seeds cannot complete germination due to the lack of final embryo growth potential increase, which can be compensated by applying GA. In contrast, seeds of GA-deficient and ABA-deficient *ga1 aba1* double mutants are capable of germinating without GA application, since the absence of ABA alleviates its negative effect in the embryo and abolishes the GA requirement. In the testa pigmentation mutant *tt4*, the GA requirement is also abolished because the restriction by the testa is much reduced. In hypothesis 2, an additional increase in embryo growth potential by the WT embryo is not required. Instead, a reduction in the mechanical resistance of the micropylar endosperm occurs (indicated by the absence of a blue arrow), for example, by degradation of its cell walls. Weakening of the endosperm is presumed to be GA inducible. Therefore, lack of germination in the *ga1* mutant can be explained by the lack of endosperm weakening. *ga1 aba1* and *ga1 tt4* double mutant seeds are still capable of germinating in the absence of GA, since restrictive forces in the embryo and testa, respectively, are absent from these mutants (denoted by blue arrows in the coat column). These hypotheses are not mutually exclusive; the increase in embryo growth potential and the decrease in the mechanical resistance can and most likely do occur simultaneously. Endo: micropylar endosperm, GP: growth potential. From Nonogaki (2006). Courtesy of the Japanese Society of Breeding

Several mechanisms are thought to be involved in the generation of embryo growth potential. Once imbibition has reached Phase II (Fig. 4.1), water uptake increases only slowly and the seed ψ is in near equilibrium with its surrounding environment. To expand and exert pressure on the tissues enclosing them, the embryonic cells must decrease their water potential (ψ) to attract more water. This is likely due the generation of solutes (e.g., sugars, amino acids) from the breakdown of polymeric reserves, such as starch, other storage carbohydrates or proteins. The accumulation of solutes lowers the osmotic potential (ψ_s) of the cell, creating a gradient for water uptake that will expand the cell volume. This expansion may be initially resisted or contained by the cell walls, resulting in increased turgor pressure or pressure potential (ψ_p), which counteracts the lower ψ_s and stops further water uptake. However, if the turgor pressure is completely contained by the embryonic cell walls, the embryo will not exert force against the tissues surrounding it. Thus, the cell walls of the embryonic tissues (or some of them; see below) must relax to lower the ψ_p and create a gradient for further water uptake. At some point, the cell walls of the expanding tissues are sufficiently relaxed that the turgor pressure is contained by the resistance of tissues external to the embryo, just as the pressure inside of an elastic bicycle inner tube is contained by the more rigid tire surrounding it. Continued solute accumulation and water uptake will increase the force exerted by the tissue against the surrounding tissues until it is great enough to allow the radicle to break through. Obviously, as in the bicycle tire analogy, this is most likely to occur at the point of least resistance by the surrounding tissues. In many cases, the tissues opposite the radicle tip are either structurally or physiologically modified to enable radicle protrusion at that point (Fig. 1.4).

The radicle–hypocotyl junction of the embryonic axis, which is adjacent to the radicle tip, specifically elongates during radicle emergence. In Arabidopsis seeds, elongation of embryonic cells occurs first in a discrete region immediately behind the radicle tip, the lower hypocotyl and transition zone (Fig. 4.19). The radicle tip is essentially pushed out by the expansion of cells behind it. The elongating cells also double in nuclear DNA content, to reach the Gap 2 (G2) stage of the cell cycle (Sect. 4.6.2), but do not undergo division. There is ample evidence from many species that completion of germination occurs in the absence of mitosis, which takes place later during subsequent growth of the radicle. Genes encoding the rate-limiting enzymes for the biosynthesis of GA are expressed in the cortex and endodermis cells of the same transition zone that exhibits early expansion to complete germination, suggesting that GA promotes the generation of growth potential by cell expansion (Sect. 6.6.1.2). GA also induces genes encoding cell-wall-modifying enzymes and other proteins, such as for xyloglucan endotransglycosylase/hydrolases (XTHs) and expansins (EXPs), which may be involved in the cell wall relaxation that is essential for subsequent cell expansion (Fig. 4.20). Expansins are cell-wall-associated proteins that can disrupt hydrogen bonds between wall components, thus reducing structural rigidity. This permits wall expansion due to the ability of the component cellulose microfibrils to rearrange. XTHs are enzymes that can break and reform bonds between the xyloglucan hemicelluloses that surround the cellulose microfibrils, apparently accommodating cell wall restructuring associated with expansion. Both types of proteins are often expressed in tissues in which cell wall modification is

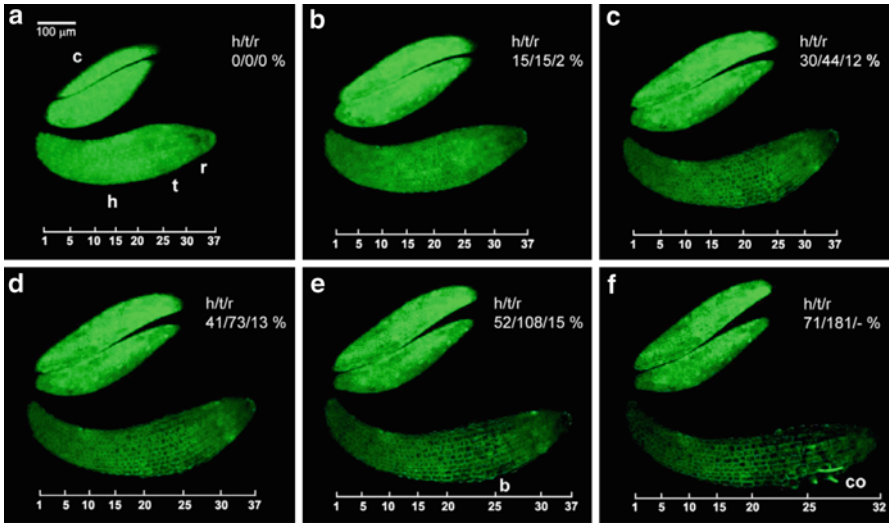

Fig. 4.19 Cell elongation during germination and early seedling growth of an isolated embryo of Arabidopsis. Seeds expressing a green fluorescent protein (GFP) illuminating the cell walls was used to obtain time-lapse fluorescent images of an isolated germinating embryo using a laser-scanning confocal microscope. (**a**) The embryo at 22 h after the start of imbibition (HAI) before elongation of the embryo commences. (**b**) Elongation of the cells of the lower hypocotyl/transition zone is evident at 38 HAI, when the testa has ruptured, and just prior to when the radicle in the intact seed emerges through the endosperm. (**c**) At 46 HAI, elongation of the hypocotyl/transition zone between cells 20–30 continues and the radicle emerges through the ruptured endosperm. At this time the radicle has elongated by only 12% and the hypocotyl by 30%, but the transition zone is 44% longer. (**d**) By 50 HAI, the radicle has emerged and has elongated by 13% whereas the hypocotyl and transition zones are 41% and 73% longer, respectively. (**e**) Further elongation occurs by 54 HAI in the hypocotyl and transition zone, with small bulges appearing in the latter. These are the initials of the collet hairs, whose formation precedes that of the root hairs. (**f**) By 62 HAI the collet hairs are visible in the early growing seedling at cells 26–28. The scale at the bottom of each embryo marks the cell number, from the point of attachment of the axis to the cotyledons to the radicle tip. Cells 31–37 are not visible in (**f**) after germination due to geotropic curvature of the radicle out of the focal plane. The region where the hypocotyl is joined to the cotyledons is also out of the focal plane of these pictures and appears as a dark area. c: cotyledons; r: radicle; h: hypocotyl; t: transition zone; b: bulge at the initiation of collet hair formation; co: collet hair. From Sliwinska et al. (2009). A movie of the changes noted above is available online through this publication. With permission of Oxford Univ. Press

occurring. It remains to be determined, however, whether production of EXPs or XTHs are essential for germination to be completed.

The opposing force that restricts radicle growth is provided by the surrounding tissues, such as the endosperm and testa. When the dispersal units are not true seeds but fruits, such as caryopses in cereals or achenes in some Composite species, the pericarp can also contribute to the mechanical resistance of the covering tissues. In many cases, the testa and pericarp are nonviable tissues in mature seeds. Therefore, the major changes in the mechanical resistance of the covering tissues during imbi-

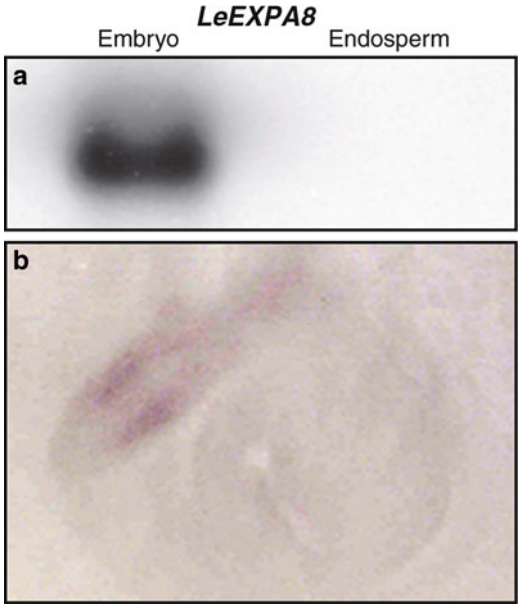

Fig. 4.20 Expression of the *LeEXPA8* gene for expansin occurs exclusively in (**a**) the embryo of germinating tomato seeds, as shown by a northern blot. (**b**) A tissue print shows the location of the transcripts of this gene in the lower hypocotyl region of the embryo prior to radicle emergence. From Chen et al. (2001). Copyright American Society of Plant Biologists

bition are observed in the living endosperm tissues. Weakening of the endosperm, especially the micropylar region of the endosperm enclosing the radicle tip (micropylar endosperm), is another important factor to allow radicle emergence (Fig. 4.18). In some species (e.g., tomato) the micropylar endosperm (also called the endosperm cap) is rigid due to the deposition of hemicelluloses such as galacto-, gluco-, or galactoglucomannans in the secondary cell wall. The physical properties of the cells walls in this region are altered through enzymatic degradation of their component polysaccharides, resulting in weakening of its resistance to penetration. In tomato and *Datura* seeds, genes encoding endo-β-mannanase, a mannan-degrading enzyme, are first expressed exclusively in the micropylar endosperm, initiating the degradation of the cell walls and thus resulting in tissue weakening (Fig. 4.21). Genes encoding other cell-wall-modifying enzymes or proteins, including *XTHs*, *EXPs*, cellulases (*CELs*), polygalacturonases (*PGs*), and pectin methylesterases (*PMEs*), are also expressed in the micropylar endosperm, suggesting cooperative roles for the encoded enzymes in the weakening events (Fig. 4.21; Table 4.2). In a number of cases, such as for endo-β-mannanase and expansins, multiple gene family members encoding functionally similar proteins are present in the genome, and different members of the gene family exhibit different tissue localization and temporal expression

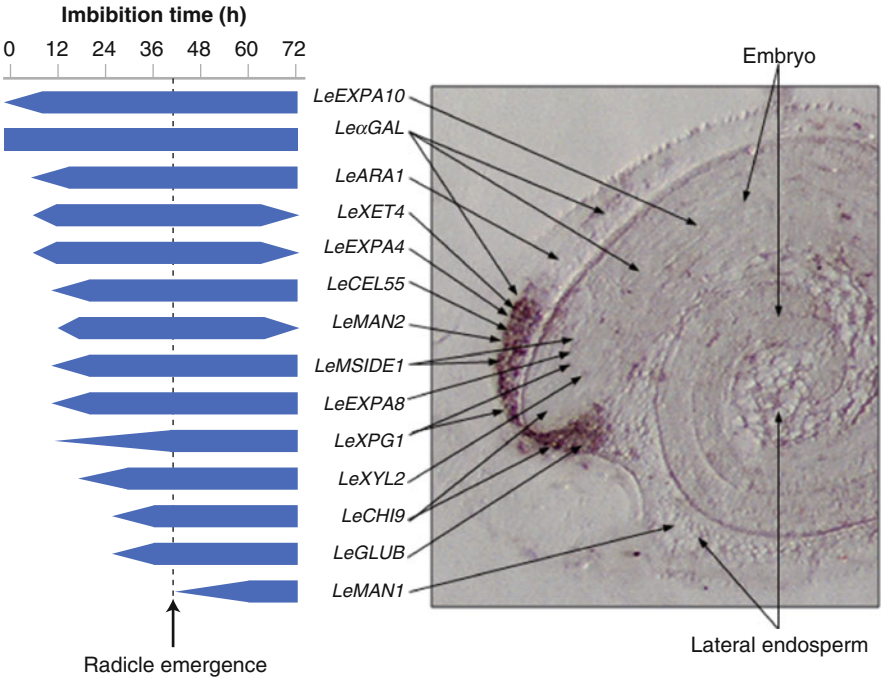

Fig. 4.21 Timing and localization of genes expressed in germinating tomato seeds. The chart to the left indicates the times at which mRNAs of the indicated genes become detectable or decline. The vertical dotted line indicates the time when radicle emergence occurs (~42 h). The image to the right shows a tissue print of a tomato seed expressing a gene specifically in the micropylar endosperm (stained region). The arrows from the genes to the image indicate the tissues in which expression is detected (the micropylar endosperm, the lateral endosperm or the embryo). For gene identifications, see Table 4.2. From Nonogaki et al. (2007). Courtesy of Wiley

patterns. This is a common mechanism in plants to regulate the development- and location-specific expression of certain proteins by gene duplication and subsequent divergence of expression patterns and function. Many pathogenesis-related genes such as β-1,3-glucanase (*βGLU*) and chitinase (*CHI*) are also expressed exclusively in the micropylar endosperm of tomato and tobacco seeds just prior to radicle emergence (Fig. 4.21). It is likely that these enzymes play protective roles in preventing microorganisms from entering the storage endosperm following germination, although their precise roles remain to be determined.

An alternative or complement to enzyme-induced weakening of the cell wall is that induced by chemical changes to its structure. In seeds of cress there is an increase in hydroxyl-radical production (·OH, a reactive oxygen species) in the apoplast of cells in the radicle and in the adjacent micropylar endosperm. The OH radicals cause breaks in the cell-wall polysaccharides, which could lead to an increased ability of the cells to elongate or stretch. ABA, which inhibits germination, causes a

Table 4.2 Genes associated with cell-wall modifications that are expressed in seeds during germination

Enzyme or protein	Gene	Tissue localization	Species
α-L-Arabinofuranosidase	*LeARA1*	LE	Tomato
Chitinase	*LeCHI9*	ME, RT	Tomato
Endo-β-mannanase	*LeMAN1*	LE	Tomato
	LeMAN2	ME	Tomato
	CaMANA		Coffee
	CaMANB		Coffee
	LsMAN1	ME, LE	Lettuce
	DfMAN	EMB, ME	*Datura*
Expansin	*LeEXPA4*	ME	Tomato
	LeEXPA8	EMB	Tomato
	LeEXPA10	EMB	Tomato
	DfEXPA1	EMB, ME	*Datura*
α-Galactosidase	*LeaGAL1*	ME, LAT, EMB	Tomato
β-1,3-Glucanase	*NtGLUB*	ME	Tobacco
	LeGLUB	ME	Tomato
β-1,4-Glucanase (cellulase)	*LeCEL55*	ME, RT, RS	Tomato
β-Mannosidase	*LeMSIDE1*	ME, LE, EMB	Tomato
Polygalacturonase	*LeXPG1*	ME, RT	Tomato
Xyloglucan endotransglycosylase/hydrolase	*LeXET4*	ME	Tomato
β-D-Xylosidase	*LeXYL2*	EMB	Tomato

Enzymes or proteins thought to have a mechanistic role in the completion of germination that have been identified as being expressed in seeds prior to radicle emergence are listed with their corresponding gene. If known, the tissue localization of expression is indicated: *ME* micropylar endosperm; *EMB* embryo; *LE* lateral endosperm; *RT* radicle tip; *RS* rest of seed (i.e., other than micropylar endosperm). Modified from Nonogaki et al. (2007)

decline in OH radical production, but this is reversed in the presence of germination-stimulating concentrations of GA.

Hypotheses 1 and 2 illustrated in Fig. 4.18 separate the increase in embryo growth potential from the decrease in the mechanical resistance of the micropylar endosperm, respectively. These two events are not mutually exclusive but are probably happening simultaneously. The scheme also explains the germination phenotypes of GA-deficient Arabidopsis seeds (*ga1*), GA-deficient and ABA-deficient double mutant seeds (*ga1 aba1*) and GA-deficient and *transparent testa* (no dormancy) double mutant seeds (*ga1 tt4*) (Sect. 6.6.1).

Many dicot seeds are epigeal, i.e., following germination the hypocotyl expands to raise the cotyledons above the soil surface (Sect. 5.1). It is not unreasonable to presume that the radicle emerges from other epigeal species by expansion of the hypocotyl/transition zone region immediately behind it, as occurs in tomato and Arabidopsis. Some dicot species are hypogeal, however, in which the cotyledons remain below the soil surface after germination; it is not known in these whether there is a specific region of the axis between the radicle and the cotyledons that

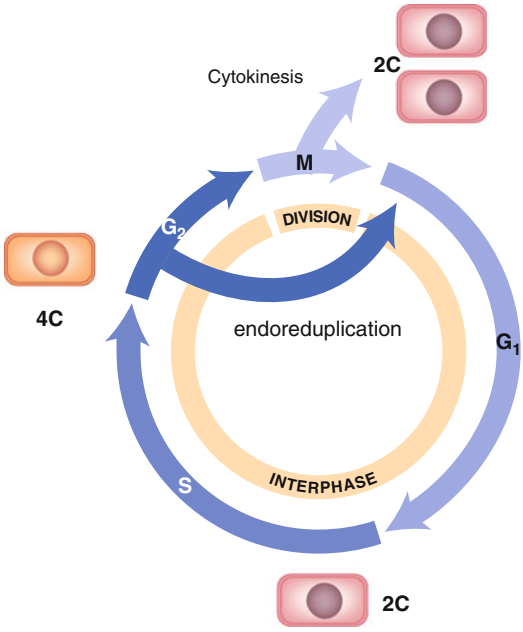

Fig. 4.22 Phases of the eukaryotic cell cycle. During Gap 1 (G_1, 2C DNA content) and Gap 2 (G_2, 4C) phases the cell can grow, in the early and late interphase stages of the cycle. DNA replication is confined to that part of the interphase termed the S phase. Mitosis (M phase) includes the division of chromosomes (which condense and become visible under a microscope), as well as subsequent cytoplasmic division (cytokinesis) to yield two daughter cells. Endoreduplication of the DNA occurs without cell division, and cells progressively increase from 4C to 8C, 16C, 32C, etc.

expands to push out the former from the seed to effect the completion of germination. The cereal embryo is structurally different from that of dicots (Fig. 1.1); which specific region of cells expands to achieve emergence of the radicle, sheathed by a coleorhiza, remains to be determined. It is not clear, for example, whether the coleorhiza expands on its own or is "stretched" by the elongating radicle inside. However, in barley, cell-wall modification in the coleorhiza is necessary for dormancy release and germination induction following ABA deactivation (Sect. 6.6.1).

4.6.2 DNA Synthesis and Cell Division (Cell Cycle)

There are two periods of DNA synthesis during germination (Fig. 4.22): the first is associated with repair of DNA following imbibition of the dry seed (Sect. 4.3.3), and the other occurs as a prelude to cell division that follows germination. The time of commencement of DNA replication can be an indication of seed vigor, since low-quality seeds need longer to complete the initial DNA repair before successful copying is possible. Synthesis of mitochondrial DNA also occurs during germination, although as a proportion of cellular DNA synthesis this is very small.

Emergence of the radicle from the seed by cell elongation is followed almost immediately by its growth due to cell division and elongation of the newly formed cells. Hence, cell division is a post-germination phenomenon, contributing to axis

growth and establishment of the seedling. β-Tubulin, a component of the microtubular cytoskeleton (mitotic spindle) that is associated with chromosome arrangement during cell division, increases in the radicle tips of tomato and sugar beet as the cell cycle proceeds during germination. In seeds that are shed with their embryo in a very immature state (Sect. 6.3.1.2), cell division will occur during the completion of their development, but this then ceases until following germination.

The cell cycle is the fundamental means by which all eukaryotic organisms increase their cell number during growth, and incorporates a period of DNA synthesis followed by mitosis (nuclear and cytoplasmic division) (Fig. 4.22). After mitosis (M), when the nuclei contain 2C DNA, there is a period of normal cell growth (Gap 1 or G_1) during which synthetic events, including those preparatory for subsequent DNA synthesis, take place. Cells generally spend the longest time of the cycle in this phase, and some cells exit the G_1 to enter a quiescent stage of the cell cycle (G_0), in which they remain metabolically active, but do not proliferate. In the active cycle, following G_1 there is DNA synthesis (S) that results in a doubling of the chromosomes (DNA content is 4C) without cell division, and a second preparative period (G_2) during which the cell can grow and conduct protein synthesis before mitosis (M). If the G_2 phase is long, then so will be the lag between DNA synthesis and cell division; some cells, in fact, synthesize DNA but never divide, whereas in others there may be a pause of many hours between synthesis and division. Following mitosis there are two diploid daughter cells each with 2C DNA content; these cells grow during the subsequent G_1 phase, and the cycle is repeated.

Cells of the mature dry embryo are frequently in the G_1 or G_0 phase (possessing 2C DNA), with some species having additionally a small proportion of cells in the G_2 phase (with 4C DNA; Table 4.3). As germination advances, the number of nuclei with 4C DNA increases in the embryo, e.g., in tomato the proportion rises from 4% in the dry state to 40% before radicle protrusion, and in maize embryos from 0 to 50%. In polysomatic species (i.e., those having endopolyploid nuclei with different C-values due to endoreduplication) such as Arabidopsis, sugar beet, and cabbage, 8C or 16C nuclei can arise in the embryo before, or as, germination is completed. Endoreduplication is mostly associated with cells that store polymeric reserves (e.g., in cotyledons and endosperms) and do not undergo mitosis, as well as the larger cells of the xylem during its formation. There are no intact nuclei in the mature starchy endosperm of cereals, and the literature contains variable claims as to the extent that the nuclei in the radicle are in the G_1 or G_2 state: reports for maize, for example, vary from 43% to the majority of the radicle as having G_1 nuclei, with ~0–46% being in G_2; reports on the wheat radicle are even more variable.

Cells of persistent mature dry endosperms, which are triploid, often have higher than the anticipated 3C nuclear content due to at least some cells undergoing endoreduplication (Fig. 4.22). This occurs when nuclei undergo DNA duplication (S) to achieve the G_2 phase, and then go directly to G_1, bypassing mitosis (M). In developing cereal endosperms, values at least as high as 192C can be achieved. These polysomatic cells with nuclei containing endoreduplicated DNA are unable to undergo mitotic divisions and are committed to the synthesis and storage of reserves. Endoreduplication also occurs in the developing cotyledons of dicots, e.g.,

Table 4.3 Nuclear DNA content of mature dry seeds of selected species expressed as C-values[a]

	Nuclear DNA %									
Species and organ/tissue	**1C**	**2C**	**3C**	**4C**	**6C**	**8C**	**12C**	**16C**	**32C**	**64C**
Lettuce										
Radicle tip		100								
Cotyledon		100								
Endosperm			100							
Pepper										
Embryo		100								
Endosperm			91		9					
Phaseolus bean										
Embryo		57		41		2				
Radicle tip		34		55		11				
Cotyledon				18		9		14	36	23
Spinach										
Embryo		61		32		7				
Radicle tip		47		29		24				
Tomato										
Embryo		96		4						
Radicle tip		89		11						
Endosperm					75		25			
Black pine										
Embryo		100								
Megagametophyte	100									
Sugar beet										
Embryo		92		8						
Cucumber										
Embryo		84		14		2				

Data based on Bino et al. (1993) and input from E. Sliwinska

[a]C-value is the DNA content of a holoploid genome with chromosome number *n*, i.e., with a meiotically reduced chromosome number, irrespectively of the degree of the ploidy of the organism

legumes such as pea, in which the cells of some cultivars can achieve 64C; following germination these cells cannot undergo mitosis.

In gymnosperms, while the mature diploid embryo cells contain nuclei that are 2C, the megagametophyte, being haploid, contains those that are 1C (Table 4.3), although in some members of the Cupressaceae 1–6C nuclei are present.

4.7 Priming and the Enhancement of Germination

As understanding of the processes involved in germination has progressed, methods to modify those processes to enhance the performance of seeds in agricultural applications have been developed. One of the most widely used of these is called seed "priming," or the prehydration of seeds to advance their germinative metabolism

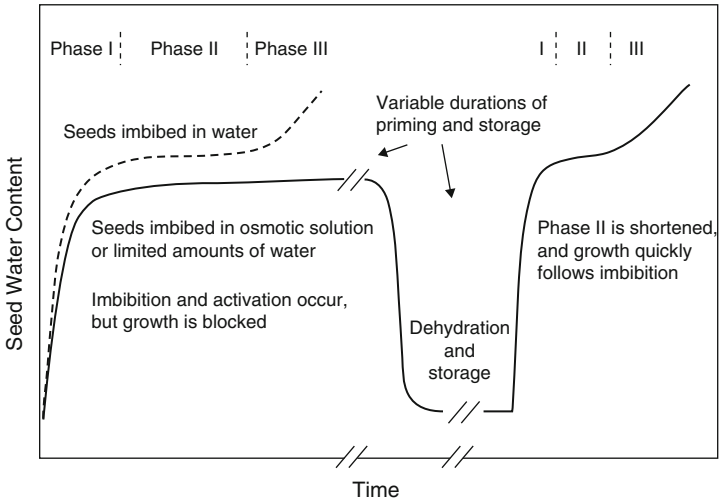

Fig. 4.23 Illustration of the process of seed priming to enhance germination. Seeds placed in water progress through the three phases of imbibition, including radicle emergence and growth (Phase III). During priming, water uptake by the seeds is restricted by imbibition in osmotic solutions or by limiting the amount of water provided to the seeds. This can extend Phase II while preventing seeds from entering into Phase III. Seeds retain desiccation tolerance and can be dried, stored and distributed for planting. When subsequently imbibed, the advancement gained during the priming treatment shortens Phase II, resulting in more rapid transition to Phase III and completion of germination

followed by dehydration prior to planting. As might be expected, such treatments result in more rapid and uniform germination and seedling emergence and can improve crop establishment under stressful conditions.

Seed priming is based upon the relationship between imbibition and water potential (ψ) illustrated previously (Fig. 4.10). When the ψ of the imbibition medium is decreased sufficiently using osmotic solutes such as polyethylene glycol (PEG) or salts (osmotic priming), or the total amount of water provided to the seeds is restricted (matric priming or hydropriming), completion of radicle emergence is prevented. However, much of germinative metabolism that occurs in Phase II can continue, including DNA and mitochondrial repair, degradation of stored mRNAs, and transcription and translation of new proteins (Fig. 4.1). Replicative DNA synthesis, cell division and embryo expansion are prevented and desiccation tolerance is maintained, allowing the seeds to be dehydrated following the priming treatment for storage, distribution and planting. The priming process is illustrated in Fig. 4.23, showing that Phase II of imbibition can be extended due to the reduced ψ beyond the time when germination would be completed in water (Phase III). Generally, if radicle emergence has not occurred, seeds retain desiccation tolerance and can be dried following the priming treatment without damage, although extending treatments too long (overpriming) can result in damage to radicle tips and poor subsequent seedling growth. Following storage and distribution, primed seeds provided with adequate water

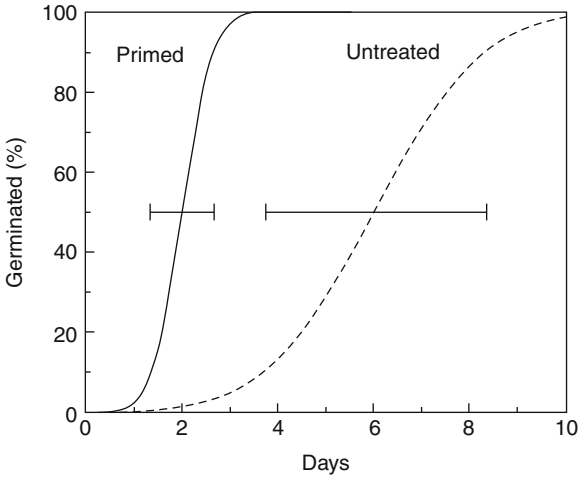

Fig. 4.24 Effect of seed priming on germination kinetics. By shortening Phase II of imbibition, priming speeds germination and also reduces the spread of germination over time, as indicated by the bars spanning the times to germination of 10–90% of the population. This improvement in germination speed and uniformity can be particularly pronounced when germination is delayed by low temperatures or other stresses

imbibe rapidly and exhibit shortened Phase II durations, moving relatively quickly from hydration to radicle emergence and growth. This considerably reduces the time from planting to seedling emergence, and also improves the uniformity (decreases the spread) of emergence over time (Fig. 4.24). These advantages have made seed priming popular for application to high-value flowers and vegetables, where rapid and uniform germination improve crop management both for the production of transplants and for direct-seeded crops. Priming can also enhance the ability of seeds to germinate under stressful conditions such as low temperatures or salinity.

An additional application of seed priming is to alleviate certain dormancy conditions. For example, light-requiring seeds must be at least partially hydrated to respond to a light signal. Seeds can be illuminated during priming to satisfy the light requirement, which is particularly useful when the seeds are to be coated inside of pellets for ease of planting, as the coatings can block the penetration of light. The leaching of germination inhibitors from sugar beet seeds (actually fruits) by soaking or "steeping" is also often combined with a priming treatment. A widespread commercial application of seed priming is to prevent the imposition of thermoinhibition at warm temperatures in crops such as lettuce. Lettuce seeds germinate readily at cool temperatures, but seeds of most commercial cultivars are inhibited from germinating at 20 to 30°C, the exact temperature depending upon the cultivar and seed production environment. This creates problems for crop establishment, particularly in desert areas where crops are planted in the warm autumn for crop production through the winter months. Priming treatments at cool temperatures enable the seeds to progress past the point at which high temperatures during imbibition can

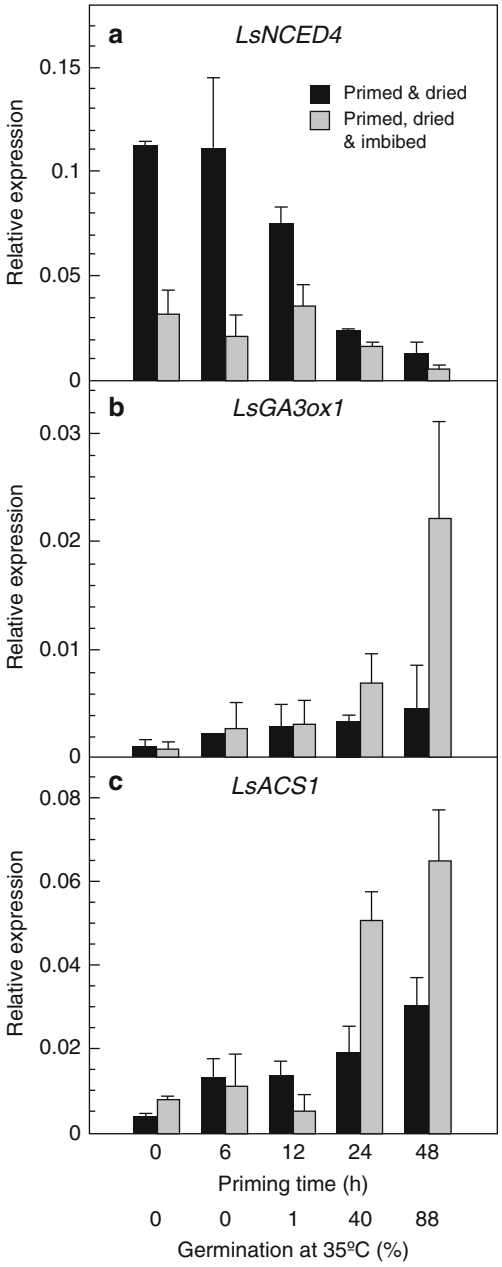

Fig. 4.25 Gene expression during and after priming of lettuce seeds. Expression (mRNA abundance) of *LsNCED4* (**a**), *LsGA3ox1* (**b**), and *LsACS1* (**c**) was measured in lettuce seeds that were primed in −1.25 MPa PEG 8000 for 0 (dry control seeds), 6, 12, 24, and 48 h at 9°C, rinsed, and rapidly dried (2 h at 32°C) (*black bars*). Expression was also measured in untreated control and primed seeds imbibed in water at 35°C for 24 h (*grey bars*), and germination percentages (after 48 h) of seeds primed for the different durations are shown at the bottom. As priming duration and germination capacity at 35°C increased, expression of *LsNCED4* decreased and of *LsGA3ox1* and *LsACS1* increased, consistent with the ability to escape thermoinhibition. From Schwember and Bradford (2010)

block germination. This is illustrated by changes in the expression of key genes encoding enzymes involved in ABA, GA and ethylene biosynthesis, which are involved in regulating dormancy and germination (see Sect. 6.6.1). In seeds imbibed under cool conditions, expression of genes involved in ABA biosynthesis (e.g., *LsNCED4* in lettuce) decreases and expression of genes involved in GA and ethylene biosynthesis increases (e.g., *LsGA3ox1* and *LsACS1*). When lettuce seeds are imbibed at high temperatures that induce thermoinhibition, these relationships are reversed, with maintenance of high *LsNCED4* mRNA levels and no expression of *LsGA3ox1* and *LsACS1*. During priming, mRNAs of *LsNCED4* decline and those of *LsGA3ox1* and *LsACS1* increase, and these trends are not reversed when the seeds are subsequently imbibed at high temperature following priming and drying (Fig. 4.25). Thus, priming enables the seeds to progress past the point that inhibitory mechanisms such as maintenance of high ABA and low GA and ethylene contents can be induced by high temperature, and thereby allows the seeds to escape from thermoinhibition and complete germination.

An undesired side effect of priming is often a reduction in the storage life of the dry seeds. This is not always the case, and some studies report extended storage life following priming treatments. Differences in results could have many causes, but drying conditions following priming may be the most significant. The rate and extent of drying following priming influence subsequent storage life, with slower or staged drying resulting in better seed storability than rapid drying. Similar types of staged reductions in seed moisture content can also restore desiccation tolerance to recently germinated seeds, suggesting that drying regimes may be influencing or reinducing desiccation tolerance mechanisms that also contribute to seed longevity (Sect. 2.5.2).

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Chapter 5
Mobilization of Stored Reserves

Abstract Mobilization of the major reserves within seed storage tissues occurs following the completion of germination to provide nutrients for the growing seedling until it becomes autotrophic. Starch, hemicelluloses, triacylglycerols (oils), and proteins are mobilized by distinct suites of enzymes, many of which are transcribed and synthesized de novo. Starch and proteins are converted to sugars and amino acids within the starch granules and protein storage vacuoles, respectively, before these catabolites are moved into the cytosol; hemicelluloses are released from cell wall polymers by specific hydrolases. Oils, in contrast, require the additional participation of two non-storage organelles within the cell, one of which, the glyoxysome, is formed de novo to accommodate the enzymes required for the catabolism of fatty acids. The final carbon product of reserve catabolism is sucrose that is translocated to the growing tissues, with proteins also yielding transportable amino acids. Regulation of starch mobilization from the endosperms of cereals, which is hormonally controlled, is well understood; in contrast, while the participation of hormones in hemicellulose mobilization in dicot endosperms is known, their role in the hydrolysis of the major cotyledon reserves is uncertain.

Keywords Mobilization • Starch • Hemicelluloses • Oil • Protein • Phytin • Hormonal regulation

5.1 Seedling Growth Patterns

The first sign that germination has been completed is usually the appearance of the radicle through the surrounding structures, followed by an increase in its length and fresh weight. In many seeds the radicle penetrates the surrounding structures as soon as elongation of the hypocotyl/transition zone (Sect. 4.6.1) commences, but in others (e.g., faba or broad bean, and other beans) there is considerable growth before the testa is ruptured. In Arabidopsis, there is initially rupture of the testa, with the

J.D. Bewley et al., *Seeds: Physiology of Development, Germination and Dormancy, 3rd Edition*, DOI 10.1007/978-1-4614-4693-4_5,

Table 5.1 Some species exhibiting hypogeal or epigeal seedling growth

	Hypogeal	Epigeal
Endopermic	Barley	Buckwheat
	Coconut	Castor bean
	Date palm	Coffee
	Hevea spp.	Fenugreek
	Maize	Lettuce[a]
	Wheat	Mung bean
	Wild oat	Onion
		Pinus spp./*Picea* spp.[b]
		Sunflower[a]
		Tomato
		Tradescantia spp.
Non-endospermic	*Aponogeton* spp.	*Arabidopsis thaliana*
	Broad bean	*Crambe abyssinica*
	Garden pea	Cucumber
	Runner bean/scarlet runner bean	Peanut
	Tropaeolum spp.	*Phaseolus* bean
		Pumpkin
		White mustard

[a]Species with an endosperm that is only one to a few cells thick, and hence has limited storage capacity
[b]Storage tissue is the megagametophyte

intact micropylar end of the endosperm being pushed through it by expansion of the embryo, and then this confining structure is ruptured to release the radicle. There are some seeds, however, from which the hypocotyl is the first structure to emerge; this occurs in some members of the Bromeliaceae, Palmae, Chenopodiaceae, Onagraceae, Saxifragaceae, and Typhaceae. In some grains (e.g., rice and barnyard grass) germinated under anoxic or hypoxic conditions coleoptile growth precedes that of the radicle (Fig. 4.14); such an unusual germination pattern is also sometimes found in wheat when grains begin to sprout on the parent plant (Fig. 2.18b).

The first hairs to be formed in the seedling following germination are in the collet region, at the junction (transition zone) between the hypocotyl and the radicle (Fig. 4.19e, f). These collet hairs have also been termed "hypocotyl hairs" or "collar rhizoids." They are important to initially anchor the seedling in its substrate (e.g., soil), to facilitate the development of geotropism and to aid in water uptake until the root hairs develop. Collet hairs arise synchronously from every epidermal cell (trichoblast) in the collet region, whereas the later-forming root hairs arise successively from alternate root epidermal cells.

Seedlings can be conveniently divided into two types on the basis of the fate of their cotyledons following germination (Table 5.1): (1) epigeal, in which the cotyledons are raised out of the soil by extension of the hypocotyl and often become

foliate and photosynthetic (Fig. 5.1a, b), and (2) hypogeal, in which the hypocotyl remains short and compact, and the cotyledons stay beneath the soil. The epicotyl expands to raise the first true leaves out of the soil (Fig. 5.2). The terms "epigeal (epigeous) germination" and "hypogeal (hypogeous) germination" are sometimes used, especially with respect to field emergence, but such use is incorrect because the phenomena relate to seedling growth, not to germination per se.

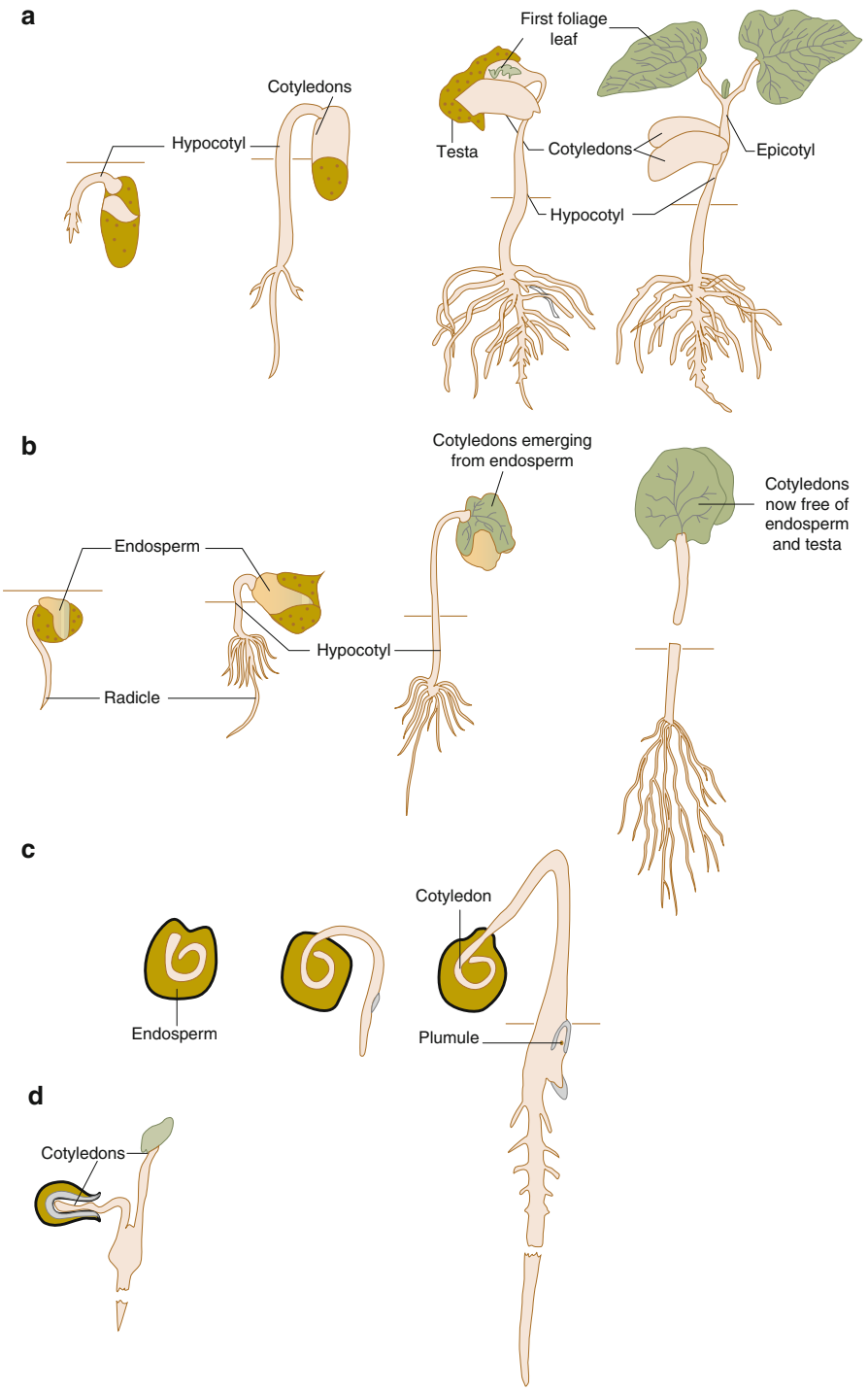
In endospermous seeds showing the epigeal mode of seedling growth, e.g., castor bean, the endosperm may be carried above ground by the cotyledons as they utilize its food stores. In onion, another epigeal type, the absorptive tip of the single cotyledon may remain embedded in the degrading endosperm, while the rest of the cotyledon turns green. The cotyledon in monocots may become highly specialized for absorption; in the Gramineae, for example, it is modified to form the scutellum, which may become extended as an absorptive haustorium (Sect. 5.5.2). Highly developed haustorial cotyledons are found in the Palmae. When the small embryo of the date palm commences growth, the cotyledon tip enlarges to form an umbrella-shaped body buried within the endosperm, from which it absorbs the hydrolyzed reserves. Likewise, the absorptive cotyledon of the coconut enlarges to invade the endosperm.

An interesting pattern of growth is shown by some *Peperomia* spp. (Fig. 5.1d) and in asparagus (both dicots), in which one cotyledon emerges from the seed, and the other remains as an absorptive organ buried within the endosperm of the seed which remains in the soil: "semi-epigeal." It has been suggested that the monocotyledonous condition evolved from this pattern of emergence, although this remains a matter for conjecture. The monocot onion seedling is a variation on the epigeal mode (Fig. 5.1c), where the single cotyledon remains within the storage tissue after its emergence from the soil.

5.2 Mobilization of Stored Reserves

The major mobilization of the polymeric food reserves present within the storage tissues of the seed commences after radicle protrusion, i.e., it is a post-germinative event. Some mobilization of these reserves can occur, often in the axis and a limited (e.g., micropylar) region of the endosperm before germination is completed; here the reserves are generally present in minor amounts, although the products of their hydrolysis might be important to support germination and early seedling establishment.

As the reserves contained within the storage tissues are mobilized, they are converted into forms that are readily transportable to the sites where they are required (usually the most rapidly metabolizing and growing organs of the seedling) for the support of energy-producing and synthetic events. Reliance on the stored reserves diminishes as the seedling emerges above the soil and becomes photosynthetically active (i.e., autotrophic). For the purpose of clarity this chapter is divided into sections, each of which covers the mobilization of one major type of storage reserve. It must be remembered, however, that storage organs usually contain substantial quantities of two or more major reserves (Table 1.2) and that hydrolysis and utilization of these occurs concurrently.


a
Cotyledons
Hypocotyl
First foliage leaf
Testa
Cotyledons
Epicotyl
Hypocotyl
b
Cotyledons emerging from endosperm
Endosperm
Hypocotyl
Radicle
Cotyledons now free of endosperm and testa
c
Endosperm
Cotyledon
Plumule
d
Cotyledons

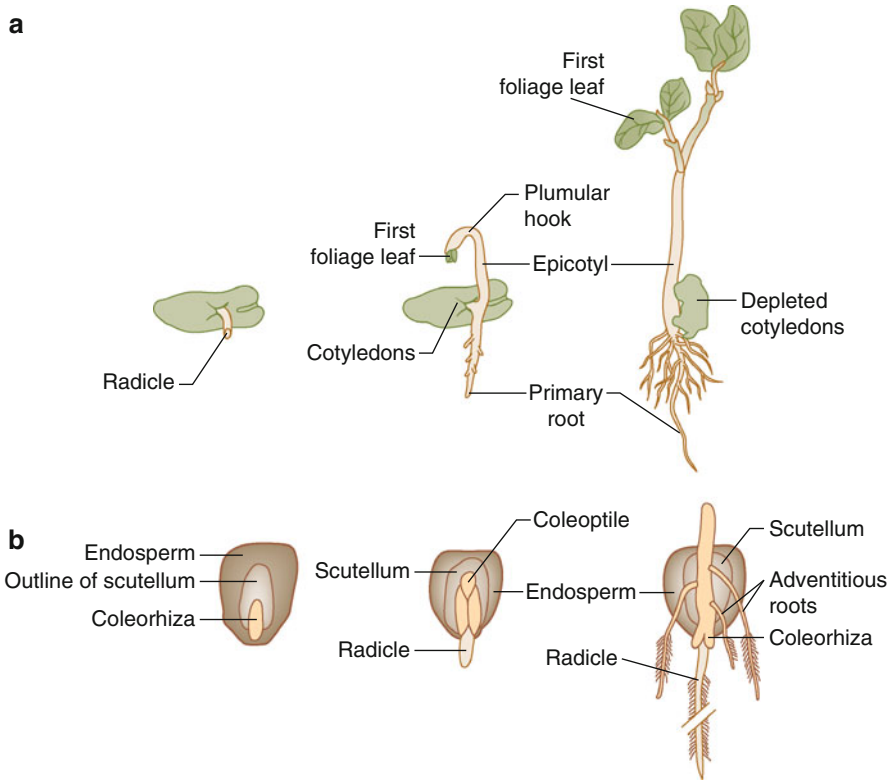


Fig. 5.2 The hypogeal type of seedling growth as shown by (**a**) the dicot faba, or broad, bean and (**b**) the monocot maize. The two bean cotyledons remain below the ground and shrivel during depletion of their reserves, eventually degenerating completely. The single cotyledon (scutellum) of maize and other cereals remains below the soil surface; in some species (e.g., wild oat) it may grow into the starchy endosperm and aid in absorption of the products of storage reserve mobilization. After depletion of the reserves the scutellum degenerates. Not drawn to scale

Fig. 5.1 The epigeal type of seedling growth in (**a**) *Phaseolus* bean. The cotyledons swell only a little and turn green; they are shed when their storage reserves are depleted. (**b**) Castor bean in which the cotyledons expand and become green and photosynthetic after their reserves are mobilized, and remain so until the first true leaves open; then the cotyledons shrivel and fall off. Both of the above are dicots. (**c**) Onion. The single cotyledon of this monocot emerges above the soil but remains embedded in the endosperm, acting as an haustorium through which the hydrolyzed storage products are imported into the growing seedling. It degenerates after the reserves are depleted. Growth of the aerial parts of the seedling is from the basal plumule. (**d**) *Peperomia peruviana*, in which one haustorial cotyledon remains embedded in the seed endosperm below soil level, and the other emerges above and becomes green. Not drawn to scale

5.3 Stored Oligosaccharide Catabolism

While there are no low-molecular-weight storage triacylglycerols or proteins in seeds, the raffinose-family oligosaccharides (RFOs, Sect. 1.3.1) represent such a storage product for carbohydrates in the embryos and storage tissues of many species of monocots and dicots, and gymnosperms. These are oligomers of sucrose and galactose (Gal) (Fig. 5.3), the most commonly present being raffinose (galactosyl-sucrose) and stachyose (digalactosyl-sucrose). Removal of the Gal units requires the enzyme α-galactosidase, and hydrolysis of sucrose is by invertase. The released Gal is presumably converted to glucose following phosphorylation, conversion to UDP-Gal and epimerization to UDP-glucose. Relatively little research has been conducted on α-galactosidases or invertases during germination. There are reports that both soluble and vacuole-associated invertases are present in the embryo and storage tissues during and following germination, with an increase in their transcripts, synthesis or activity particularly during early seedling growth; but correlations of activity with RFO or sucrose mobilization are weak or absent.

α-Galactosidase is generally synthesized during development and is present in mature dry seeds, along with RFOs. This raises the question as to where the enzyme is sequestered when the seed is developing to prevent it from hydrolyzing its substrates. In the seeds of tomato, some legumes including soybean and pea, and date

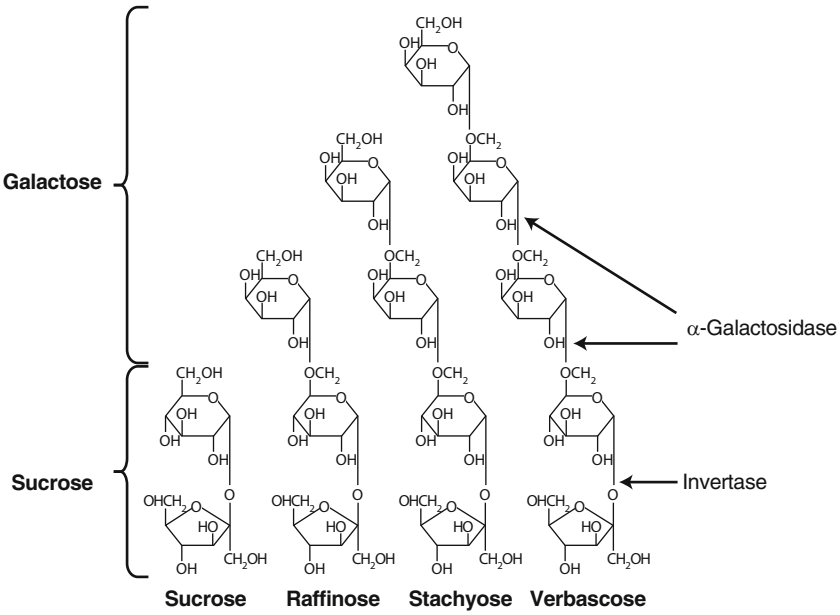


Fig. 5.3 Sucrose and raffinose-family oligosaccharides (RFOs) from mono- to tri-galactosyl sucrose (raffinose to verbascose). Shown are the enzymes required to break the links between the galactose units and between galactose and sucrose (α-galactosidase), and to convert sucrose to glucose and fructose (invertase)

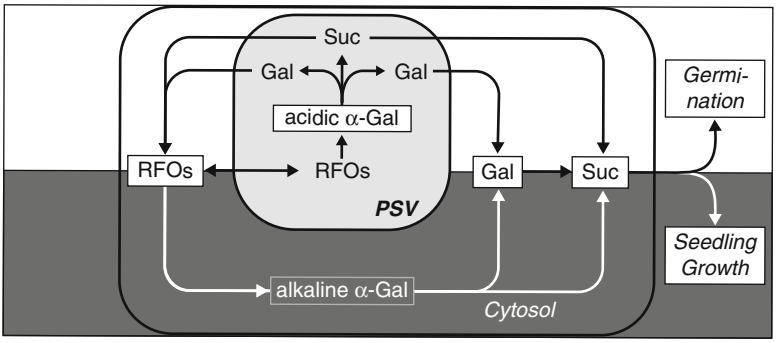


Fig. 5.4 Proposed model for the hydrolysis of RFOs in pea seeds during and following germination. During germination (*top panel*) RFOs are imported into the protein storage vacuole (PSV) from the cytoplasm and hydrolyzed to galactose (Gal) and sucrose (Suc) by an acidic α-galactosidase (α-Gal), the activity of which is initiated by acidification of the vacuole, presumably by H^+ ion pumps in the membrane. The sugars are released from the PSV and utilized by the germinating seed. Following germination (*lower panel*) there is de novo synthesis of a cytoplasmic alkaline α-galactosidase to provide for further hydrolysis of the RFOs. After Blöchl et al. (2008)

palm, α-galactosidase and RFOs are spatially separated, and in the dry seed the enzyme is present in protein storage vacuoles (PSVs), while the oligosaccharides lie within the cytoplasm. How the enzyme and substrate come together during and following germination is explained in a model for RFO hydrolysis in the pea seed (Fig. 5.4). Here, during germination, there is initially hydrolysis by an acidic α-galactosidase of RFOs imported from the cytoplasm into the intact PSV, the enzyme being synthesized and subsequently sequestered therein as the seed is developing. Following germination there is the expression of genes for, and the synthesis of, an alkaline α-galactosidase, which is located in the cytoplasm, and this also acts to hydrolyze the RFOs therein, in concert with that in the PSVs. Germinating pea seeds contain anabolic enzymes of the RFO pathway, as well as the catabolic ones, although activities of the latter predominate. Under stress conditions, however, when germination is slowed or impeded, temporary resynthesis of RFOs may occur.

The importance of RFOs as an early source of sugars to produce energy during germination has been questioned, at least in some species, e.g., inhibition of α-galactosidase activity in imbibed soybeans does not delay their germination, nor does the addition of sucrose or Gal improve it in the absence of enzyme activity. In contrast, impairment of RFO breakdown during germination of pea seeds considerably delays its completion. α-Galactosidase also plays a role in the mobilization of Gal-containing cell wall hemicelluloses, e.g., galactomannans, following germination (Sect. 5.6.2).

5.4 Pathways of Starch Catabolism

There are two catabolic pathways of starch: one hydrolytic and the other phosphorolytic.

The amylose and amylopectin in the native starch granule are first hydrolyzed by α-amylase, an endohydrolase that breaks the α(1→4) glycosidic links between the glucose residues randomly throughout the chains. The released oligosaccharides are further hydrolyzed by α-amylase (or with the cooperation of α-glucosidase—see below) until glucose and maltose are produced.

$$\text{Amylose} \xrightarrow{\alpha\text{-Amylase}} \text{Malto-oligosaccharides} \xrightarrow{\alpha\text{-Amylase}} \text{Glucose} + \text{Maltose}$$

Multiple forms of this enzyme occur in germinated seeds of many species. Wheat, for example, contains over 20 α-amylase isoenzymes that fall into two groups separated by isoelectric focusing on the basis of their specific isoelectric point (pI, the pH at which a protein loses its electrical charge). Similarly, two groups of α-amylases occur in grains of other cereals, such as rice and barley; in the latter HvAMY1 (*Hordeum vulgare* α-amylase 1) has a stronger affinity for linear malto-oligosaccharides, whereas HvAMY2 plays a larger role in initial starch degradation. But α-amylases cannot hydrolyze the α(1→6) branch points of amylopectin, and hence highly branched cores of glucose units, called limit dextrins, are produced.

$$\text{Amylopectin} \xrightarrow{\alpha\text{-Amylase}} \text{Malto-oligosaccharides} \xrightarrow{\alpha\text{-Amylase}} \text{Glucose} + \text{Maltose} + \text{Limit dextrin}$$

The small branches must be released by enzymes specific for the α(1→6) link (debranching enzyme, limit dextrinase) before being hydrolyzed to the monomer.

$$\text{Limit dextrin} \xrightarrow{\text{Debranching enzyme}} \text{Malto-oligosaccharides} \xrightarrow{\alpha\text{-Amylase}} \text{Maltose} + \text{Glucose}$$

$$\text{Malto-oligosaccharides} \xrightarrow{\alpha\text{-Glucosidase}} \text{Glucose}$$

Another amylase, β-amylase, is an exohydrolase that cannot hydrolyze native starch granules; rather it cleaves away successive maltose units from the nonreducing end of large oligomers released by prior α-amylolytic attack. Again, amylopectin cannot be completely hydrolyzed, and the involvement of a debranching enzyme is essential. The importance of β-amylase in the mobilization of starch in cereals has been questioned, for some barley cultivars completely lack this enzyme yet grow into normal seedlings.

The disaccharide maltose, produced by α- and β-amylase action, is converted by α-glucosidase (maltase) to two glucose molecules. This enzyme can also cleave glucose from low-molecular-weight malto-oligosaccharides. There are several different α-glucosidases in the endosperm of a particular cereal, although the possibility exists that instead of being hydrolyzed there, maltose is transported instead, or also, into the growing embryo via the scutellum for cleavage to glucose therein.

$$\text{Maltose} \xrightarrow{\alpha\text{-Glucosidase}} \text{Glucose}$$

Starch phosphorylase releases glucose-l-phosphate (Glc-1-P) by incorporating a phosphate moiety, rather than water, across the α(1→4) linkage between the

penultimate and last glucose at the nonreducing end of the polysaccharide chain. Complete phosphorolysis of amylose by this exohydrolase is theoretically possible, and amylopectin can be degraded to within two or three glucose residues of an $\alpha(1\rightarrow6)$ branch linkage; it is more likely to act upon polymeric chains released by α-amylase, however. The enzyme cannot attack starch granules, which first must be partly degraded by other enzymes.

$$\text{Amylose / amylopectin} + \text{Pi} \xrightarrow{\text{Starch phosphorylase}} \text{Glc-1-P} + \text{Limit dextrin}$$

To what extent this pathway of starch degradation occurs in seeds is unclear, although it is important in the mobilization of temporary starch in the plastids of leaves, and in potato tubers. It is unlikely to operate efficiently in the cereal endosperm because the storage cells are nonliving, and hence, there is no means to provide for the constant supply of required Pi. However, there is pronounced phosphorylase activity in the cotyledons of some germinated legumes during starch mobilization, e.g., pea.

5.4.1 Synthesis of Sucrose

The products of starch (and triacylglycerol) catabolism eventually are transported as sucrose into the growing root and shoot of the seedling. Glc-l-P released by phosphorolysis can be used directly as a substrate for sucrose synthesis, but glucose released by amylolysis first must be phosphorylated to glucose-6-phosphate (Glc-6-P) and then isomerized to Glc-l-P. This combines with a uridine nucleotide (UTP) to yield pyrophosphate (PPi) and the nucleotide sugar uridine diphosphoglucose (UDPGlc), which in turn transfers glucose to free fructose or to fructose-6-phosphate.

$$\text{Glc-l-P} + \text{UTP} \xrightleftharpoons{\text{UDPGlc pyrophosphorylase}} \text{UDPGlc} + \text{PPi}$$

$$\text{UDPGlc} + \text{Fructose} \xrightleftharpoons{\text{Sucrose synthase}} \text{Sucrose} + \text{UDP}$$

$$\text{UDPGlc} + \text{Fructose-6-P} \xrightleftharpoons{\text{Sucrose - 6 - P synthase}} \text{Sucrose-6-P} + \text{UDP}$$

It is generally accepted that this latter reaction is the predominant, if not the only one involved in sucrose synthesis, whereas the sucrose synthase is important for sucrose catabolism. The phosphate moiety is cleaved from sucrose-6-P by sucrose phosphatase. In the seedling tissues, sucrose can be hydrolyzed to free glucose and fructose by invertase (β-fructofuranosidase, sucrase), or converted to UDPGlc and fructose by sucrose synthase.

5.5 Mobilization of Stored Starch in Cereal Grains

Although studied in all agronomically important cereals, much is known about starch mobilization and its control in germinated barley, in part because of the central role of this process in the production of malt for beer production.

An initial event is the release of cell wall-degrading enzymes, β-glucanases, from the scutellum into an intermediate layer of crushed cells (Fig. 1.1) that lies between the scutellar epithelium and the starchy endosperm. The digestion of this layer facilitates the release and passage of α-amylase from the scutellum into the starch-storing cells to commence digestion of this reserve. The initial production of this enzyme invariably occurs in the region of the scutellum, in the epithelial layer of this organ (e.g., rice), or the entire scutellum (e.g., sorghum), or in the few aleurone layer cells that penetrate the peripheral regions of the scutellum (e.g., barley). Later the enzyme is usually synthesized within the aleurone layer, the only living cells in the storage tissue, which lie to the outside of the mature cereal endosperm (in barley it is three cell-layers thick, in maize and wheat only one, and rice one to several), and is then secreted into the starchy endosperm. Thus, although α-amylase synthesized in, and released from the scutellum is important during the early stages of starch mobilization, e.g., in barley, wheat, rye and oat, most of the later hydrolysis is effected by enzyme from the aleurone layer. In rice, synthesis of α-amylase in the scutellum precedes that in the aleurone layer and is at least as important for starch hydrolysis; in maize, the scutellum is persistently a major source of the enzyme.

5.5.1 Synthesis and Release of α-Amylase and Other Hydrolases from the Aleurone Layer

Although the hydrolysis of starch by amylases is central to its mobilization, there is also collaborative activity of other enzymes to aid in the synthesis and movement of the major enzyme, α-amylase, from the living cells of the scutellum and aleurone layer to the nonliving starch-storing endosperm cells, and in the breakdown of the starch granule. Synthesis of α-amylase in the scutellum and aleurone layer requires the transcription of several genes for this hydrolase, and their subsequent translation. The hormonal regulation of this is considered in Sect. 5.5.3. In the aleurone layer cells of barley, about 60% of the newly synthesized protein is α-amylase, and therefore, a supply of amino acids is required to sustain this high level of production. This is achieved by the hydrolysis of stored proteins (mostly globulins, but also albumins and minor amounts of prolamin) present in the protein storage vacuoles (PSVs) of the mature aleurone layer cells (Fig. 5.5a, b). Aleurone layer cells, and those of the scutellum, are also rich in triacylglycerols that are sequestered in oil bodies (Fig. 5.5b), and these are mobilized by lipases, with the resultant fatty acids being converted to sugars (Sect. 5.7) as a source of energy for synthetic events, and

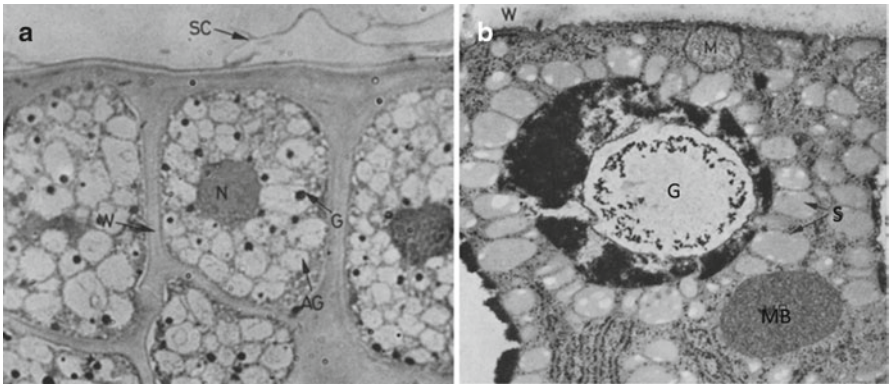


Fig. 5.5 (**a**) Light micrograph of mature aleurone layer cells of a barley grain, showing the presence of protein storage vacuoles (also called aleurone grains, AG), which are not stained for protein, containing dark regions (phytin globoids, G). Cell wall (W), nucleus (N), outer seed coat (SC). (**b**) Electron micrograph of a protein storage vacuole of a barley aleurone layer cell containing protein (dark areas) and a globoid (G), with surrounding oil bodies (S). Cell wall (W), mitochondrion (M), microbody (glyoxysome, MB), involved in stored oil utilization. From Jones (1969)

for the synthesis of membrane lipids. Phytase is also required for mobilization of the phytin-containing globoid.

A variety of enzymes involved in the mobilization of the carbohydrate reserves in the starchy endosperm are also synthesized in the aleurone layer, some facilitating the release of α-amylase, and others accompanying it to ensure the hydrolysis of starch and other stored reserves. These include limit dextrinase and α-glucosidase (maltase) to effect breakdown of the starch to glucose (Sect. 5.4) and enzymes to hydrolyze the starchy endosperm cell walls (e.g., β-1→3, 1→4 glucanases to hydrolyze the mixed-linkage glucans that make up about 75% of these walls in barley). In wheat the starchy endosperm cell walls are rich in arabinoxylans; rice and maize endosperm cell walls are also hemicellulose- as opposed to glucan-rich. To degrade these arabinoxylan-rich walls, and also those of the aleurone layer cells of barley and other cereals, pentosanases such as β-xylanase and α-arabinofuranosidase are synthesized and released from the aleurone layer, thus facilitating the passage of the hydrolases from this region to the starchy endosperm (there are no plasmodesmatal connections between the aleurone layer and the starchy endosperm), and through the cells of this storage tissue. Other enzymes synthesized in the aleurone layer and released into the starchy endosperm include endo- and exo-peptidases (Sect. 5.8) to hydrolyze the predominantly prolamin storage proteins, and phosphatases and nucleases for dephosphorylation of macromolecules and hydrolysis of remnant nucleic acids.

An exception to the above pattern of synthesis and release is shown by β-amylase, at least in barley, rye, rice, sorghum and wheat grains. This enzyme is synthesized in the starchy endosperm during its development, is present in the mature dry grain as up to 1% of total protein therein, and becomes bound to proteins on the periphery

of the starch granules, and perhaps to other endosperm proteins, during maturation drying. It is activated when released by selective protein-cleaving hydrolases synthesized and released from the aleurone layer, or by reduction of disulfide bonds by which it is attached to other proteins. Maize grains do not accumulate β-amylase during their development, however; rather this is de novo synthesized in the aleurone layer following germination.

5.5.2 Starch Breakdown and the Fate of the Products of Hydrolysis

During synthesis of starch to form the granule there are abundant channels formed through this structure, extending from pores on the surface to the interior. When there is mobilization of the starch these channels become widened and the pores become deeply pitted before the surface of the granule has been degraded (Fig. 5.6a); these are the paths by which α-amylase, and presumably other hydrolases, penetrate into the granule as the starch is hydrolyzed. To further aid access of hydrolases to the starch, the membrane surrounding the amyloplast, the organelle in which the starch granules are synthesized, disintegrates; this may occur during drying of the mature endosperm, and/or upon subsequent imbibition of the grain. The products of starch degradation, glucose, maltose and small malto-oligosaccharides, along with the hydrolytic products of proteins and cell walls, are taken up into the scutellum for modification and transport into the growing embryo. In some cereal grains (e.g., those of the oat family, but not of barley or wheat), the scutellum elongates into the endosperm as digestion proceeds, thus presenting a much-increased surface area for absorption of the hydrolytic products into the growing embryo. The cells of the epithelial layer of the scutellum elongate and separate to form finger-like projections into the starchy endosperm (Fig. 5.6b, c). They are metabolically very active, with many mitochondria present, and there are numerous transporters in the plasma membrane of these cells, for sugar, amino acid and peptide uptake (Sect. 5.8.2.1). Within the scutellum reside the enzymes for the hydrolysis of di- or oligomeric sugars to glucose, and those for the synthesis of sucrose; there is a vascular conducting system that is continuous from the scutellum into the growing embryo through which this sugar is transported. In rice, when there is an excess of sugar flowing into the scutellum, it is temporarily converted to starch, in granules, in the cells around the vascular tissues. This is then hydrolyzed, converted back to sucrose, and loaded into the phloem by sucrose transporters for distribution in the embryo. A temporary deposition of starch also occurs in the micropylar region of the endosperm of some dicot seeds (e.g., celery and tomato) when stored oils and proteins in this region are mobilized during germination.

Malting is a manipulated variation of the mobilization of starch in barley grains to produce the maximum amount of fermentable sugars for the brewing of beer and distilling of liquors. Successful malting requires considerable technological and biological understanding of reserve mobilization, garnered from centuries of

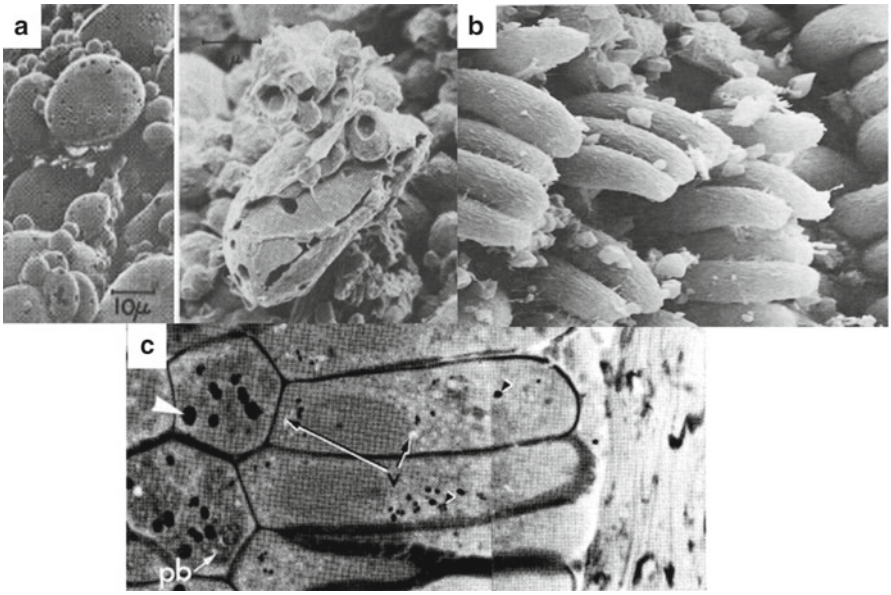


Fig. 5.6 (**a**) Two scanning electron micrographs at low and high magnification that show the degradation of starch granules during hydrolysis in the endosperm of wheat. Scale bar *left* 10 μm, *right* 1 μm. From Dronzek et al. (1972). Courtesy of American Association of Cereal Chemists. (**b**) Scanning electron micrograph of the epithelial layer of cells of the extended scutellum of wild oat to show the extent of their expansion, increasing the surface area over which reserve breakdown products are absorbed from the starchy endosperm into the embryo. Courtesy of J. Sargent and M. Negbi. (**c**) Light micrographs that show the swelling and extension of the epithelial cells of the scutellum of wheat grains into the depleted cells of the intermediate layer (*right*) and the beginning of their separation, prior to major reserve mobilization. Protein storage vacuoles (pb) are present but are being depleted, forming smaller (*black arrowheads*) or empty vesicles (v) as the proteins are hydrolyzed to provide amino acids for the synthesis of hydrolases released into the starchy endosperm. Large white arrow: starch granule. From Swift and O'Brien (1972). Courtesy of CSIRO Publishing

research and experience; the steps involved are described only very superficially here. Imbibition (steeping) of the grain under tightly controlled conditions of hydration and temperature is necessary to initiate the synthesis of enzymes in the scutellum and aleurone layer while limiting root growth (chitting) to prevent utilization and hence loss of sugars. Barley grains are then transferred to germination beds on which humidification and temperature are again tightly controlled; during this stage there is the major synthesis of hydrolytic enzymes to mobilize cell walls (mixed-linkage glucanases) and storage proteins (proteinases) within the starchy endosperm, thus allowing easier access to the starch of the necessary degrading enzymes. β-Amylase is also activated. α-Amylase is synthesized in the aleurone layer and released into the starchy endosperm, but at this stage its ability to break down starch is restricted: only about 10% of the starch is hydrolyzed during malting. In some malting procedures the hormone gibberellin (GA) may be sprayed onto the grain

during germination to enhance enzyme production in the aleurone layer, although germination of the grain during steeping is important to initiate hormone synthesis and release from the scutellum (Sect. 5.5.3). The next stage is kilning, in which the grain is heated from the 16°C germination temperature to about 60°C, and then briefly higher to 82°C for lager malts or 100°C for ale malts. Water is driven from the grain, to about 5%, the malt increases in color (the longer the kilning, the darker the malt), and heat-labile enzymes such as the glucanases, proteases and β-amylase are destroyed; α-amylase is heat resistant. The final result of malting is a grain with a friable endosperm that can be crushed; it has an altered composition of cell walls and proteins, starch that has undergone only limited degradation, and a high amount of α-amylase that has been released from the aleurone layer into the modified starchy endosperm. This malted product is then sold in the dry state and utilized by the brewers and distillers; mashing of the malt under appropriate conditions results in the release of sugars from the starch by α-amylase; their fermentation by yeast produces the required ethanol for beverages.

5.5.3 Hormonal Control of Starch Mobilization

There has been a large amount of research on the regulation of synthesis of starch-degrading enzymes, particularly of α-amylases in barley grains, for which Himalaya has been the cultivar of choice. Its advantages are that it is hull-less, the aleurone layer can be readily isolated from the starchy endosperm, and the former has an almost absolute requirement for GA to induce hydrolytic enzyme synthesis. While this is a good model system in which to understand hormonal regulation, many barley cultivars, and those of other cereals, do not respond so clearly to applied hormone, perhaps because the mature grains already contain considerable amounts of GAs that were imported from the parent plant during their development, and/or because there is considerable and rapid hormone synthesis and distribution from the embryo upon imbibition. Applied GA may speed up enzyme production, however, an advantage in the barley malting industry.

The aleurone layer is essentially a secretory tissue that responds to a hormone signal (GA) released from the embryo (scutellum) (Fig. 5.7). This hormone induces a number of profound changes in the metabolism of the aleurone layer, which lead to the synthesis and secretion of α-amylase and other enzymes (Sect. 5.5.1) to effect mobilization of the contents of the starchy endosperm. When GA is applied to isolated aleurone layers of barley there is the induction of transcripts for α-amylase within 2–3 h, which is followed by its translation to produce the enzyme (Fig. 5.8). At the time of its maximum synthesis, about 25% of the transcripts in the aleurone layer are for this enzyme.

While α-amylase is discussed here as if it were a single enzyme, in fact in barley and wheat it consists of several different isoforms (posttranscriptional/translational variants from the same gene) and isozymes (forms encoded by different genes of a multi-gene family) separable from each other on the basis of their isoelectric

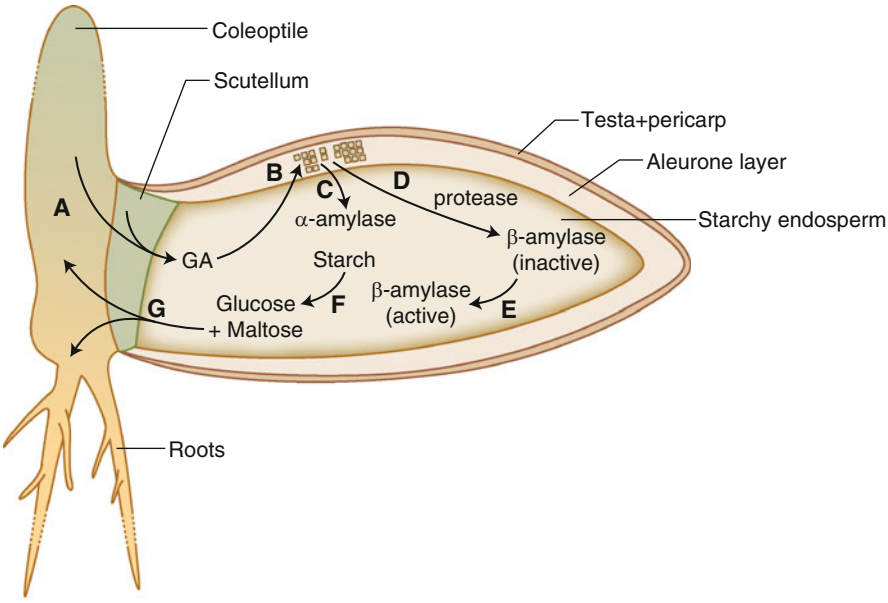


Fig. 5.7 A generalized diagram of a barley grain to show the relationship between embryo and aleurone layer in effecting the hydrolysis of starch by α- and β-amylase. Gibberellin (GA) is synthesized in the embryo and released from the scutellum (A). When it reaches the aleurone layer (B) it stimulates the synthesis of α-amylase that diffuses into the starchy endosperm (C) where it initiates the hydrolysis of starch (F). Activation of β-amylase in the starchy endosperm (E) follows the release of proteases de novo synthesized in the aleurone layer (D); this enzyme hydrolyzes starch polymers (F) released initially by α-amylase. The products of starch hydrolysis, mostly glucose and maltose, are absorbed by the scutellum (G), converted to sucrose and distributed to the growing seedling

points (pI). In wheat and barley most are members of either the low pI (4.5–5.5, AMY1) or high pI (5.9–6.9, AMY2) group, each being encoded on different chromosomes; fewer variants are present in maize, sorghum, rice or oats and most are in the low pI category. In barley there are ten α-amylase genes expressed in the aleurone layer, of which six are for the high pI forms; these genes are transcribed more and earlier in response to GA. It is likely that the different enzymes have different affinities to bind to granules and the released polymers during starch degradation due to variations in the structure of their carbohydrate-binding sites.

Because the de novo synthesis of α-amylase requires a ready supply of amino acids (Sect. 5.5.1), an early response to GA is the hydrolysis of storage proteins within the aleurone layer. Proteases are present within the PSVs of mature dry and early-imbibed cells, but they have little or no activity because the internal pH of this organelle is above optimal for these enzymes. A decline in pH from approx. 7 to 5 or less is then achieved by GA-induced active pumping of H^+ ions into the PSVs through their surrounding membrane (Fig. 5.9); this activates the proteases, thus resulting in the hydrolysis of the storage proteins and the release of amino acids to

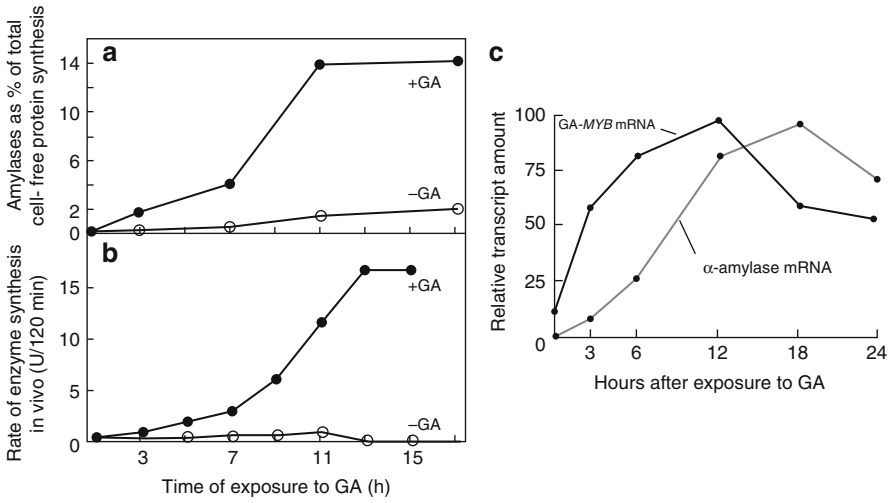


Fig. 5.8 (**a**) Increase with time in the amount of translatable transcripts for barley α-amylase in isolated aleurone layers incubated in water (–GA) or gibberellin (+GA). The mRNA for the enzyme was extracted from the aleurone layers at the various times indicated and its quantity measured as that supporting the synthesis of α-amylase in vitro. (**b**) Synthesis of the enzyme in vivo in response to GA. U: units of α-amylase activity. After Higgins et al. (1976). (**c**) Increase in GA-induced transcripts for GA-MYB precedes those for α-amylase, as part of the progression of events detailed in Fig. 5.10. After Gubler et al. (1995). Copyright American Society of Plant Biologists

the protein-synthesizing complex in the cytoplasm. Other hydrolases, e.g., lipases, nucleases and phosphatases also are activated.

The signalling pathway for GA (the active form being GA_1), from its reception at the surface of the aleurone layer cell to the transcription of the α-amylase genes, is shown in Fig. 5.10. GA_1 may be first detected by an appropriate receptor complex in the plasma membrane of the cell (step 1), which initiates two separate signal transduction pathways (step 2). Of these, the Ca^{2+}-independent pathway leads to the transcription of the genes for α-amylase (steps 3–10) and other GA-induced hydrolases, while the other promotes a two- to threefold increase in steady-state cytosolic Ca^{2+} concentrations (steps 11, 12), perhaps by its import from the apoplast; this cation is important for enzyme activation (see later).

In the Ca^{2+}-independent pathway GA_1 binds to a soluble GA receptor protein (GID1) in the nucleus (step 3), which causes a change in its configuration, facilitating its binding to a DELLA-GRAS protein complex (depicted in blue and yellow, respectively, in step 4). An F-box protein (F-box proteins contain an F-box domain of amino acids that encourages protein–protein interactions) enters the nucleus and binds to this complex, and this allows for the addition of several ubiquitin molecules (ubiquitination) to the GRAS protein (step 5). The DELLA-GRAS proteins incorporated into GID1 (step 4) come from the upstream promoter region of a gene for GA-MYB, where they form a repressor-protein complex preventing its

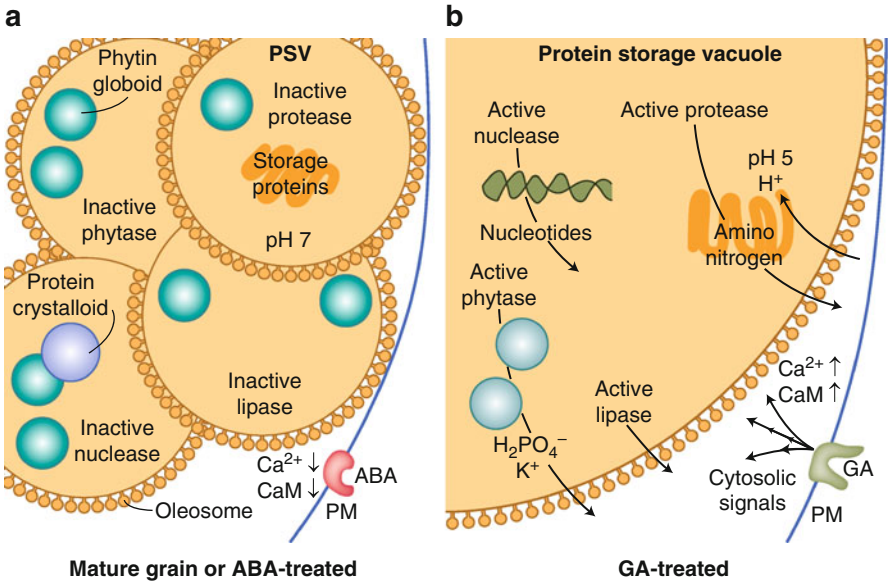


Fig. 5.9 A model for changes that occur in protein storage vacuoles (PSVs) of the barley aleurone layer following exposure to GA. (**a**) The inactive enzymes, and their substrates, in the mature aleurone layer cell (and those exposed to the inhibitor ABA) are depicted for convenience as being in separate vacuoles; in vivo they are together in the same PSVs. (**b**) The hormone is perceived at the plasma membrane (PM) surface of the cell, and cytosolic signals including Ca^{2+} and its binding protein calmodulin (CaM) promote coalescence of the PSVs, acidification of the vacuole lumen (the decline in pH being due to an influx of H^+ ions), activation of the hydrolytic enzymes and release of the degradation products into the cytosol. This model is based on studies using isolated protoplasts obtained by enzymatic removal of the cell walls of the aleurone layer. From Bethke et al. (1998). With permission of Oxford Univ. Press

transcription. DELLA proteins (so named because they all contain the conserved amino acid sequence DELLA: aspartic acid, glutamic acid, leucine, leucine, alanine) have been identified in many plant tissues as negative regulators of GA responses, by blocking the promoter region of GA-responsive genes. Their removal is necessary for GA to induce the promoter. This occurs when the ubiquinated DELLA-GRAS protein is targeted and degraded by a specific set of proteases present within a hydrolytic proteasome complex within the nucleus (step 6).

Thus, with the repressor protein complex removed, the *GA-MYB* gene can now be transcribed (step 7) (Fig. 5.8c) and its mRNA migrates to the protein synthesizing complexes in the cytosol where the GA-MYB transcription factor is translated, followed by its import into the nucleus. It now binds to a specific GA-response element (GARE) in the promoter region of α-*amylase* gene (step 8) and other GA-induced genes to effect their transcription (step 9). The transcripts leave the nucleus and are translated on endoplasmic-reticulum-associated polysomes; the resultant proteins enter into the lumen of this rough endoplasmic reticulum (RER) and are transported through the endomembrane system via the default pathway to the

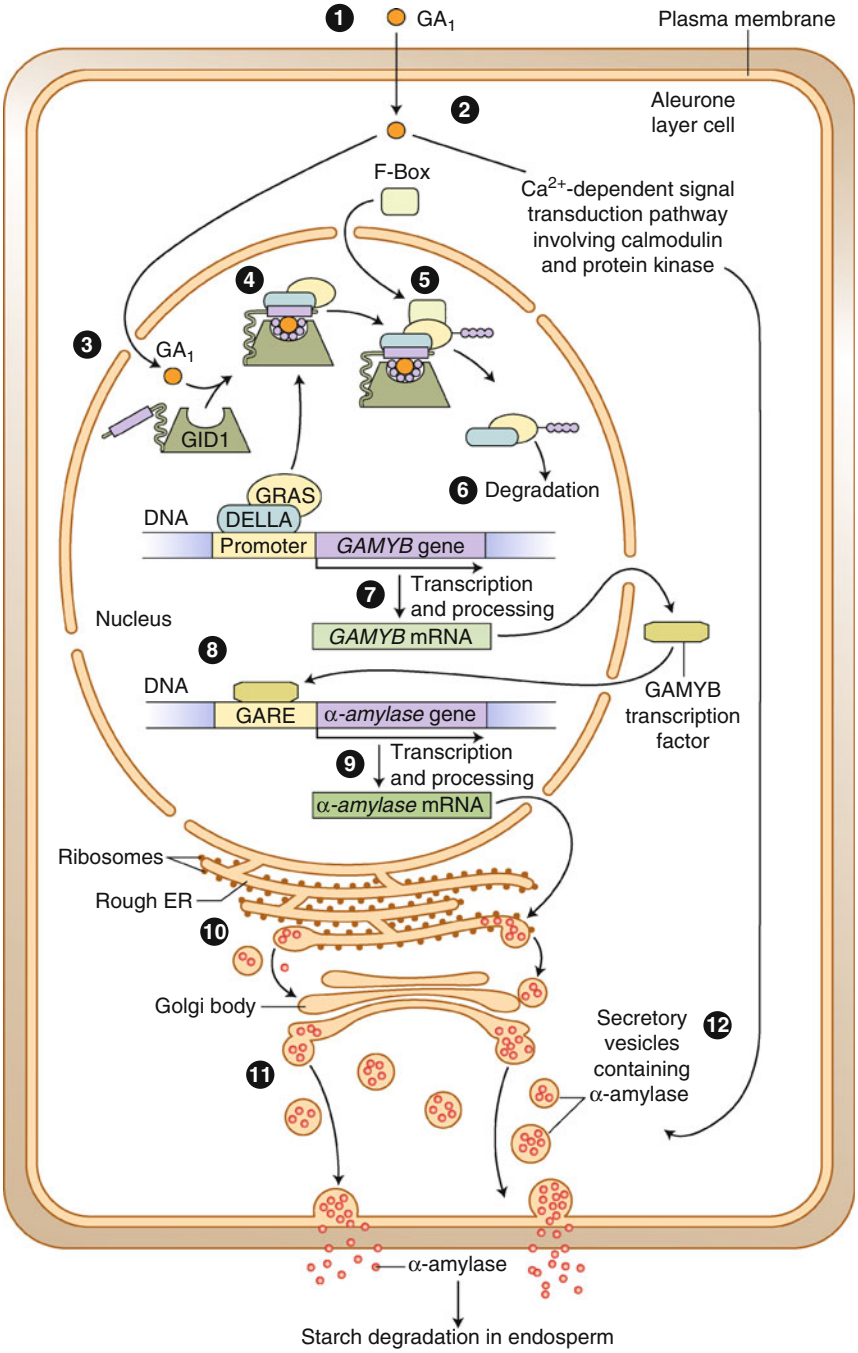


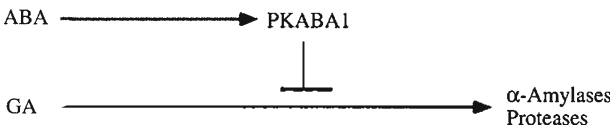
Fig. 5.10 Diagrammatic representation of the induction of α-amylase synthesis in a barley aleurone layer cell by GA (GA_1). A calcium-independent pathway (steps 1–11) induces the transcription of α-amylase (and other hydrolases) whereas activation and secretion of this enzyme requires a calcium-dependent pathway (step 12). The steps are explained in the text. From Taiz, L. and Zeiger, E., *Plant Physiology,* 5th Edition. Sinaur Associates, Sunderland, Mass., with permission

Golgi (step 10). As this occurs the enzyme proteins may undergo posttranslational modifications. The enzymes are packaged into secretory vesicles (step 11) that migrate to the plasma membrane, with which they fuse to release the enzymes from the aleurone layer cell. As noted in Sect. 5.5.1 the walls of the aleurone layer cells can impair the movement of α-amylase into the starchy endosperm; GA-induced synthesis and secretion of degrading pentosanases results in the formation of channels in the intervening walls, and their eventual total digestion.

The Ca^{2+}-dependent signal transduction pathway (step 12) plays an important role in the activation of α-amylase; the enzyme is a Ca^{2+}-containing metalloprotein that must bind this cation while in the lumen of the ER or Golgi in order to be active when secreted. A GA-stimulated Ca^{2+}-calmodulin (CaM)-dependent pathway also plays a role in ensuring secretion of the enzyme. In addition there are complex signal-transduction pathways involving Ca^{2+}-sensors such as CaM, and Ca^{2+}-activated protein kinases, as part of a second messenger complex coupled to hormone induction. These also play a role in the regulation of GA-induced cellular changes.

While the main focus of this section has been on the positive influence of GA on the induction of synthesis of α-amylase, this is suppressed in the presence of abscisic acid (ABA). For example, the acidification of the PSVs is prevented by ABA (Fig. 5.9a), and there is a suppression of transcription of genes for α-amylase and other hydrolases. Both hormones are synthesized by cereal embryos and diffuse to the aleurone layer; hence, it is likely that a balance in influence of the two is important in regulating the extent of hydrolase production and secretion. Whether or not such a balance influences α-amylase synthesis in the scutellum is unclear. In the aleurone layers of both barley and wheat there are numerous changes in transcript production under the influence of ABA of GA. Many more genes are up-regulated by ABA than are down-regulated, the number of the former being more or less equal to the number up-regulated by GA; but a larger number are down-regulated by ABA. This points to a complex interaction between these antagonists at the genome-, and consequently the cellular/metabolic-level.

The activity of ABA in barley aleurone layer cells involves synthesis of an ABA-induced protein kinase (PKABA1), which acts as a suppressor at some point along the signal transduction pathway for GA. Hence, as a result of this cross-talk at the intersection of the two hormonal pathways, there is inhibition of GA-induced expression of the genes for low and high pI α-amylases and the proteases responsible for mobilization of the storage proteins in the starchy endosperm, as simplified below:



In reality, this interaction is considerably more complex, and involves several proteins including transcription factors that are negative regulators of GA signalling, and regulatory proteins such as those of the 14-3-3 class (a conserved family

of proteins that bind to diverse signalling proteins, including kinases and transmembrane receptors). Information obtained from Arabidopsis indicates that PKABA1 itself needs to be phosphorylated to be active. This key phosphorylation event is performed by a kinase involved in ABA signal transduction, such as SNF1-related protein kinase 2 (SnRK2). However, this kinase is suppressed by a phosphatase, such as ABI1 and ABI2, in the absence of ABA. This suppression of the kinase by the phosphatase is eliminated upon the perception of ABA by an ABA receptor, such as PYR1 (PYRABACTIN RESISTANCE1), because the receptor protein inactivates the phosphatase and activates the SnRK2 and PKABA1. This scheme is also very important in the regulation of germination by ABA (Sect. 6.6.1.1).

5.5.4 Programmed Cell Death of the Aleurone Layer and Other Tissues

Upon completion of mobilization of the reserves from the starchy endosperm of cereal grains the aleurone layer undergoes PCD, as does the scutellum. As a result there is autolysis of their cells from which nutrients are mobilized and transferred to the growing embryo. PCD of the aleurone layer commences in the cells nearest to the embryo and then extends to the more proximal ones. The demise of the aleurone layer is stimulated by GA, but is considerably delayed or prevented by ABA. Nitric oxide (NO), which is synthesized from NO_2^- in the apoplast of aleurone layer cells, can also delay the onset of PCD by acting as an antioxidant.

Cell death is as a result of oxidative stress, which is stimulated by GA in two ways: (1) It promotes the breakdown of oils stored in the aleurone layer and during their conversion to sugars. β-Oxidation of the fatty acids (Sect. 5.7) in the glyoxysome releases hydrogen peroxide, a reactive oxygen species (ROS) (Sect. 8.4.1) that inflicts damage on macromolecules. Additional ROS are produced in the mitochondria. (2) It suppresses the expression of genes for enzymes that are able to defend the cells against the ROS attack, e.g., superoxide dismutase, catalase and ascorbate peroxidase. Death of the cells occurs when they become highly vacuolated accompanied by a loss of plasma membrane integrity; this results in loss of turgor and cytoplasmic collapse. In contrast, ABA maintains or promotes high expression of the genes for the defensive enzymes, prevents the hydrolysis of stored oils and stimulates mitochondria to minimize ROS production. In the aleurone layers of barley and wheat, in the presence of GA there is also an accumulation of nucleus-located nucleases late during PCD; DNA is not degraded in GA-insensitive mutants (wheat) or when ABA is present (barley).

PCD is a common phenomenon in plants, involved in events from cell differentiation to senescence. With respect to seed initiation and development it is operative, for example, during megaspore determination in the embryo sac, in the release of pollen from the anthers, in controlling the death of the nucellus and the suspensor during embryogenesis. It also causes the loss of metabolic integrity of the cells of

the starchy endosperm of cereals so that it is nonliving at maturity, of the endosperms of castor bean and tomato as they become depleted of reserves following germination, and likewise in the expended cotyledons of germinated dicot seeds.

The regulation of PCD in the developing cereal endosperm is tightly controlled, so that there is the completion of at least most of the synthesis of the storage reserves before entry into the cell death program. This appears to be influenced by the hormones ABA and ethylene, the former delaying the program, and the latter accelerating it; therefore, progression of PCD could be regulated by ABA through its effect on ethylene synthesis. Why the aleurone layer, the only region of the endosperm that remains living in the mature grain, is immune from ethylene-induced PCD during late development is unknown; this hormone does not appear to play a role in its post-germination PCD either.

See also Sect. 5.8.3 for information on PCD in reserve tissues of dicots.

5.6 Mobilization of Stored Carbohydrate Reserves in Dicots

In contrast to the large amount of research on triacylglycerol (TAG) mobilization in dicots (Sect. 5.7), there have been relatively few studies on starch utilization, and mostly in legumes. In non-endospermic legumes the endosperm is broken down as a source of nutrients during seed development, being either residual in (e.g., soybean), or absent from (e.g., peas, *Phaseolus* bean) the mature seed; the cotyledons assume the role as the major storage organ. These may contain predominantly starch or TAGs. Endospermic legumes of the tribe Trifolieae retain a substantial endosperm at maturity (e.g., fenugreek, carob, guar) and it becomes the site of stored carbohydrate reserves, as hemicelluloses, whereas the cotyledons are the site of storage proteins.

The regulation of dicot reserve mobilization is discussed as a separate Sect. 5.10 following individual accounts of the mobilization of each of the reserves because there is only a limited amount of information on any one of them.

5.6.1 Starch-Storing Non-endospermic Legumes

Hydrolysis of starch reserves in the cotyledons commences after germination is completed. Their depletion in pea cotyledons is biphasic, an initial slow rate, during which starch phosphorylase is the dominant hydrolase, being followed by a more rapid starch loss as activity of amylases (probably both α- and β-) increases (Fig. 5.11a, b). To what extent the phosphorylase can attack the native starch granule is unknown; a role in the degradation of soluble glucans released by amylolytic attack, achieved by the relatively low amount of amylase activity at this time, is more likely. Mobilization is aided by disintegration of the amyloplast membrane, exposing the starch to cytosolic enzymes, which appear to include a limit dextrinase

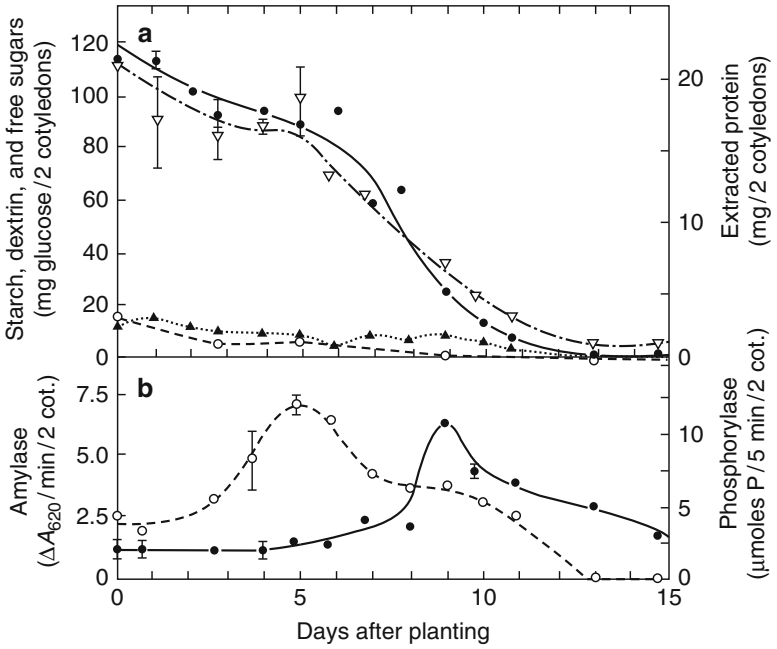


Fig. 5.11 (**a**) Changes in the amount of starch and dextrin (●), oligosaccharides (○), free sugars (▲), and extracted protein (▽) in the cotyledons of pea, cv. Early Alaska. (**b**) Changes in starch phosphorylase (○) and amylase (●) activities. After Juliano and Varner (1969)

and the phosphorylase. Conversion of released malto-oligosaccharides by the latter enzyme to Glc-1-P likely occurs in the cytosol, and this in turn is converted to sucrose and exported to the growing seedling; free sugars and dextrins do not accumulate in the cotyledons (Fig. 5.11a). In studies of amylolysis of starch in chickpea, mung bean and *Phaseolus* bean seeds, increases in α-amylase activity in the cotyledons have been reported following germination. Starch mobilization in black gram seeds is purported to involve the import of the starch granules into lytic vacuoles, formed from protein storage vacuoles, for hydrolysis (Sect. 5.8.3).

5.6.2 Hemicellulose-Storing Endospermic Legumes

In many species of the Trifolieae a well-developed endosperm, with thick-walled cells containing the storage carbohydrate galactomannan, lies between the seed coat and the cotyledons. In fenugreek extensive deposition of this hemicellulose polymer to the inside of the primary walls during seed development results in the gradual occlusion of the living contents until in the mature seed the cells are dead (Sect. 3.2.2). The outermost region of the endosperm is the aleurone layer that is made up of a unilayer of living thin-walled cells devoid of galactomannan (Fig. 5.12a).

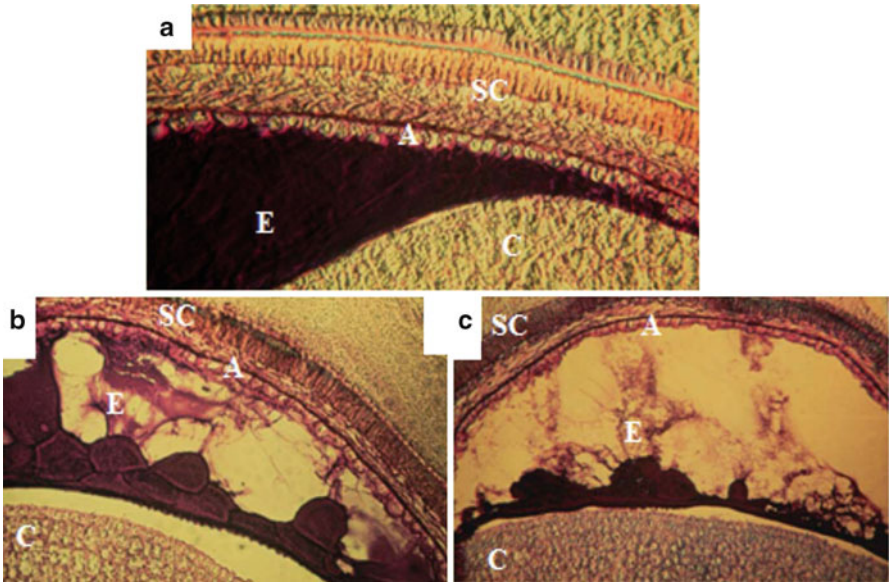


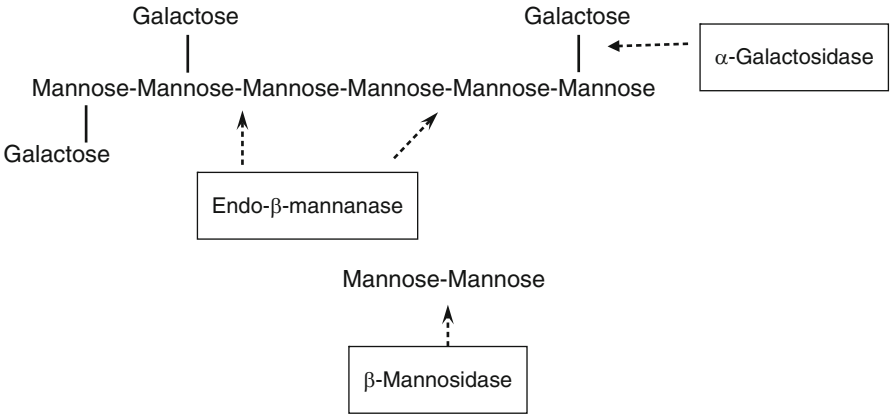
Fig. 5.12 Light micrographs of the outer region of a fenugreek seed in the lateral endosperm region: (**a**) During germination and before mobilization of the endosperm reserves. The three-layered seed coat (SC), a small part of the cotyledon (C), and the endosperm layer (A and E) are shown. The aleurone layer (A) is the outer living single-cell layer of the endosperm, the rest (E) being composed of large cells with thin primary walls to the inside of which is deposited the dark-staining galactomannan secondary cell wall that appears to completely fill the cell. (**b**) Following germination, when the galactomannan-rich cell walls in the endosperm are being dissolved. The dissolution zones (clear regions in the endosperm) begin at the aleurone layer, the source of hydrolytic enzymes, and spread toward the cotyledons. (**c**) The endosperm is almost depleted and only a remnant remains between the seed coat and the cotyledon. The aleurone layer is still present, but will soon disintegrate. Starch granules (stained *blue*) are present in the cells of the cotyledon. Courtesy of J.S.G. Reid, Univ. Stirling. For original micrographs see Reid, J. S. G. 1971. *Planta* 100, 131–142

In most endospermic legumes, however, the endosperm cell walls do not completely occlude the cytoplasm, and all cells have living contents at maturity (e.g., Chinese senna, Fig. 1.4).

In fenugreek seeds, and perhaps those of other endospermic legumes also, the endosperm plays a role in addition to that as a carbohydrate storage reserve. The high affinity of galactomannans for water (when imbibed, many become mucilaginous) allows the endosperm to regulate the water balance of the embryo during germination; this may be important to plants in their native habitat, since many members of the tribe Trifolieae have their origins in the dry regions of the eastern Mediterranean.

After emergence of the radicle, the galactomannan in the endosperm begins to be mobilized. There is a wave of hydrolysis in the fenugreek seed commencing close to the aleurone layer and moving toward the cotyledons (Fig. 5.12b) until the reserves are depleted (Fig. 5.12c). This is due to the synthesis and release from this

layer of three critical enzymes: α-galactosidase, β-mannosidase (exo-β-mannanase), and endo-β-mannanase. α-Galactosidase is an exopolysaccharidase that cleaves the α-(1→6) link between the unit Gal side chains and the Man backbone.



Endo-β-mannanase is an endoenzyme that hydrolyzes oligomers of Man (tetramers or larger) to mannobiose or mannotriose, and β-mannosidase then converts these to Man. The latter enzyme might also act as an exo-mannopolysaccharidase and hydrolyze single Man residues from the oligomannan chain. Mannan breakdown by phosphorolysis appears not to occur.

The released Gal and Man are absorbed by the cotyledons, Gal by passive diffusion, but Man requires active uptake utilizing a carrier-specific component. Neither sugar accumulates in the cotyledons, but instead they are metabolized further, perhaps by initially being phosphorylated to Gal-1-P and Man-6-P. If not used directly for energy metabolism, they are transformed to sucrose and then to starch, which is remobilized when the sucrose content of the cotyledons falls after its transport to the axis. This sequestering of sugars as a large polymer is a convenient strategy for the removal of potentially osmotically damaging monomers, and for the retention of useful metabolites. Not surprisingly, an increase in α-amylase activity within the cotyledons coincides with starch hydrolysis. A summary of the events involved in galactomannan breakdown in endospermic legumes is shown in Fig. 5.13. However, not all the enzymes required for the conversion of Man to sucrose and starch have been located within the cotyledons, although it is reasonable to assume that they are there.

Arabinogalactans are present in the thickened cell walls of lupin cotyledons, composed of β(1→4)-linked Gal residues with α(1→5)-arabinose side chains; these hemicelluloses are degraded at the same time as the stored reserves (proteins and oils) within the cells are mobilized. During cell wall utilization there is a transient increase in starch in the cotyledons, as in fenugreek, presumably because cell wall mobilization outstrips the ability of the cotyledons to export sucrose, the final product of arabinose and Gal conversion, to the growing axis. Cotyledons of seeds of the tropical legume tree *Hymenaea courbaril* contain xyloglucans in their cell walls as storage polysaccharides. During their mobilization there is an increase in

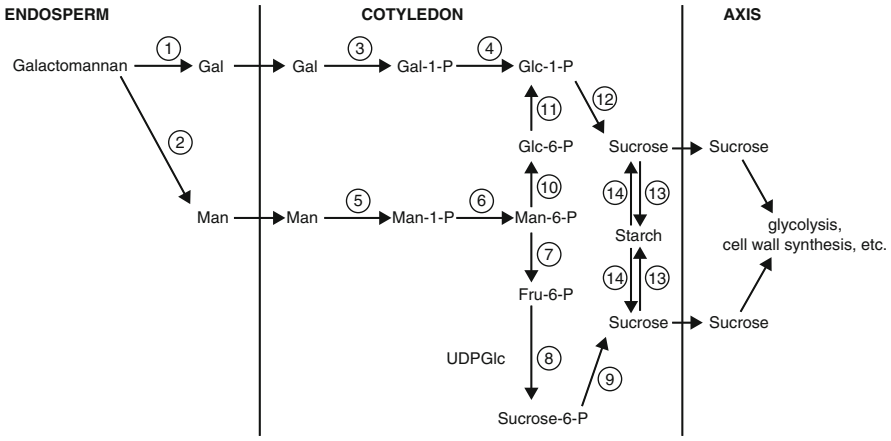


Fig. 5.13 Flow diagram to illustrate the potential fate of the products of galactomannan mobilization in endospermic legumes. Enzymes: (1) α-galactosidase; (2) endo-β-mannanase and β-mannosidase; (3) galactokinase; (4) hexose phosphate uridyl transferase (a group of three enzymes that convert Gal-1-P + UTP → UDPGal → UDPGlc → Glc-1-P + UTP); (5) mannokinase; (6) phosphomannomutase; (7) phosphomannoisomerase; (8) sucrose-6-P synthetase; (9) sucrose phosphatase; (10) C_2 epimerase; (11) phosphoglucomutase; (12) sucrose synthase or sucrose-6-P synthase (see Sect. 5.4.1); (13) see Sect. 3.2.1; (14) see Sects. 5.4, 5.4.1. Gal, galactose; Man, mannose; Glc, glucose; Fru, fructose

xyloglucan hydrolases, free sugars, and a transient increase in starch in the cotyledons when transport of the sugars is slower than the rate of their production.

5.6.3 Hemicellulose-Containing Seeds Other than Legumes

A number of nonleguminous plants also store mannans, although few have received much attention as far as mobilization of their reserves is concerned. The role of hormones in the mobilization of cell wall galactomannans in tomato and lettuce seeds is detailed in Sect. 5.10.1.

Hydrolysis of polysaccharides in the endosperm of date palm (89% Man deposited in the secondary walls; much of the rest is cellulose) occurs when a haustorial projection from the hypogeal seedling (Sect. 5.1) grows into it. This results in preformed hydrolytic enzymes being released from protein storage vacuoles in the endosperm, which come into contact with the wall following loss of outer membrane integrity. The galactomannan is degraded to its constituent monomers, which are absorbed by the haustorium and transported to the growing axis; there they are converted to sucrose.

Mobilization of galactomannans from the cell walls of the lettuce seed endosperm commences when endo-β-mannanase activity increases within the endosperm itself, immediately after germination is completed. α-Galactosidase is

present as a constitutive enzyme within the endosperm. The products of hydrolysis diffuse to the cotyledons, and small oligomannans are cleaved further by β-mannosidase located in their cell walls; the resultant Man residues are taken up by the cotyledon cells (summarized in Fig. 5.30). The breakdown of galactomannans within the endosperm of tomato also requires the synthesis of two isozymes of endo-β-mannanase, one in the micropylar endosperm, which is involved in the completion of germination (Sect. 4.6.1), and one in the lateral endosperm that mobilizes the cell walls following germination.

While the walls of the hard endosperm in seeds such as of coffee are very thick, and may account for over 50% of the cell volume, there is still some cytoplasm present in the mature cells, and they are capable of producing the appropriate hydrolases; there is no peripheral aleurone layer. Initially in the imbibed coffee seed there is the synthesis and expression of transcripts for endo-β-mannanase and β-mannosidase in the micropylar region of the endosperm during germination, presumably to facilitate radicle emergence, and later endo-β-mannanase in particular increases in the lateral endosperm as this major area of mannan reserves is mobilized. Following germination the cotyledons remain embedded in the endosperm until it is depleted and emerged above the soil, thus allowing for continued import of cell-wall-derived sugars into the growing seedling.

Some seeds store hemicelluloses other than mannans, and degrade them as a carbohydrate source following germination. The cell walls of nasturtium cotyledons contain "amyloids" that stain with iodine in a starch-like reaction. However, the walls are composed of (galacto)xyloglucans; these are degraded initially by xyloglucan endotransglycosylase and β-galactosidase to form oligomers, which are then converted to free monosaccharides by α-xylosidase and β-glucosidase; of these, the first three enzymes increase in activity in the cotyledons following germination, but the glucosidase is present in the dry seed and remains constant in activity following imbibition.

5.7 Stored Triacylglycerol Mobilization

Seed oil- (TAG) catabolism, like that of its synthesis involves many enzymes located in several organelles within the storage cell. An overview diagram is shown in Fig. 5.14. Initial TAG hydrolysis (lipolysis) is by lipases, enzymes that catalyze the three-stage hydrolytic cleavage of the fatty acid ester bonds, ultimately to yield glycerol and free fatty acids (FFAs). The latter enter the peroxisome (often called the glyoxysome in seeds) for conversion to oxaloacetic acid (OAA), which then passes into the mitochondrion, and finally into the cytosol for conversion to sucrose, the sugar that is transported from the storage cotyledons to the growing regions of the seedling, or from the storage endosperm to the cotyledons and thence throughout the seedling.

In more detail (Fig. 5.15), the FFAs released by lipases (step 1) are utilized in oxidation reactions in the glyoxysome to yield compounds containing fewer carbon

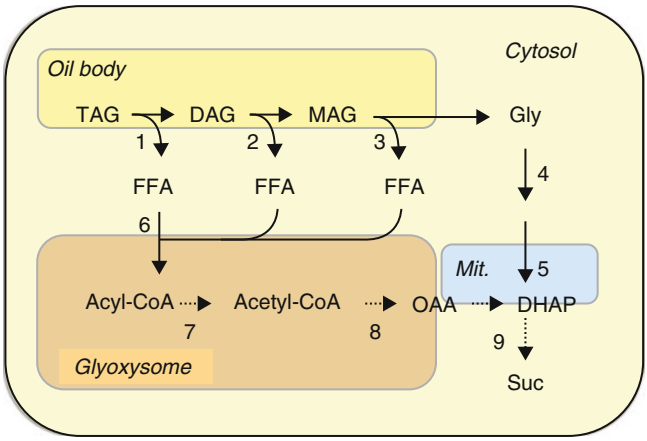


Fig. 5.14 Generalized schematic of triacylglycerol (TAG) mobilization in reserve tissues of seeds following germination. The TAGs in the oil body are hydrolyzed to free fatty acids (FFA) and glycerol (Gly), through the diacylglycerol (DAG) and monoacylglycerol (MAG) forms, possibly by the sequential action of several lipases (steps 1–3). Gly is converted to dihydroxyacetone phosphate (DHAP) by glycerol kinase (4) and Gly-3-P dehydrogenase (5). FFAs are transported to the glyoxysome and activated to acyl-CoAs (6) and enter the β-oxidation spiral (7). The acetyl-CoA product is converted to organic acids by the glyoxylate cycle and subsequent steps result in products such as oxaloacetic acid (OAA) in the mitochondrion (Mit) and the cytosol (8), which along with DHAP is converted by gluconeogenesis (9) to sucrose (Suc)

atoms. The predominant oxidation pathway is β-oxidation, in which the FFA is first esterified with coenzyme A (CoA) in a reaction requiring ATP, and then, by a series of steps involving the successive removal of two carbon atoms this acyl CoA is broken down to acetyl CoA (steps 2–6). This requires that the enzymes in each step of β-oxidation sequentially accept substrates that are progressively 2C shorter in length; thus they either have multiple isoforms with different chain-length specificities, or they have broad substrate specificity.

Saturated fatty acids with an even number of carbon atoms yield only acetyl CoA. Chains containing an odd number of carbon atoms, if completely degraded by β-oxidation, will yield the two-carbon acetyl moieties (acetyl CoA) and one three-carbon propionyl moiety (propionyl CoA, CH_3CH_2CO-S-CoA). This, in turn, can be degraded in a multistep process to acetyl CoA. The acetyl moiety may be completely oxidized in the citric acid cycle to CO_2 and H_2O or utilized initially via the glyoxylate cycle for carbohydrate synthesis (steps 8–11). This latter process is the most important during seedling establishment.

The oxidation of unsaturated fatty acids (e.g., oleic acid; 18:1Δ9*cis*) is by the same general pathways, although some extra steps are required. The double bonds of naturally occurring unsaturated fatty acids may be in the *cis* configuration,

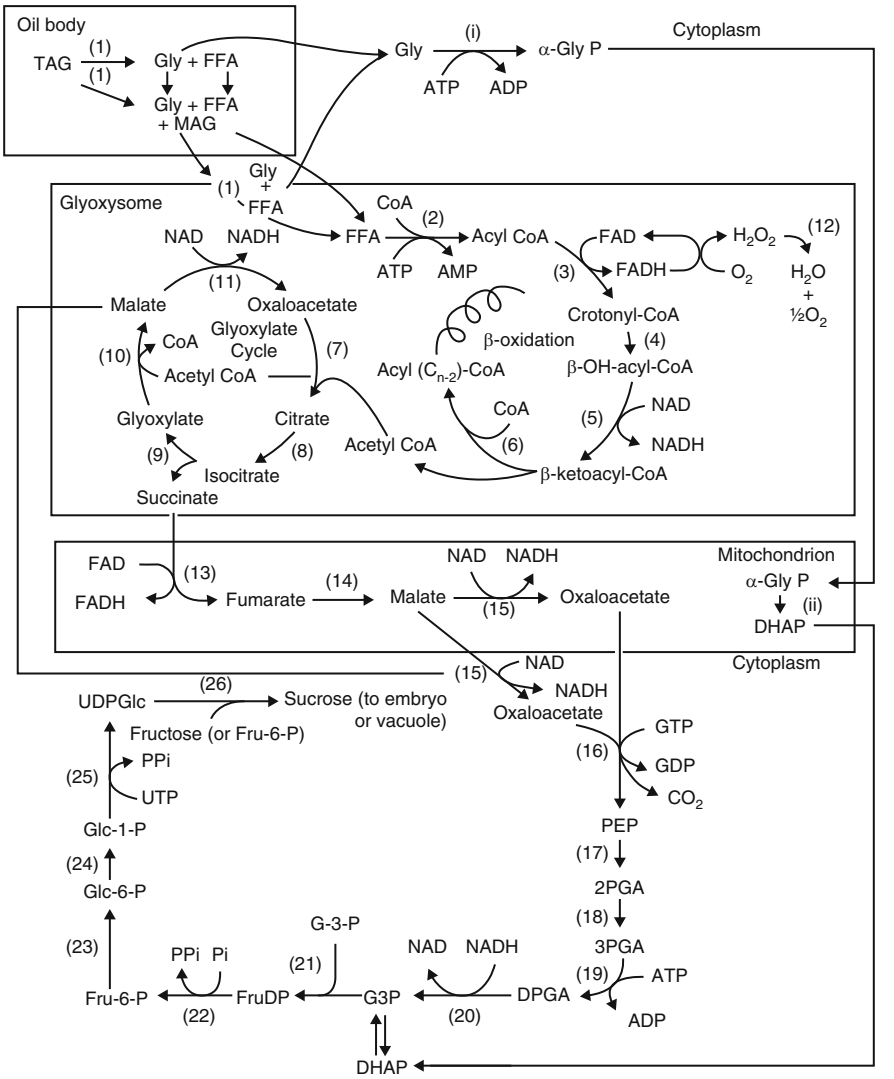
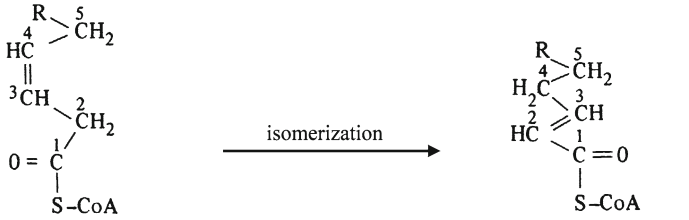


Fig. 5.15 Detailed pathways of TAG catabolism and sucrose synthesis. Enzymes: (1) lipases, e.g., SDP1; (2) fatty acid thiokinase; (3) acyl CoA dehydrogenase; (4) enoyl CoA hydratase (crotonase); (5) β-hydroxyacyl CoA dehydrogenase; (6) β-ketoacyl thiolase; (7) citrate synthase; (8) aconitase*; (9) isocitrate lyase; (10) malate synthase; (11) malate dehydrogenase**; (12) catalase; (13) succinate dehydrogenase; (14) fumarase; (15) malate dehydrogenase; (16) phosphoenolpyruvate carboxykinase; (17) enolase; (18) phosphoglycerate mutase; (19) phosphoglycerate kinase; (20) glyceraldehyde-3-phosphate dehydrogenase; (21) aldolase; (22) fructose-1,6-bisphosphatase; (23) phosphohexoisomerase; (24) phosphoglucomutase; (25) UDPG1c pyrophosphorylase; (26) sucrose synthase or sucrose-6-P synthase and sucrose phosphatase. (i) Glycerol kinase; (ii) α-glycerol phosphate oxidoreductase. Substrates: TAG, triacylglycerol; MAG, monoacylglycerol; Gly, glycerol; FFA, free fatty acid; PEP, phosphoenolpyruvate; 2PGA, 2-phosphoglyceric acid; 3PGA, 3-phosphoglyceric acid; DPGA, 1,3-diphosphoglyceric acid; G3P, glyceraldehyde-3-phosphate; FruDP, fructose-1,6-bisphosphate; Fru-6-P, fructose-6-phosphate; Glc-6-P, glucose-6-phosphate;

which is a block to β-oxidation. Thus, for step 4 to occur, they must be in the *trans* position. Hence, in a reaction involving at least four enzymes (three isomerases and a reductase) the FFA is converted to its oxidizable form:



$\Delta^{3,4}$ *cis* Enoyl CoA $\Delta^{2,3}$ *trans* Enoyl CoA

which is the normal substrate for the next enzyme in the β-oxidation pathway, enoyl CoA hydratase (step 4). Polyunsaturated fatty acids containing two or more double bonds (e.g., linoleic acid, 18:2; linolenic acid, 18:3) cannot be degraded simply by β-oxidation either, but the appropriate enzymes (2,3 enoyl CoA isomerase, 3-OH acyl CoA epimerase and 2,4 dienoyl CoA reductase) that are required for the continuation of β-oxidation are present within the glyoxysome. For β-oxidation of ricinoleic acid (12-OH 18:1), the C_8-intermediate (2-hydroxy 8:0) fatty acid requires conversion by an α-hydroxy acid oxidase and oxidative decarboxylation to circumvent the metabolic barrier caused by the hydroxyl group. The heptanoyl CoA so formed can be catabolized further by β-oxidation. Analyses of over 7,000 plant species has revealed that there are hundreds of different minor fatty acids in seed oils, some of which are family-, genus-, or even species-specific; their variation in structure and substitutions is extensive, but presumably for each there is an appropriate enzyme or enzymes in the glyoxysome to ensure that they are efficiently catabolized.

A by-product of β-oxidation is hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) that is damaging to macromolecules such as proteins and nucleic acids. This is broken down in the glyoxysome to water and molecular oxygen by catalase (step 12). In addition, there is a glyoxysome-membrane-bound H_2O_2-eliminating set of enzymes.

Directly coupled to the β-oxidation pathway is the glyoxylate cycle, which takes the acetyl CoA and, in a series of enzymatic reactions, links this to the glycolytic pathway, which then operates to produce hexose. The key enzymes for forging this link are malate synthase (MLS) and isocitrate lyase (ICL), which are unique to the

Fig. 5.15 (continued) Glc-1-P, glucose-1-phosphate; UDPG1c, uridine diphosphoglucose; α-Gly P, α-glycerol phosphate; DHAP, dihydroxyacetone phosphate. Coenzymes and energy suppliers: FAD/(H), flavin adenine dinucleotide/(reduced); NAD/(H), nicotinamide adenine dinucleotide/(reduced); GTP, guanosine triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate; GDP, guanosine diphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; CoA, coenzyme A. *located in the cytosol; ** present in the glyoxysome and cytosol, but in the latter location is part of the glyoxylate cycle

glyoxylate cycle. Acetyl CoA is first converted to citrate (in the same manner as initiates its entry into the citric acid cycle: step 7), then to isocitrate, which is cleaved to produce succinate and glyoxylate. Another acetyl CoA is incorporated into the cycle (step 10) and is condensed with glyoxylate by MLS to yield malate. With each turn of the cycle one molecule of succinate is released (step 9) and is converted to oxaloacetate by citric acid enzymes in the mitochondria (steps 13–15), and then into the glycolysis pathway as phosphoenolpyruvate (step 16) for conversion to sucrose. For simplicity, the location of all glyoxylate cycle enzymes in Fig. 5.15 is depicted as being within the glyoxysome; however, two of the five enzymes involved, aconitase (step 8) and malate dehydrogenase (step 11) are present in the cytosol (although the recycling of NADH to NAD occurs within the organelle). This requires that there be present in the glyoxysome membrane efficient shuttling mechanisms so that intermediates in the cycle can readily pass into the cytosol and back again.

An important enzyme in the completion of gluconeogenesis is a pyrophosphatase (V-H^+PPase), which is located in the vacuolar membrane and converts cytosolic pyrophosphate produced during steps 22 and 25 to phosphate (PPi to Pi). A mutant (*fugu5*) of Arabidopsis lacking this enzyme exhibits poor seedling establishment due to a decrease in sucrose synthesis in the cotyledons, where the oil is stored, although it can complete glyoxysome-related steps beyond β-oxidation. The cytosolic accumulation of PPi likely results in a feedback reaction suppressing steps 22 and 25, thus interfering with the completion of gluconeogenesis and resulting in a poorer supply of sucrose essential for seedling growth.

Glycerol, produced when the TAG is stripped of its fatty acids, enters the glycolytic pathway after its phosphorylation by glycerol kinase in the cytosol and is oxidized in the mitochondrion to the triose phosphate dihydroxyacetone phosphate. This is released into the cytosol and after conversion to glyceraldehyde-3-phosphate (G-3-P) is condensed by aldolase to another G-3-P in the reversal of glycolysis to yield hexose units (step 21), and ultimately sucrose (step 26). Alternatively, the triose phosphates may be converted to pyruvate and then oxidized through the citric acid cycle in the mitochondrion.

5.7.1 Mobilization of TAGs from Oil Bodies

While more research has been conducted on mobilization in the cotyledons and endosperms of oil-storing dicot seeds, the pattern of TAG utilization is similar in many ways in cereal grains (where the oil is mostly in the scutellum) and in gymnosperm megagametophytes.

Lipases are usually only detected following germination and are located in the oil body membrane as well as that of glyoxysomes; the close association between these organelles (Sect. 5.7.2) is consistent with an interaction between them to ensure lipolysis and the transfer from the former to the latter of FFAs for further modifications. Several lipases, with different pH optima, have been identified in oil-storing seeds, and their genes cloned, but in many instances their importance in the

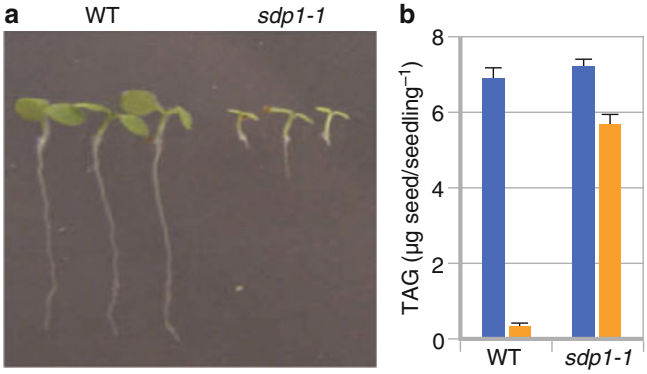


Fig. 5.16 (**a**) Phenotypic differences between 5-day-old seedlings of wild-type Arabidopsis and a lipase-deficient mutant *sdp1-1* (*sugar-dependent1-1*) showing poor growth of the latter. (**b**) Triacylglycerol (TAG) content of wild-type (WT) and mutant at 0 (*blue bars*) and 5 days (*orange bars*) from the start of imbibition; the failure to mobilize most of the storage oil results in poor growth of the seedling. After Quettier and Eastmond (2009). Courtesy of Elsevier

release of FFAs from TAGs has not been established. However, the Arabidopsis mutant *sdp1* (*sugar-dependent1*) is defective in a lipase associated with the oil body membrane, and has considerably less ability to mobilize TAGs in the cotyledons, resulting in seedlings that exhibit poor growth compared to those of the wild type (Fig. 5.16a, b). The SDP1 enzyme likely initiates TAG mobilization in this species; because it shows preference for TAGs over DAGs or MAGs, other lipases may well be involved in completing lipolysis. The FFAs are imported into the glyoxysome by special membrane-associated transporters.

5.7.2 Role and Formation of the Glyoxysome

The overall process of conversion of FFA to glucose is termed gluconeogenesis, by definition the production of this sugar from a non-carbohydrate source. The FFAs are provided from the oil bodies, and subsequent steps require the participation of glyoxysomes and mitochondria, with the final synthesis of glucose; the subsequent formation of the transport sugar sucrose occurs in the cytosol. Consult Sect. 5.7 for details. Because of the metabolic collaboration between the three organelles it is not surprising to find them in juxtaposition within the cell (Fig. 5.17).

Glyoxysomes are a special class of peroxisomes (previously referred to as microbodies) that contain all of the enzymes of the β-oxidation spiral, and also the glyoxylate cycle with the unique enzymes ICL and MLS. The glyoxylate cycle resembles the citric acid cycle, except that the decarboxylation steps between isocitrate and succinate are circumvented by the action of ICL, thus avoiding the loss of carbon as CO_2. Like all peroxisomes they do not contain nuclear material or a protein-synthesizing complex; they are bounded by a single membrane and are

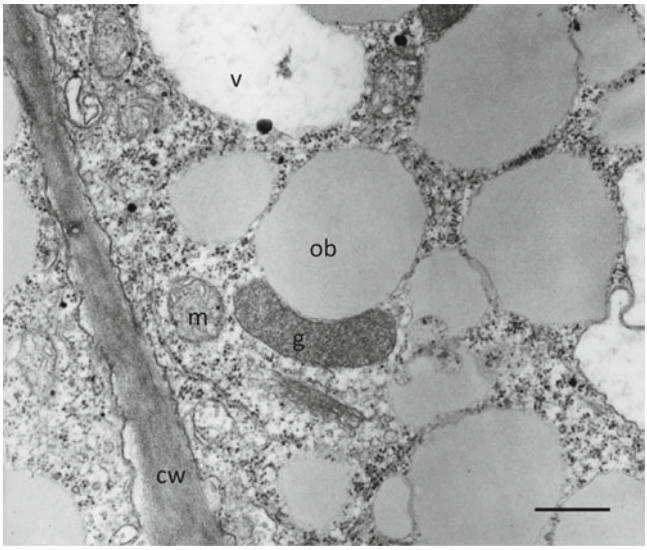


Fig. 5.17 Electron micrograph of an oil-storing cell in the cotyledon of a dark-grown cucumber seedling. The glyoxysome (g) is in close proximity to the oil body (ob) and mitochondrion (m). The vacuole (v) and cell wall (cw) are also marked. Bar 0.5 μm. Courtesy of R.N. Trelease, Arizona State Univ. and R.T. Mullen, Univ. Guelph

slightly denser than mitochondria. Some are formed during mid- to late- stages of development in the oil-storing cells, and in mature seeds they are small, with a diameter of ~0.2 μm and while they contain some of the component enzymes, these are insufficient in amount. By the time the glyoxysomes are fully active in the processing of FFAs in the germinated seed they have become 10–20 times larger and all of the enzymes are appropriately present; also, new organelles are formed. For this to be achieved there must be an import of new materials into their membranes, and of enzymes into the matrix.

A general, but simplified model for glyoxysome biogenesis follows. During seed development, in cells of oil-storage tissues, there is the formation of distensions in specific regions of the endoplasmic reticulum (peroxisomal ER, pER) where there is insertion of a certain subset of glyoxysomal membrane proteins, all of which are synthesized on free cytosolic polysomes (Fig. 5.18a). The resultant pre-glyoxysomal vesicles are stable in the mature dry seed, and following germination more are produced in a similar manner. Their enlargement to form mature glyoxysomes occurs by the addition (posttranslationally) of nascent matrix proteins, and other membrane proteins, followed by, or concomitant with, their fusion with other newly formed pre-glyoxysomal vesicles; fusion can also occur with preexisting mature glyoxysomes.

The mature glyoxysomes themselves may divide to form incomplete daughter glyoxysomes; this involves first their elongation (the membrane material for this being ER/pER-derived), then constriction in defined regions, followed by fission to form new organelles (Fig. 5.18b). As already indicated, there are two potential routes

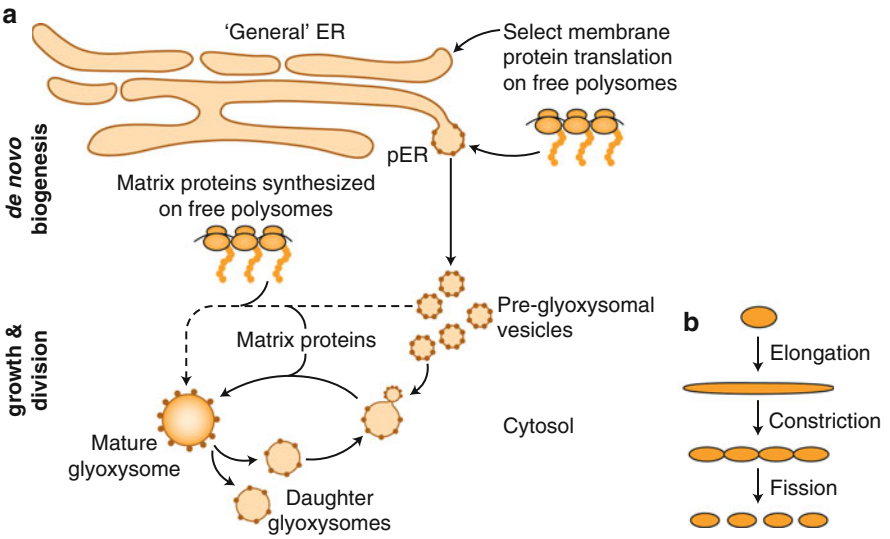


Fig. 5.18 (**a**) Schematic representation of the steps involved in the biogenesis of glyoxysomes. Pre-glyoxysomal vesicles containing certain membrane proteins are budded off from a specific region of the ER (peroxisomal ER, pER). Select membrane proteins are synthesized in the cytosol on free polysomes and imported into the pER. Alternatively, membrane proteins may be imported into the ER at sites other than the pER ("general" ER) and migrate to this region. The released pre-glyoxysomal vesicles increase in size by fusion with preexisting immature glyoxysomes, or with daughter glyoxysomes formed by fission of mature glyoxysomes. As the glyoxysomes are increasing in size, matrix proteins and additional membrane proteins are imported from free polysomes within the cytosol. Based on information in Ma et al. (2011). Also see diagrams in Mullen, R.T. and Trelease, R.N., *Biochim. Biophys. Acta* 1763, 1655–1668 (2006). (**b**, *inset*) Model to explain glyoxysome proliferation. This occurs through sequential elongation, constriction and fission. The steps require the association of several proteins with the membrane at each stage, e.g., peroxins (PEX proteins) for elongation, and dynamin-related proteins (DRP-family proteins) for fission. After Kaur and Hu (2009). Courtesy of Elsevier

by which the necessary membrane proteins are added to the pre-glyoxysome as it grows. In both cases the proteins are synthesized on free polysomes within the cytosol: one route has them inserted directly into the pre- or daughter glyoxysome, and the other is for them to be inserted in the general ER and/or pER, from where nascent pre-glyoxysome vesicles bud and fuse with a daughter glyoxysome.

Similarly to the proteins incorporated into the glyoxysome membrane, those destined for the matrix or interior of the organelle are nuclear encoded, synthesized on free cytosolic ribosomes, and targeted posttranslationally. One of at least two types of conserved amino acid sequences is present on proteins destined for transport into the glyoxysome; these are the type 1 and type 2 peroxisomal targeting signals (PTSs). The PTS1 is most commonly present on matrix-bound proteins; it is located at the C-terminus of the protein as a terminal tripeptide, usually as a small-basic-large and hydrophobic amino acid motif, (e.g., serine-lysine-leucine: SKL). The PTS2 has a nonapeptide motif (arginine-valine-5 variable amino acids-histidine-phenylalanine: RV[X5]HF) near to the N-terminus, and is present on matrix proteins that are usually

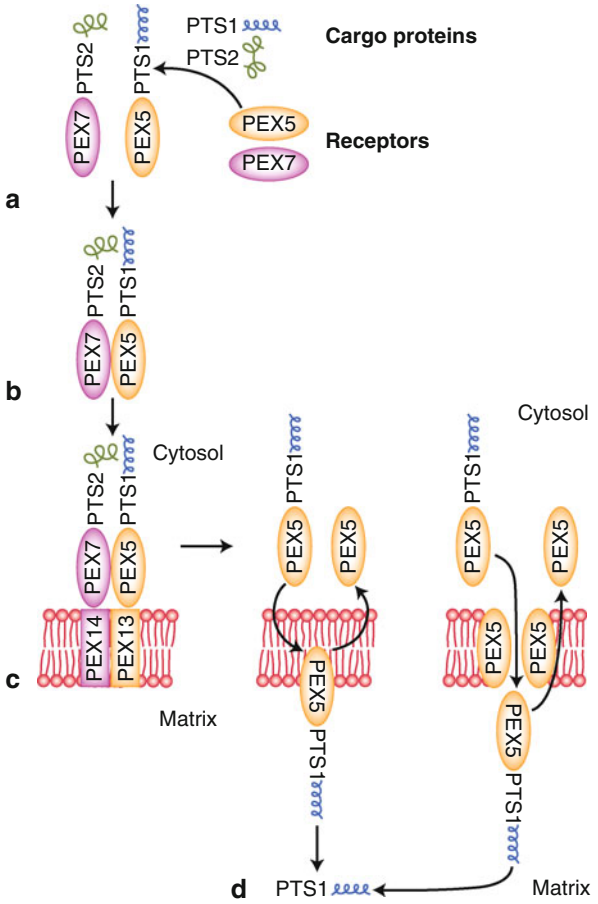


Fig. 5.19 A model for the import of matrix proteins into the peroxisome/glyoxysome. (**a**) Following their synthesis on free cytoplasmic polysomes, matrix-destined (cargo) proteins with carboxy-terminal PTS1 or amino-terminal PTS2 sequences are recognized by their appropriate (cognate) PEX receptor proteins. (**b**) PEX5 can act as a co-receptor for PEX7 and (**c**) they travel to the glyoxysomal membrane where they are recognized by specific peroxisomal membrane-bound PEX docking proteins. (**d**) Two possible mechanisms of transfer of the cargo protein into the matrix are by a simple shuttle (*left*) or an extended shuttle (*right*) mechanism. For simplicity the docking proteins and PEX7-cargo protein are omitted from this step, although the latter follow the same pathway. Also not shown are the membrane-associated PEX proteins involved in the recycling of the receptor into the cytosol. Modified from diagrams in Laynton-Hogg et al. (2010). See also Ma et al. (2011)

proteolytically processed after import into the organelle. As shown in the simplified model in Fig. 5.19, import of all matrix proteins can be divided into four stages: initial binding of the protein to be imported (termed the cargo protein) to its cognate receptor; transport and docking of the receptor-cargo complex at the glyoxysome membrane; translocation of this complex across the membrane into the matrix with release of the cargo; recycling of the receptor.

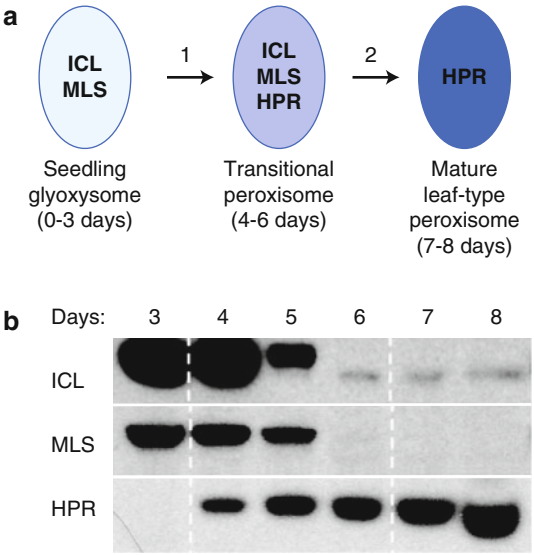


Fig. 5.20 (**a**) Representation of the changes occurring as the glyoxysome is transformed into a peroxisome in the cotyledon cells of Arabidopsis following germination. Representative enzymes of the glyoxysome: isocitrate lyase (ICL) and malate synthase (MLS), and of the peroxisome: hydroxypyruvate reductase (HPR). (**b**) Western blot showing the decline in ICL and MLS, and the increase in HPR as seedling development proceeds. There is an overlapping period of about 2 days when enzymes for both functions of the same organelle are present. Days signify time from imbibition of the seed. From Lingard et al. (2009). Courtesy of the National Academy of Sciences, USA

Genes that are involved in peroxisome/glyoxysome biogenesis and maturation are called *PEROXIN* (*PEX*) genes, which are conserved in plants, animals and yeast; several PEX proteins play a role in the import of cargo proteins into glyoxysomes. The PTS regions in the proteins to be imported are recognized by receptor proteins in the cytosol (PEX5 or PEX7) (Fig. 5.19). These now combine and travel to the surface of the organelle. For a protein to enter into the glyoxysome matrix the receptors must first be recognized by docking proteins, which are a group of PEX proteins, the majority of which are called peroxisomal membrane proteins (PMPs) present on the glyoxysomal membrane outer surface. The next step is the translocation of the cargo protein across the membrane and its subsequent release into the matrix. This particular step is incompletely understood, but two types of mechanisms are proposed: (1) a simple shuttle mechanism operates allowing the receptor-cargo complex to pass through the membrane before the cargo is released and the receptor is returned to the cytosol; (2) the extended shuttle model proposes that the receptor protein remains in the membrane to form a pore through which other receptor-cargo proteins can pass, the cargo being released and the receptor recycled.

In species whose mode of seedling growth is epigeal the cotyledons turn green as they emerge from the soil into the light. During greening the glyoxysomes undergo a gradual loss of function as they are converted into peroxisomes (Fig. 5.20a); key

FFA-catabolizing enzymes such as those of the glyoxylate cycle (ICL, MLS) and β-oxidation spiral decline within the organelle, while others, e.g., catalase and malate dehydrogenase, are retained and increase as new enzymes that are photorespiration-associated, e.g., hydroxypyruvate reductase (HPR), are imported (Fig. 5.20b). Coincidental with these changes in enzyme content during glyoxysome-peroxisome transformation is the cessation of expression of genes for FFA utilization, and an increase in expression of those necessary for peroxisome function. There is a brief period of overlap where enzymes for both functional activities are present. A number of specific PEX proteins play a role in the removal of the glyoxysomal enzymes, helping in their transport out of the evolving peroxisome so that they are destroyed in the cytosol, by the proteasome (a complex of proteases that degrade unneeded or damaged proteins).

In oil-storing seeds where the cotyledons remain below ground, and in endosperms or megagametophytes that also become depleted of reserves, the glyoxysome degrades as the expended storage organ undergoes programmed cell death. While TAGs are present in the starchy endosperm of oat grains, it is not known if or how they are mobilized: the tissue in which they are stored is nonliving, there are no mitochondria or glyoxysomes, and the aleurone layer does not secrete lipases into it.

5.7.3 Utilization of the Products of TAG Catabolism

As with the products of starch and hemicellulose catabolism, those of TAG mobilization are a vital source of carbon and energy to support growth of the seedling. FFAs and glycerol are to a large extent converted to hexose, and finally to sucrose, by a sequence of reactions outlined in Fig. 5.15. Castor bean endosperms contain high amounts of sucrose-6-P synthase, sucrose phosphatase, and also sucrose synthase (Sect. 5.4.1). Here the major product of TAG mobilization is sucrose, which is taken up by active transport into the cotyledons. More than 80% of this sucrose is redistributed to the growing axis. If the embryonic axis is removed, there is temporary storage of sucrose in the endosperm, in vacuoles that develop as the storage products are degraded. Sucrose uptake by the cotyledons is thereby drastically reduced. Thus, as far as the growing seedling is concerned, removal or damage to the sink (axis) alters replenishment at the source (cotyledons).

The cotyledons of some seeds (e.g., pumpkin, watermelon, sunflower) can utilize acetyl CoA arising from β-oxidation of fatty acids for amino acid synthesis via partial reactions of the glyoxylate and citric acid cycle. The usual products are glycine, serine, glutamic acid, glutamine, and γ-amino butyric acid.

5.8 Storage Protein Mobilization

Hydrolysis of storage protein (polypeptides) in the protein storage vacuoles (PSVs) to their constituent amino acids requires a class of enzymes called proteases, some of which effect total hydrolysis whereas others produce small polypeptides that must

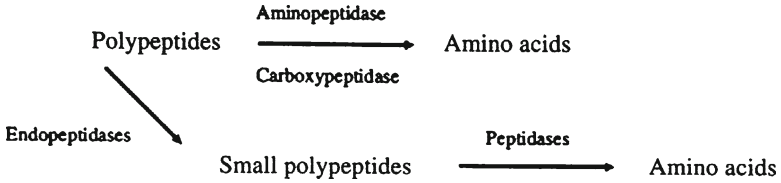
be degraded further by peptidases. The proteases can be categorized as follows in relation to the manner in which they hydrolyze their substrates:

(1) Endopeptidases: these cleave internal peptide bonds within the protein to yield smaller polypeptides. These can be classified into four major groups: (a) serine endopeptidases, which have a serine in their active site where the peptide bond is broken; (b) cysteine endopeptidases, which have a cysteine in the active site; (c) aspartic endopeptidases, with two aspartates in the active site; and (d) metalloendopeptidases, which have a metal ion (usually Zn^{2+}) in the active site.

(2) Aminopeptidases: these sequentially cleave the terminal amino acid from the free amino end of the polypeptide chain. There are multiple forms of these, located in the cytosol; they are active at neutral or slightly alkaline pHs.

(3) Carboxypeptidases: as (2), but single amino acids are sequentially hydrolyzed from the carboxyl end of the chain. There are multiple forms of these located within PSVs, and all contain serine in their active site; thus they are serine carboxypeptidases.

Both (2) and (3) are exopeptidases, and many are relatively nonspecific with respect to the amino acids that they cleave from the terminus of a polypeptide.



The liberated amino acids may be reutilized for protein synthesis or be deaminated to provide carbon skeletons for respiratory oxidation or conversion to other metabolites. Ammonia is produced by deamination, but this is prevented from reaching toxic concentrations by fixation into glutamine and asparagine, two commonly transported forms of amino acid.

5.8.1 Protein Mobilization During Germination

Whereas there are alternative sources of sugars (as oligosaccharides) to those from starch, hemicelluloses or TAGs for the embryo to utilize during germination (Sect. 5.3), such is not the case for amino acids, which are not present in sufficient amounts in the mature dry seed to sustain protein synthesis until the time of radicle emergence and subsequent post-germinative storage protein mobilization. Hence, there is germination and post-germination mobilization of proteins, which are mediated by different enzymes in different parts of the seed. This has been studied extensively in vetch and buckwheat seeds, although it is likely to represent the general pattern of proteolysis in leguminous and nonleguminous seeds; less detailed studies on cucumber, rapeseed, soybean, and *Phaseolus* bean support this. During germination the storage globulin proteins in the PSVs of both the axis and cotyledons are subjected to proteolysis, and the amino acids are reutilized to make more proteins in the

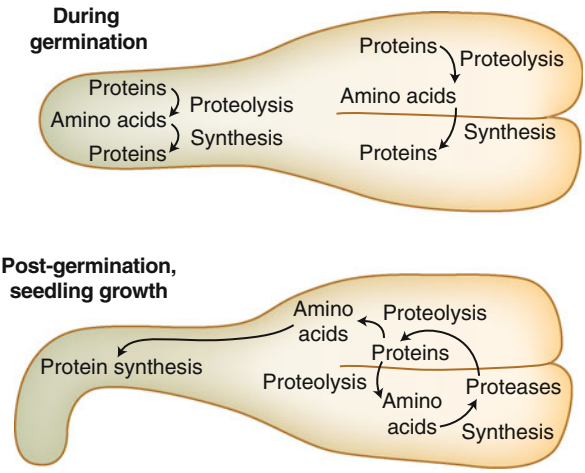


Fig. 5.21 Comparisons of the patterns of storage protein mobilization in the axis and cotyledons of a dicot seed during and following germination. During germination the released amino acids from storage proteins in the axis and cotyledons, by the action of proteases already present in the dry seed, are reutilized for protein synthesis within the same region. Following germination, when the protein reserves in the axis have been depleted, the sole source of amino acids for seedling growth is the cotyledons; most amino acids are transported therefrom to the growing regions, although some are retained for the synthesis of proteases and other hydrolases required for the mobilization of the major storage reserves. Based on information in Müntz et al. (2001)

same regions (Fig. 5.21). The enzymes involved in the mobilization of the proteins during germination are stored within the PSVs and are present in the dry seed, being activated following imbibition. By the time seedling growth is underway, the proteins in the axis are depleted and the source of amino acids for protein synthesis is now exclusively those exported from the cotyledons, although some are retained there for use in the synthesis of proteases and other hydrolases necessary for mobilization of the starch or oil reserves, for example. The post-germination synthesis of new proteases is essential for protein utilization in the cotyledons.

In vetch seeds the proteases to mobilize the globulins in the PSVs of the axis are synthesized during late embryogenesis; why they do not hydrolyze the storage proteins at that time is unknown, but it could be because the enzymes are in an inactive form, at an incorrect pH, or their substrate has to undergo some structural changes to permit them to be effective. Five different cysteine proteases are present in the axes of germinating seeds, located in the PSVs, and one additional type in the cotyledons, which appears not to be in this storage organelle. The mRNAs for at least three of the proteases are known to be present in the developing axes and cotyledons of the maturing embryo, and remain there during subsequent imbibition, to be replaced with transcripts for the same or different proteases as germination and seedling growth proceed.

5.8.2 *Protein Mobilization Following Germination of Cereals*

Reserve proteins are present in two separate regions of the cereal grain: in the aleurone layer and starchy endosperm (Sect. 1.3.3), with a minor amount being present in the scutellum and axis, which may be hydrolyzed to supply amino acids for protein synthesis during germination and early seedling growth prior to mobilization of the major endosperm reserves.

Proteases in the aleurone layer are discussed in Sect. 5.5.3 with respect to their role in providing amino acids for the synthesis of key enzymes for starch mobilization. Those necessary for the hydrolysis of the major protein reserves stored in the PSVs in the starchy endosperm are synthesized and secreted there from the aleurone layer, which also releases malic acid, acidifying the storage cells to about pH 5, thus optimizing the conditions for enzyme activity. During maturation drying of the endosperm the membranes of the PSVs tend to lose their integrity, aiding in the exposure of the storage proteins to their hydrolases upon subsequent rehydration.

The number of proteases involved in the mobilization of starchy endosperm proteins is generally large. In maize kernels at least 15 different endopeptidase activities are detectable during the first 6 days after the start of imbibition (DAI). Four groups of enzymes have been identified, based on the time of their appearance. Group I is present in the dry seed; it contains two metalloendopeptidases that decline in activity soon after imbibition. They appear not to be involved in the initial mobilization of zein, the major storage protein. Group II endopeptidases increase following germination and reach peak activity after 3 DAI. These are SH-(cysteine) endopeptidases and have a high affinity for γ-zein, the form of storage protein that is located peripherally in the PSVs, and thus is the first to be subjected to proteolysis. Group III enzymes achieve maximum activity at 5 DAI and are mostly cysteine endopeptidases that cleave α-zein, the form located internally within the PSVs. Group IV enzymes increase in activity only after 3 DAI, and their specificity is for α-zein. They are unable to hydrolyze γ-zein, but by the time they are present in the endosperm this form of zein is likely to have been completely mobilized. The site of synthesis of the endopeptidases that hydrolyze zein is either the scutellum or the aleurone layer. In addition to these groups of endopeptidases, it is likely that there are also several amino- and carboxy-peptidases involved in completing proteolysis, as well as the oligopeptide-degrading peptidases.

Consistent with these observations on maize, a general pattern of protein hydrolysis in both cereals and dicots seems to be emerging in that metalloendopeptidases are present first, then a series of cysteine-endopeptidases, followed by the terminal-acting (amino- and carboxy-) peptidases and the enzymes that hydrolyze the resultant oligopeptides, the peptidases. The different substrate specificities of the enzymes, as they arise, could account for the order in which storage proteins and their component forms are mobilized.

An even greater complexity of proteases occurs in germinated barley grains, where there are 42 distinct enzymes, the majority (27) being cysteine endopeptidases, along with serine endopeptidases (8), aspartic endopeptidases (4) and metalloendopeptidases

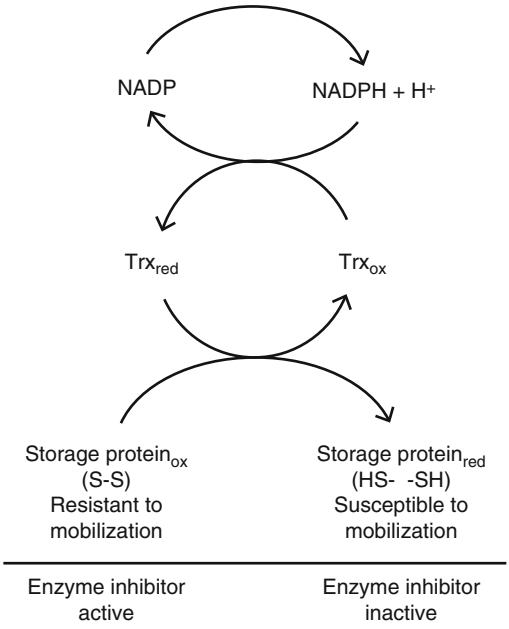


Fig. 5.22 Regulation of storage protein mobilization and enzyme inhibitor activity in wheat endosperms by thioredoxin (Trx). Reduction of Trx requires the transfer of reducing power from NADPH + H^+. The reduced form of Trx in turn reduces the storage protein, rendering it available for proteolytic cleavage. In the case of the enzyme inhibitor α-amylase/subtilisin the reduction of the disulfide bonds in its constituent cysteine residues to unlinked cystines renders it inactive. red, reduced; ox, oxidized

(3). In barley, but more so in other cereals such as wheat, proteolysis of the starchy endosperm storage proteins is aided by their reduction by thioredoxin.

Thioredoxins (Trx) are small (12 kDa) oxido-reductive enzymes that contain a dithiol-disulfide active site (–HS SH– ↔ –S–S–). In plants there are six well-defined types that reside in different cell compartments and function in an array of metabolic events. Trx*h* is located in the cytosol and following imbibition of the wheat grain reduces the redox-active disulfide groups of gliadin and glutenin storage proteins to the sulfhydryl state (Fig. 5.22); this increases both the solubility of the proteins and their susceptibility to proteolysis. These changes are also accompanied by activation of thiocalasin, a Ca^{2+}-dependent serine protease, which is then able to hydrolyze the reduced storage proteins. Thioredoxin may also play an indirect role in activating other hydrolytic enzymes in cereal grains, including some involved in starch hydrolysis such as α-amylase and a debranching enzyme, pullulanase. This is achieved by inactivating protein inhibitors (Sect. 5.8.4) of these enzymes, such as α-amylase/subtilisin inhibitor, which is not functional in its thioredoxin-reduced state. Whether or not thioredoxin plays a similar role in enhancing protein mobilization in dicot seeds is not known, but there is evidence that it can act as a reductant of protease inhibitors and 2S storage proteins. In addition to its role in post-germinative reserve mobilization, Trx positively influences germination through unidentified mechanisms; suppression of *Trx* gene expression prevents precocious germination (Sect. 2.4.2).

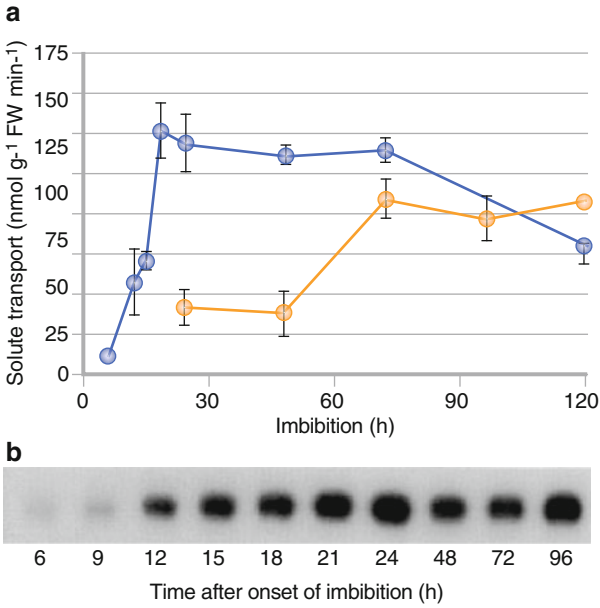


Fig. 5.23 (**a**) Development of the capacity for the transport of dipeptides and amino acids into barley scutella isolated from the grain at different times following the start of imbibition. Scutella were isolated at the times indicated and imbibed on a medium containing the dipeptide Ala-[^{14}C] Phe (blue) or amino acid [^{14}C]Ala (orange) for 10 min to determine their uptake. (**b**) Increase in the expression of the *HvPtr1* gene for a membrane peptide transporter; the northern blot shows an increase in transcripts present in scutella dissected following imbibition of the barley grain from 12 to 96 h. From West et al. (1998). Courtesy of Wiley

5.8.2.1 Uptake of Amino Acids and Peptides into the Embryo

Proteolytic activity within the starchy endosperm of cereal grains results in the production of amino acids, dipeptides, and a number of small oligopeptides. These soluble products are rapidly absorbed by the embryo, via the scutellum, with di- or tri-peptides being taken up more efficiently than free amino acids. Although this uptake of peptides does not appear to involve or require their hydrolysis, they are eventually cleaved by peptidases within the scutellum, and only free amino acids accumulate to any extent in the growing embryo. Active uptake mechanisms within the plasma membrane of the scutellum can distinguish between peptides and amino acids. Isolated scutella of barley, for example, can import the dipeptide alanine-phenyalanine several days sooner than alanine alone (Fig. 5.23a). The capacity of the scutellum to transport the dipeptide is acquired very early following imbibition, at the time when the whole grain is still germinating. During germination there is the synthesis of a plasma-membrane-associated transport protein, HvPTR1, in the scutellum from newly formed transcripts (Fig. 5.23b). HvPTR1 is a peptide transporter, which has a broad specificity for peptides with 2–4 amino acids and broad

tolerance of their amino acid composition. In the presence of amino acids its transport capacity is inhibited, whereas in the presence of glucose it is enhanced. Such interactions between hydrolysis products and transporters may be important in balancing the flux of nitrogen and carbon into the embryo during and following germination. The inactivation of HvPTR1 by the presence of amino acids is the result of post-translational phosphorylation of the serine residues in this protein.

A number of transporters have been identified in cereal grain scutella, several of which are synthesized de novo before or at the time that protein mobilization commences within the endosperm. In wheat and barley scutella there are at least four transporters for amino acids alone: two are nonspecific, one is specific for proline, and another for basic amino acids. Maize and rice also possess multiple uptake systems, but with some differences in specificity. The efficiency with which scutella take up certain peptides, compared to amino acids, also varies among species.

5.8.3 Protein Mobilization Following Germination of Dicots

Mobilization of storage proteins during germination is discussed in Sect. 5.8.1; here post-germination utilization of the major reserves in the storage tissues is followed. The large polymeric storage proteins (11S legumins and 7S vicilins) tend to be insoluble in the PSVs in which they are stored. Initially they need to undergo limited proteolysis by endopeptidases at exposed positions on their surface to effect structural changes, thus rendering them more susceptible to further enzymic degradation (Fig. 5.24). Subsequent hydrolysis by endo- and carboxy-peptidases results in the production of small peptides and amino acids, which are then transferred from the PSVs into the cytosol by active transporters; the membranes of these organelles remain intact following maturation drying, unlike those in the cereal endosperm. After transport into the cytosol the small peptides are subjected to amino-, di- and tri-peptidases to yield free amino acids.

The identities of the initiating endopeptidases vary with the type of storage protein or its particular subunit, as demonstrated in soybean cotyledons. Mobilization of the α and α′ subunits of the vicilin β-conglycinin, which have a 93% amino acid sequence identity to each other, is initiated by a serine endopeptidase, Protease C1. This enzyme does not hydrolyze the smaller β-subunit of β-conglycinin, however, which in contrast to the α and α′ subunits lacks a segment of 179 amino acids from the N-terminal end, and likely contains the target site for the Protease C1. To initiate hydrolysis of the β subunit there is requirement for a cysteine endopeptidase, Protease C2, which hydrolyzes the products resulting from the activity of Protease C1. Papain-like cysteine proteases of the C2 type are commonly present in the cotyledons of different legume species that contain the same type of storage protein.

In soybean cotyledons, within the same PSVs as the globulin β-conglycinin are Kunitz- and Bowman-Birk trypsin inhibitors (Sect. 5.8.4); their degradation is initiated by a different cysteine endopeptidase, Protease K1. In mung bean, however, the

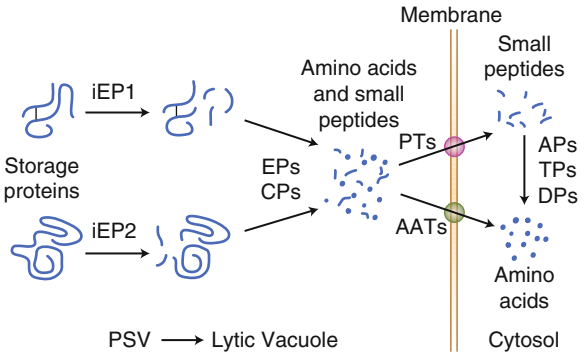


Fig. 5.24 Depiction of the pathways for the degradation of the major storage proteins (legumins and vicilins) in the PSVs of legume seeds. Storage protein degradation initially requires their limited hydrolysis by specific endopeptidases (iEP1, iEP2) to make them more susceptible to proteolytic degradation. The partially modified proteins are now available for further hydrolysis by these and other endopeptidases (EPs), as well as serine carboxypeptidases (CPs) to small peptides and amino acids. These are transported across the PSV membrane into the cytosol by amino acid (AATs) and peptide (PTs) transporters; degradation of the small peptides to amino acids is completed by aminopeptidases (APs), tripeptidases (TPs), and dipeptidases (DPs). The PSVs transform into lytic vacuoles during and following protein hydrolysis. From Wilson (2006). With permission of CAB International

Bowman-Birk type trypsin inhibitor is first hydrolyzed by a serine endopeptidase, Proteinase F.

The proteases involved in the mobilization of the storage proteins may be synthesized and sequestered in PSVs during seed development. There are several reasons why the proteases are inactive until after germination: (1) The pH inside of the vacuole is unfavorable for activity of the enzymes, and must be changed in order for them to become catalytic. In developing mung bean cotyledons, for example, there is the accumulation of both a Bowman-Birk type trypsin inhibitor and Proteinase F in the same PSVs. The enzyme is inactive during maturation and germination because the pH inside the vacuole is too high; following germination H^+ ions are introduced into the PSVs by proton pumps, lowering the internal pH, resulting in enzyme activation. Soybean Protease C1 is also activated following germination by acidification of the PSVs in which it is present along with its target storage proteins at seed maturity. (2) The enzyme is incapable of hydrolyzing the storage protein until after it has been modified by a different protease. In mature and imbibed seeds of mung bean and barley the storage proteins and carboxypeptidase are present in the same PSVs. However, this enzyme, being an exopeptidase, does not have a substrate to act upon until after the storage protein has been modified by an endopeptidase (as in Fig. 5.24), which is synthesized following germination. (3) There is an inhibitor of the protease that must be inactivated to allow the enzyme to hydrolyze the storage protein. This is rare, but occurs in buckwheat seeds (Sect. 5.8.4).

The cellular changes that precede and accompany proteolysis have been studied most thoroughly in the cotyledons of mung bean, although what occurs there is likely representative of events in the storage tissues of many dicots. In mung bean the major storage protein is a vicilin, which comprises 70–80% of the total, and the major enzyme responsible for its hydrolysis is a cysteine endopeptidase, vicilin peptidohydrolase (Vpase), with some participation by a carboxypeptidase. Dry and early-imbibed cotyledons contain tubular ER (T-ER), which is dismantled approx. 12–14 h after the start of imbibition. Although, overall, there is a net loss of membrane, at the same time there is a proliferation of a new type of cisternal ER (C-ER), with ribosomes attached (Fig. 5.25a). The Vpase is synthesized de novo from newly produced transcripts on polysomes attached to the C-ER, and the enzyme is inserted into the ER lumen and packaged into vesicles. These are transported to the PSVs, and degradation of the vicilin therein commences only after the peptidohydrolase has been inserted (Fig. 5.25b). Initially the vicilin is cleaved from 50- to 63-kD components to 20- to 30-kD components, and these are then hydrolyzed more slowly. As protein digestion proceeds, the emptying PSVs fuse to form a large vacuole containing an array of hydrolytic enzymes, thus becoming a lytic vacuole (Fig. 5.25c). Digestion of cell contents by the enzymes in this vacuole is achieved when vesicles are internalized by an autophagic process in which a portion of the cytoplasm is engulfed and sealed off by the PSV membrane (Fig. 5.25d). Although mung bean exhibits epigeal seedling growth, the first true leaves expand as the cotyledons appear above the soil, and hence, the latter do not become photosynthetic. Their cells are completely depleted of contents due to lytic vacuole activity, the result of programmed cell death (PCD) (Sect. 5.5.4).

A similar mode of hydrolysis occurs in black gram seeds. Here the PSV becomes a lytic vacuole by the import of cysteine proteases, and as the storage protein is being hydrolyzed autophagic bodies enveloping cell contents, such as mitochondria, are ingested, as part of PCD. An apparent additional feature is that α-amylase is synthesized and targeted to the PSV, and starch granules are imported into this organelle for breakdown as it morphs into a lytic vacuole.

A variation on this mode of PCD occurs in the castor bean endosperm. Here, following mobilization of the protein and lipid reserves, an inactive papain-type cysteine endopeptidase (CysEP) is synthesized by polysomes on the ER and released from there as ribosome-associated vesicles called ricinosomes. The final stage of degeneration of the endosperm cells is marked by destruction of the nucleus and DNA fragmentation, breakdown of lytic vacuoles releasing proteases and nucleases and causing acidification of the cytosol, and disruption of the ricinosomes releasing the CysEP that becomes activated (achieved by removal of an N-terminal propeptide and a C-terminal KDEL [lysine-asparagine-glutamine-leucine] sequence) to participate in the hydrolysis of the remaining proteins. Similar KDEL-tailed CysEPs are involved in a variety of tissues undergoing PCD; they are euphemistically termed "corpse-processing proteases," and are known to be active for example in depleted hypogeous cotyledons of vetch seedlings and megagametophytes of white spruce seedlings, as well as in a variety of plant- and seed-developmental events, e.g., during

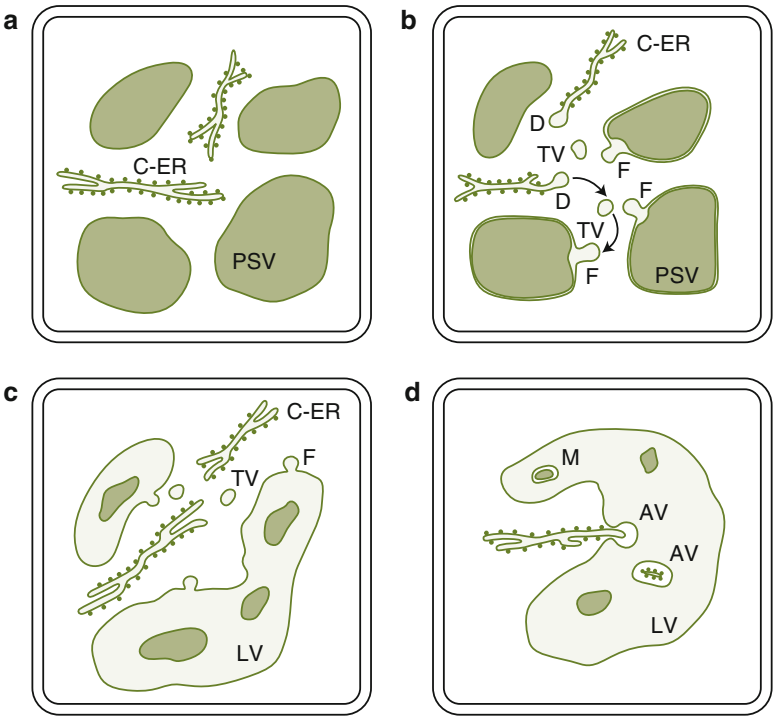


Fig. 5.25 Representation of a storage-protein-storing cell from a mung bean cotyledon illustrating the changes undergone by the protein storage vesicles (PSVs) and ER during reserve hydrolysis and cell autolysis. (**a**) Starting 12–24 h from imbibition. Formation of cisternal endoplasmic reticulum (C-ER) with ribosomes attached. PSVs contain storage proteins (*shaded*). (**b**) Three to five days from start of imbibition. Vicilin peptidohydrolase is synthesized on polysomes attached to newly formed C-ER and inserted into the lumen. Dilations (D) of the cisternae form containing the enzyme; these break off as transport vesicles (TV) and carry the peptidohydrolase to the PSVs, with which they fuse (F). The proteinase commences hydrolysis of the vicilin. (**c**) As proteins are hydrolyzed the PSVs coalesce to form lytic vacuoles (LV). Other proteases and hydrolytic enzymes, e.g., ribonucleases, phospholipases, start to be inserted into the PSV after synthesis on the C-ER. (**d**) Autophagic vacuoles (AV) form, engulfing cell contents such as the C-ER and mitochondria (M). More PSVs fuse to form a large LV containing many autolytic enzymes. Organelles and cell structures other than the ER and PSVs are omitted for clarity. Cell contents not drawn to scale. Based on the studies of M.J. Chrispeels and co-workers, e.g., Herman, E.M., Baumgartner, B. and Chrispeels, M.J., Eur. J. Cell Biol. 24, 226–235 (1981)

nucellus degeneration in developing castor bean seeds. Ricinosomes are likely involved in the final destruction of the reserve storage cells in many seeds, complementing the activity of lytic vacuoles. Their presence in the remnant cells of the micropylar endosperm of the tomato seed following germination is shown in Fig. 5.26, as PCD proceeds.

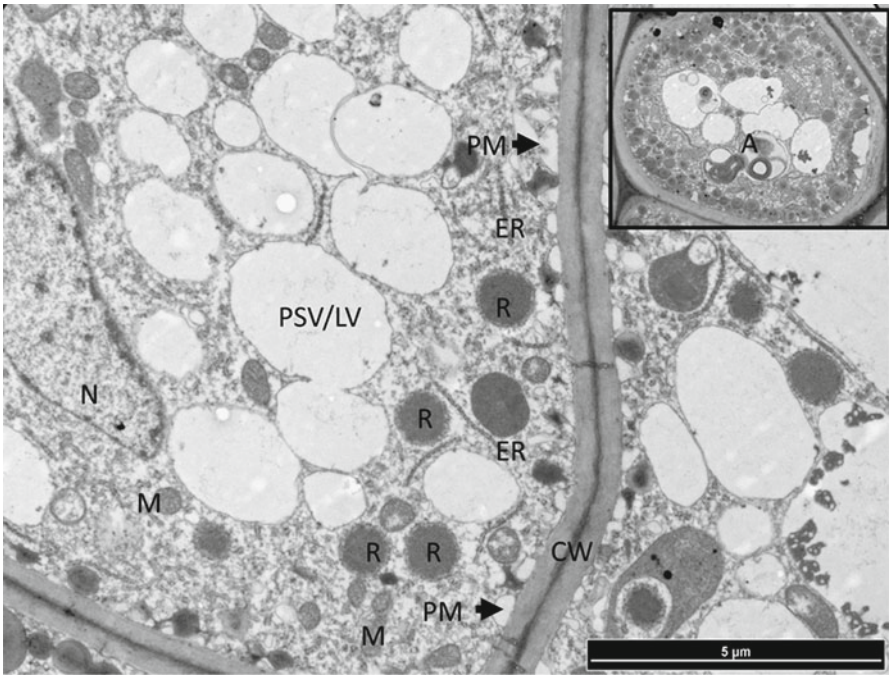


Fig. 5.26 Remnant cells of the micropylar endosperm of tomato, at 144 h after the start of imbibition, undergoing programmed cell death (PCD) following protrusion through it of the radicle. The depleted protein storage vacuoles have become lytic vacuoles (PSV/LV) that are autophagic and ingesting cytosol contents. Present in the cell are numerous ricinosomes (R) exhibiting a rough surface, the source of proteolytic enzymes released into the cytosol. The blebbing of the plasma membrane (PM, *arrowed*) away from the cell wall (CW) is an indication that PCD is proceeding. Also marked are mitochondria (M), nucleus (N) in which there is chromatin condensation (*not visible*), and endoplasmic reticulum (ER). Inset: the inclusion of autophagic vesicles (A) in depleted PSV/LVs. For more micrographs showing the progression of PCD in the tomato lateral and micropylar endosperm see DeBono, A.G. and Greenwood, J.S. Can. J. Bot. 84, 791–804 (2006). Micrographs courtesy of J.S. Greenwood, Univ. Guelph

5.8.4 Protease Inhibitors

Within both monocot and dicot seeds are proteins that specifically inhibit the action of proteases in animals and, to a lesser extent, in plants. There are several families of protease inhibitors, including the following: (1) Bowman-Birk inhibitors, which are small proteins (8–9 kDa) with many intra-chain disulfide bridges; they are common in legumes, and in the aleurone layer and embryo of rice, and bind to and inactivate serine proteases. They can account for up to 5% of total seed protein in some legumes, e.g., soybean. (2) Kunitz-protease (Kunitz-family trypsin) inhibitors, which target serine proteases. They are approx. 21 kDa in mass and are widely present in

legume seeds, accounting usually for about 3–5% of seed protein (e.g., soybean, *Acacia* spp.), and also in the embryo and aleurone layer of cereal grains. A subfamily of these is the bifunctional cereal trypsin/α-amylase inhibitors, some of which conduct only the protease inhibition. (3) Phytocystatins are inhibitors of cysteine proteases (e.g., papain) and are part of a larger family that targets this protease type; they are widespread in legume seeds and cereal grains.

Several functions have been suggested for seed protease inhibitors: (1) Storage protein. Because they can constitute between 5 and 15% of total protein in the mature seed of some species of cereals and legumes, they may play a role as storage albumins, being more readily mobilized than the insoluble globulins or prolamins during and following germination. Inhibitor proteins are also generally rich in sulfur-containing amino acids. (2) Seed inhibitors generally do not inhibit proteases within the seed itself. Therefore, their role is more likely in the protection or dissuasion from predation by insects or other pathogens. Some inhibitors can inhibit proteolytic digestive enzymes of insect pests or the secreted proteases of invading fungi and other microorganisms; in addition there are α-amylase inhibitors, and those that are bifunctional for amylases and proteases, thus providing a broad range of protection. (3) There is scant evidence that inhibitors influence the activity of proteases within the seed itself. Sometimes, as in mung bean, following germination there is a decline in the inhibitor content in the storage cotyledons as protease activity increases, but the two phenomena are not causally related. The only exception would appear to occur in buckwheat seeds. These contain several serine protease inhibitors that can suppress bacterial, fungal and insect proteases (and have been used in transformation experiments to improve resistance of tobacco and potato plants to biotic stress), as well as one that interacts with the native seed enzyme. A metalloendopeptidase exists in the PSVs of dry buckwheat seeds, inactivated by being complexed with an inhibitor. This enzyme-inhibitor complex can be disrupted in vitro by divalent cations (Zn^{2+}), allowing protease activity. It is suggested that during the early stages of storage protein hydrolysis in the germinated seed, Zn^{2+} is released from phytin as this is mobilized. The cation then binds to the inhibitor and inactivates it, allowing metalloprotease activity to increase, thus initiating the mobilization of the major legumin storage protein.

5.8.5 Utilization of Liberated Amino Acids in Dicot Seedlings

The major transported forms of amino acids from the storage organs into and throughout the growing seedlings are the amides, i.e., asparagine and/or glutamine. Hence, the amino acids liberated from storage proteins must be further metabolized, including the conversion of amino nitrogen to amido nitrogen. The synthesis of asparagine involves the donation of an amino group from glutamine in a reaction catalyzed by the ATP-dependent enzyme asparagine synthase (AS):

$$\text{Aspartate} + \text{glutamine} + \text{ATP} \xrightarrow{\text{Asparagine synthase (AS)}} \text{Asparagine} + \text{Glutamate} + \text{AMP} + \text{PPi}$$

Glutamine itself is formed from glutamate:

$$\text{Glutamate} + \text{NH}_3 + \text{ATP} \xrightarrow{\text{Glutamine synthase (GS)}} \text{Glutamine} + \text{ADP} + \text{Pi}$$

and glutamine can donate its amino group to form glutamate from α-ketoglutarate using the enzyme GOGAT (glutamate synthase).

$$\text{Glutamine} + \alpha\text{-ketoglutarate} + \text{NADPH} \xrightarrow{\text{GOGAT}} 2\ \text{Glutamate} + \text{NADP}$$

Alternatively, glutamate dehydrogenase (GDH) can add ammonia to α-ketoglutarate to yield glutamate in the presence of NADPH. Glutamate can also be synthesized by transamination of α-ketoglutarate utilizing the amino group of other amino acids.

The fates of the amino acids released from storage proteins in relation to their transport and subsequent utilization in the growing seedling are summarized in Fig. 5.27.

In cotyledons of various legume seeds there is an increase in the activity of the enzymes involved in glutamate, glutamine, and asparagine synthesis at a time when the major protein reserves are being hydrolyzed. Hence, as amino acids are being liberated from the stored form they undergo the appropriate conversions to the readily transportable asparagine. GDH, GS, and AS are present only in low amounts in the cotyledons of mature dry cotton seeds but increase appreciably during the first 2 days from the start of imbibition (likely by de novo synthesis), peaking approximately at the time of, or just prior to the commencement of, protein mobilization. In this seed, as in the legumes, the major transport form of amino acid is asparagine. The pattern of amino acid metabolism in pea cotyledons is unusual in that a major transported form of amino acid, and the one that accumulates in the storage tissue is homoserine, which is synthesized from aspartate. Glutamine is another transport form of amino acid, but not asparagine to any great extent.

In the gymnosperm loblolly pine and other *Pinus* spp., arginine is the major component of the free amino acid pool in the megagametophyte as the arginine-rich protein reserves are being mobilized. This amino acid is also high in amount in the growing seedling, and thus may be exported to there from the reserve tissue without prior conversion. Arginase, which converts arginine to ornithine and urea in the urea cycle, increases in activity in the shoot region of the embryo coincidentally with the influx of its substrate.

The predominant form of transported nitrogen from the endosperm of castor bean is glutamine, although some of the amino acids released from the mobilized storage proteins, e.g., aspartate, glutamate, alanine, glycine, and serine, can be converted to sucrose and transported as the sugar. The amide nitrogen derived from the deamination of these gluconeogenic amino acids is probably used in the production of glutamine. By comparison, amino acids that are not gluconeogenic are probably transported unchanged to the growing seedling; some might undergo modifications of their carbon skeleton to form glutamate.

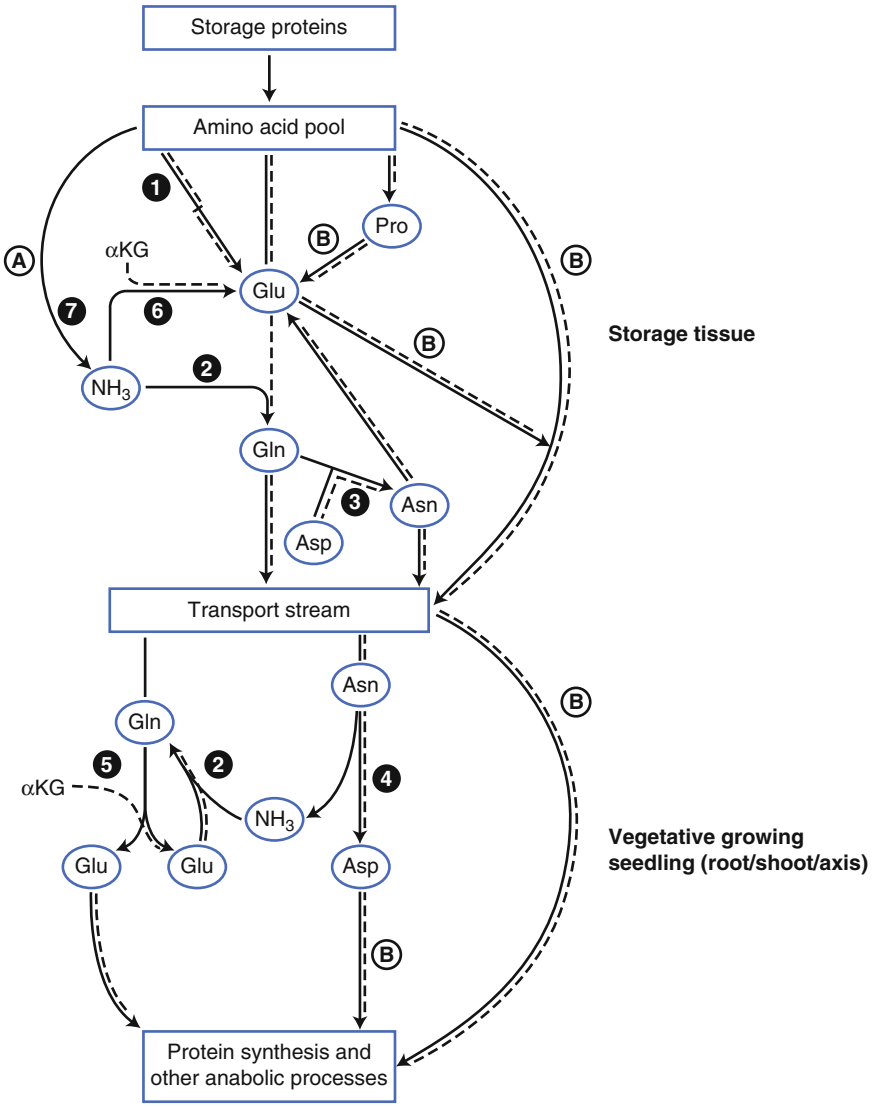


Fig. 5.27 The fate of amino acids liberated by storage protein hydrolysis, with emphasis on the fate of glutamine and asparagine, the major transport amino acids within the vascular tissue of the growing seedling. Enzymes: (1) aminotransferase; (2) glutamine synthase; (3) asparagine synthase; (4) asparaginase; (5) GOGAT; (6) glutamate dehydrogenase; (7) deaminase. Reactions: (A) deamination of amino acids to yield NH_3; (B) direct interconversions of amino acid skeletons (Pro to Glu), or direct transport and utilization of amino acids without interconversion. Compounds: Glu, Glutamic acid; Gln, glutamine; Asp, aspartic acid; Asn, asparagine; NH_3, ammonia; Pro, proline (high in the amino acid pool of cereals when storage prolamins are broken down); α-KG, α-ketoglutaric acid. *Solid lines* show the path of N, and dashed lines the path of C in or from amino acids. Shown is the fate of amino acids in cotyledons and their transfer to the growing regions via the vascular transport stream. For proteins mobilized in the endosperm or megagametophyte there must be the transport of amino acids from this tissue to the growing embryo and into its vascular system. Based on Miflin et al. (1981)

Amino acids from seeds whose major site of storage is the endosperm are taken up into the cotyledons by active transporters in the membranes of the outermost cells, e.g., glutamine in castor bean. In species where the cotyledons are the storage organs, these are connected to the growing regions by a network of vascular conducting tissue, and the products of hydrolysis are translocated to the axis largely within the phloem. Loading of amino acids into the translocation stream might be aided in some species, e.g., broad bean, by the presence of transfer cells that border the xylem and phloem; these are specialized cells with an increased surface area to aid the transport of solutes over short distances (Fig. 3.2d). In other species, e.g., mung bean, there are no transfer cells, but the parenchyma cells adjacent to the phloem have extensive evaginations of the plasmalemma to form fine tubules (plasmalemmasomes): these also serve to increase the surface area for transport.

The import of amino acids and peptides into the embryo of cereal grains is discussed in Sect. 5.8.2.1.

5.9 Phytin Mobilization

Phytic acid (*myo*-inositol hexaphosphate) is the major phosphate reserve in many seeds, and in its storage form is a mixed salt with such elements as K^+, Mg^{2+} and Ca^{2+} (Sect. 1.3.4).

Phytase, a specific phosphatase, hydrolyzes the phytin to release phosphate, its associated cations, and *myo*-inositol. Breakdown of the phytin is rapid and complete following germination, for *myo*-inositol phosphate esters with fewer than six phosphate groups do not accumulate within seeds. The released *myo*-inositol may be used by the growing seedling for cell wall synthesis, since this compound is a known precursor of pentosyl and uronosyl sugar units normally associated with pectin and certain other cell wall polysaccharides. When the ions become separated from the phosphate is not clear, although it is likely that they are cleaved together from the *myo*-inositol ring, and then the ions are released, perhaps by exchange with H^+, as the phosphate is utilized in cell metabolism.

There are two pathways by which the phosphates are removed from the phytin, the major difference being whether initially it is the phosphate in position 4 of the *myo*-inositol ring that is removed, or in position 3 (Fig. 5.28). In cereals such as rye, barley, rice and oat, the former pathway operates, with the next phosphate being removed from position 3; additionally, there may be less active phytases that operate via the alternative position-3 pathway. In legumes, phytase removes the phosphate from position 3 first, with the next one being removed from position 4; again there may be low-activity phytases that act via the alternative position-4 pathway. There are also minor variations in the steps following the first position-4 dephosphorylation, accomplished by phytases of low activity (Fig. 5.28). Many phytases and phosphatases (acidic and basic) have been reported in storage tissues of legumes and cereals, and it is evident that the role they play in phytin mobilization is variable.

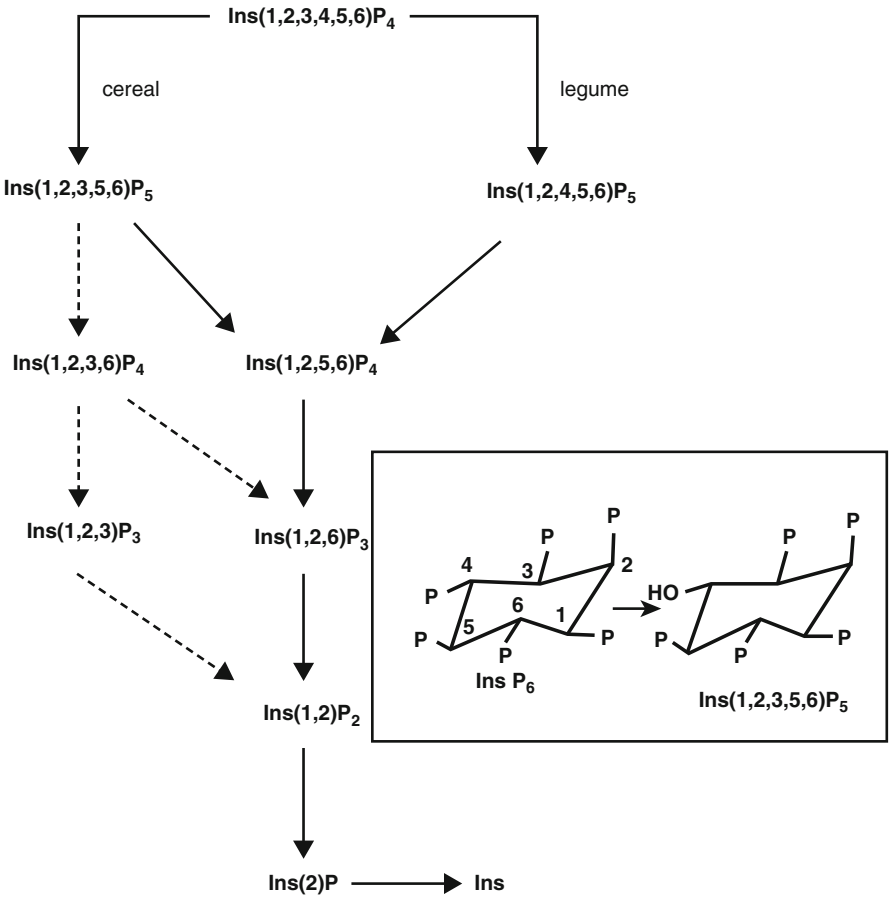


Fig. 5.28 Pathways by which phosphate is removed from phytin during its mobilization in cereal grains and legume seeds. From Ins (1,2,3,4,5,6)P_6 (phytin) the phosphate is either removed from position 4 (*left side*) or position 3 (*right side*) of the *myo*-inositol ring, in cereals or legumes, respectively, and then sequentially from positions 3 or 4 to yield a common intermediate, the tetraphosphate Ins (1,2,5,6)P_4. This is then converted to the P_3, P_2, and finally the P_1 form, inositol phosphate, Ins(2)P, from which the phosphate in position 2 is cleaved to release free *myo*-inositol. Minor phytases also initially remove the first phosphate via an alternative pathway in cereals and legumes. Also, in both there is an alternative pathway for removing subsequent phosphates (*dotted lines*), but this is low in activity compared to the major pathway (*solid lines*). Inset is an illustration to show the removal of the first phosphate from position 4 on the *myo*-inositol ring. Ins, *myo*-inositol. Based on information in Greiner et al. (2002) and references therein

Maize is unusual among cereals in that the majority of phytin is stored in the scutellum, rather than the aleurone layer, although small amounts may also occur in the coleoptile and radicle. Two phytase genes (*PHYTI* and *II*) have been isolated from this species, and their transcripts increase during and following germination in the tissues where phytin is present, at the time of its mobilization. In mature dry

barley aleurone layers the PSVs contain phytin globoids and also acid phosphatases with high phytase activity, which increase over several days following imbibition. It is possible that they are activated by the influx of H^+ ions into the PSVs, in the same manner as the proteases stored therein (Sect. 5.5.3), although there is synthesis of a different phytase following germination. Phosphate and ions released from the aleurone layer are presumably absorbed by the scutellum and distributed in the growing seedling.

The pattern in the cotyledons and axes of dicots is such that there is an increase in activity of phytases (or phosphatases) as the phytin reserves decline. Usually phytase activity is low in, or absent from dry seeds, and increases following germination, coincidentally with the hydrolysis of storage proteins. The released phosphate is transported to the growing axis. Surprisingly, the germinated seed may retain its capacity to synthesize phytin, for when isolated embryos from mature castor bean seeds are incubated in phosphate solutions there is an increase in phytin, especially within the cotyledons. The site of phytin deposition is not known, however, but it is not within PSVs. Temporary storage of phosphorus as phytin might be a way of conserving this important metabolite during early seedling establishment.

Other forms of phosphate, such as lipid, protein, and nucleic acid phosphate occur in smaller amounts in seeds. Phospholipids associated with membranes and phosphoproteins are probably dephosphorylated during their hydrolysis (acid phosphatases may play a role here); this is frequently associated with destruction of the cell for recycling of its contents to the growing regions during programmed cell death. The free phosphate is translocated to the growing axis, but the lipid and protein moieties are catabolized in situ.

5.10 Control of Reserve Mobilization in Dicots

While there is considerable information on the control of mobilization of stored reserves in cereal grains, particularly with respect to the hormonal influences on hydrolytic enzyme production in the aleurone layer (Sect. 5.5.1), much less is known about the control mechanisms in dicot seeds. There are several reports that hormones stimulate reserve mobilization, but this is largely the result of enhancing germination and/or seedling growth, and is not related directly to the control of production of the enzymes involved. Removal of the axis also influences the mobilization of the stored reserves; this is discussed further is Sect. 5.10.2. Of course, if this occurs in nature, such damage will be fatal, but if after germination the exposed axis in the intact seed is subjected to abiotic stresses such as water deficit, salinity, or cold temperatures its growth will temporarily slow down or cease, influencing the mobilization of reserves in the storage tissues.

Mobilization of stored reserves is affected by two processes: enzyme formation and enzyme activity; hence, its regulation can be achieved by controlling either or both. However, it is not always possible to determine which is responsible simply by

measuring the overall rates of loss of the reserves. Furthermore, measurements of the activity of extracted enzymes do not necessarily reflect their in vivo activity, and storage tissues that have the same extractable enzyme activity may be mobilizing reserves to quite different extents. Therefore, an understanding of the regulation of mobilization should include knowledge about rates of reserve utilization, rates of enzyme formation, and in vitro and in vivo enzyme activity.

5.10.1 Regulation in Endospermic Dicots

Structurally, the endospermic legume fenugreek is closest to the cereal grain in that there is an aleurone layer surrounding a nonliving storage endosperm in which reserve galactomannans are present as thickened cell walls. The enzymes endo-β-mannanase, β-mannosidase, and α-galactosidase are released from the aleurone layer to effect cell wall hydrolysis (Sect. 5.6.2). How mobilization of reserves within the fenugreek endosperm is controlled by the embryo is not clear, but it is assumed that at least some temporal control exists because the hydrolytic enzymes do not increase in activity, nor are they released from the aleurone layer, until after germination is completed. Abscisic acid (ABA) is present in the endosperms of intact seeds; if this hormone is leached out from isolated endosperms, then endo-β-mannanase activity increases. Reapplication of ABA to leached endosperms prevents any increase in enzyme activity. Diffusible saponin-like substances are also present in the fenugreek (and carob) endosperm, and these strongly inhibit any increase in α-galactosidase activity. These, and perhaps ABA, may play a role in limiting galactomannan hydrolysis until germination is completed, although it remains unknown how their inhibitory effects are overcome, and whether some role is played by the germinated embryo. There is no known requirement for a stimulatory hormone such as GA.

More is known about the regulation of cell wall-degrading enzymes in the endosperms of germinated lettuce and tomato seeds. In both species the major stored reserves are lipid and protein within the cotyledons, although the early post-germinative mobilization of the endosperm cell walls may provide sugars for early seedling growth. In the lettuce seed, there is mobilization of galactomannans in the endosperm cell walls by endo-β-mannanase following red-light- or GA-stimulated germination. The endosperm in the dry and imbibed seed contains ABA, which prevents the synthesis of this enzyme during germination. If the endosperm is isolated at this time and placed in a buffer solution, however, there is a strong increase in endo-β-mannanase activity (Fig. 5.29a) because the inhibitor is leached out of this tissue into the surrounding medium; reapplication of ABA to washed isolated endosperms prevents an increase in enzyme activity (Fig. 5.29b). This leaching cannot occur in the intact seed because it is prevented in the presence of the outer fruit coat. Therefore, another mechanism is required to overcome the inhibitory effect of ABA; this is controlled by the embryo, as summarized in Fig. 5.30. During germination there is the release of a stimulus from the axis, possibly GA and/or cytokinins (CK) that initially must be transported into the cotyledons. What then occurs in the cotyledons is unknown, but

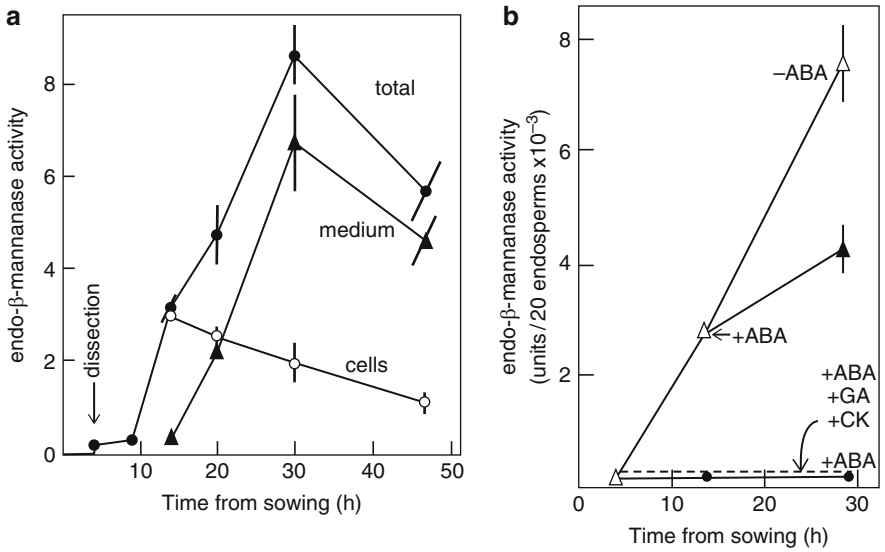


Fig. 5.29 (**a**) Increase in endo-β-mannanase activity in isolated endosperms of lettuce following their dissection after 4 h of germination and incubation in a citrate-phosphate buffered medium for up to 48 h. Initially there is a build-up of enzyme in the endosperm cells, followed by a decline after 12 h of incubation as it is released into the cell walls and surrounding medium. (**b**) Inhibition of endo-β-mannanase production by ABA (1 μM) in isolated endosperms dissected after 4 h of germination and placed for a further 26 h on buffered medium (●). Control endosperms –ABA incubated only on buffered medium produced high enzyme activity (Δ). Enzyme production was repressed (▲) when ABA was added to control endosperms 10 h after dissection. Incubation of ABA (1 μM)-treated endosperms with GA (100 μM) or the cytokinin benzyladenine (BA, 50 μM), singly or together, did not result in any increase in endo-β-mannanase activity (*dotted line*). After Halmer and Bewley (1979)

as the result of the passage of the stimulus from the axis through them there is the production of a positive signal that is released into the endosperm following germination, and there it represses the negative influence of ABA on enzyme production. Direct application of GA and/or CK to the endosperm does not induce endo-β-mannanase activity in the presence of ABA (Fig. 5.29b). Hydrolysis of the cell wall galactomannans produces small oligomannans from which the galactose side chain is removed by α-galactosidase in the endosperm; this enzyme increases somewhat in activity following germination, again influenced by the axis. The free galactose and oligomannans are taken up by the cotyledons, the latter being converted to mannose by β-mannosidase that is constitutively present in its cell walls, being synthesized and sequestered there during seed development.

5.10.2 Regulation in Non-endospermic Dicots

It is clear that mobilization is frequently influenced by the embryonic axis, i.e., the radicle/hypocotyl and plumule, or, where the endosperm is the storage tissue, by

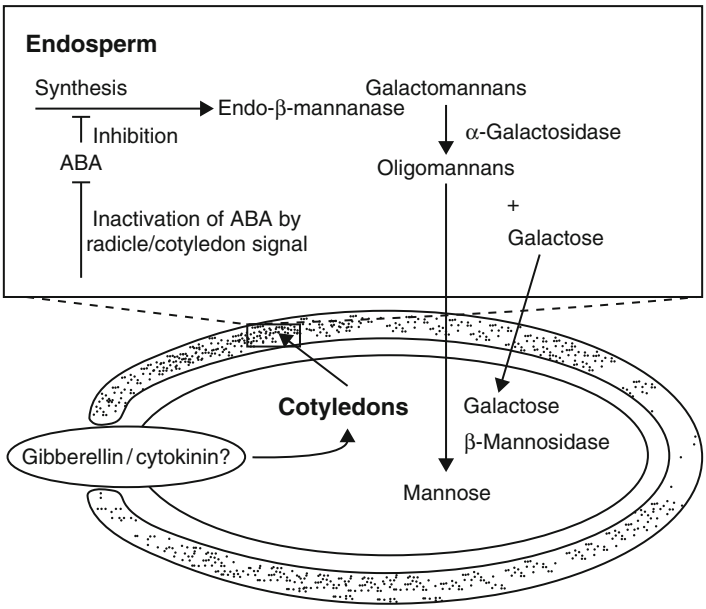


Fig. 5.30 Model to explain the regulation of the breakdown of galactomannan-rich cell walls in endosperms of germinated lettuce seeds. During germination a signal passes from the axis through the cotyledons and then to the endosperm where, following germination, it overcomes the negative effect of the endogenous inhibitor ABA on endo-β-mannanase synthesis. α-Galactosidase activity also increases and the products of cell wall-galactomannan degradation are taken up by the cotyledons. Small oligomannans are then hydrolyzed to mannose residues by β-mannosidase already present within the cotyledon cell walls. Mannose and galactose are likely quickly converted to glucose, and then to sucrose for transport and utilization within the growing seedling as an energy source until mobilization of the lipid reserves commences within the cotyledons. From Bewley (1997). Courtesy of Elsevier

the embryo as a whole. This point has been established by means of surgical experiments in which the axis (or embryo) is excised at different times during germination and seedling growth. An illustration of the role of the axis is provided by a study on protein mobilization in the cotyledons of mung bean seeds. In the 6 days following the start of imbibition, the seed completes germination and seedling growth ensues. During this time, the amount of storage protein in the cotyledons falls by about 75%, a change that is accompanied by a rise in activity of extractable proteinase (vicilin peptidohydrolase) (Fig. 5.31a, b + axis). The enzymatic breakdown of stored protein leads at first to an accumulation of free amino acids in the cotyledons, but these decrease after about 3 days as they, or their products, are transported into the growing axis (Fig. 5.31c + axis). The pattern is quite different, however, in isolated, imbibed cotyledons, from which the axis was previously removed. Here, the rates of protein hydrolysis and the increase in peptidohydrolase activity are reduced by about 75%, and the amino acids that arise from the limited protein breakdown accumulate in the cotyledons (Fig. 5.31a–c-axis). Hence, the mung bean

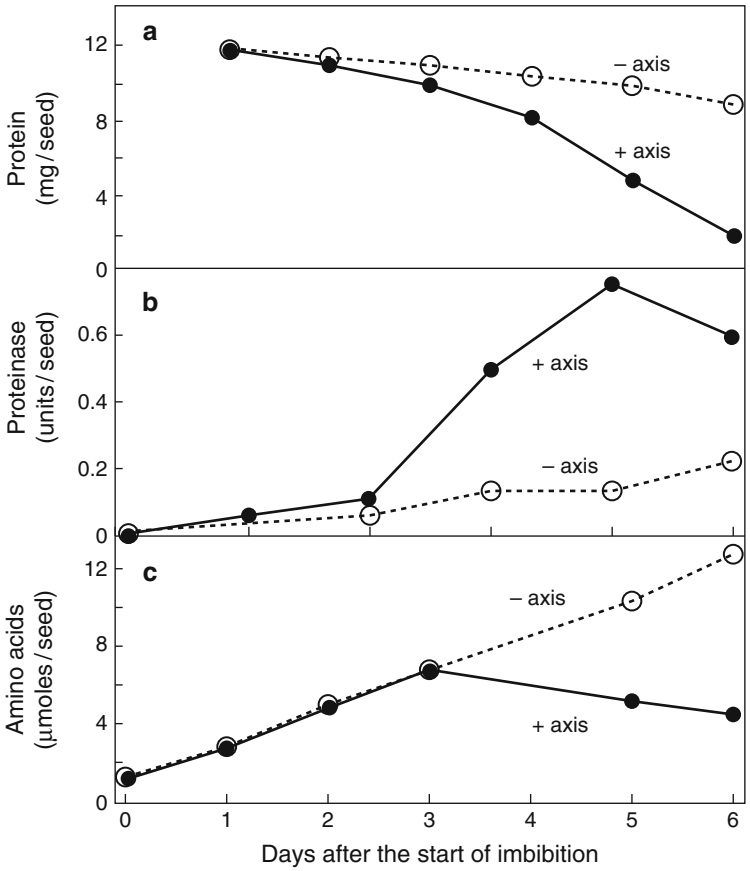


Fig. 5.31 Influence of the axis on protein mobilization in the cotyledons of mung bean seeds. The embryonic axis was removed from dry seeds and the cotyledons imbibed in moist sand (-axis, *dotted line*), or intact seeds were germinated in moist vermiculite (+ axis, solid line) in darkness. (**a**) Storage protein. (**b**) Vicilin peptidohydrolase activity. (**c**) Free amino acids. After Kern and Chrispeels (1978)

axis appears to regulate the breakdown of the proteins stored in the cotyledons, at least partially, by controlling the formation of peptidohydrolase.

Similar effects of the axis on reserve mobilization and changes in enzyme activity in the cotyledons, or of the embryo on these events in endosperms or megagametophytes, have been observed in the seeds of quite a number of species. Some examples are in Table 5.2. But there are also some cases where removal of the axis has little or no effect on enzyme activity, and so the tissues containing the enzyme seem to be autonomous. To add to the complexity, even within the same species (e.g., pea) some researchers find effects of the axis while others do not! It is important to note, however, that a change in extractable enzyme activity in the absence of the axis does not necessarily reflect a change in activity within the storage tissue itself.

Table 5.2 Influence of the presence of the axis on extractable enzyme activity in the storage organs of some dicot seeds (cotyledons or endosperm*)

Species	Enzyme	Effect of axis
Mung bean	Peptidohydrolase	+
	Glutamine synthase	–
	Asparagine synthase	–
Castor bean*	Proteases	+
	Catalase	+
	Isocitrate lyase	–
Lettuce*	Endo-β-mannanase	+
	α-Galactosidase	+
Squash	Protease	+
	Isocitrate lyase	+
Chickpea	Protease	+
	α-Amylase	+
Pea	Protease	+
	α-Amylase	+
Dolichos lablab	Proteases	+

+ Signifies that extractable enzyme activity increases in the presence of the axis compared to in its absence
– No promotive effect of the axis

Some enzymes are sensitive to product inhibition, and their activity ceases when the products of even limited reserve hydrolysis accumulate, a situation that would occur if exit of the breakdown products was impeded by the absence of the growing axis (see later for a discussion of source–sink effects). Thus, an enzyme could be inactive in vivo but active when extracted and assayed in vitro.

An interesting case is the cucumber seed, which illustrates the important regulatory role of the tissues enclosing its storage organs, the cotyledons. After germination of an intact seed, the triacylglycerols (TAGs) stored in the cotyledons rapidly become depleted, but if the axis is removed from a newly imbibed seed, there is very little mobilization of these reserves (Fig. 5.32a, curve a). Removal of the testa and the inner membrane around the axis-free cotyledons allows substantial TAG breakdown to proceed, however, indicating that these enclosing tissues normally inhibit mobilization (Fig. 5.32a, curve b). The inhibition is probably due to a limitation of oxygen entry, affecting both enzyme synthesis and the oxidation of fatty acids, for which molecular oxygen is needed. The coat is also important in intact seeds that have germinated normally. TAG breakdown in the cotyledons of these seeds does not begin when the axis elongates but only when the testa is pushed off as a result of its being wedged against a peg of tissue on the elongating hypocotyl (Fig. 5.32a, curve d, Fig. 5.32b). Moreover, the start of TAG mobilization is advanced when the testa is experimentally removed from newly germinated seeds (Fig. 5.32a, curve c). In cucumber, therefore, both the axis and the testa have important regulatory influences in the mobilization of TAGs by the cotyledons; mobilization declines when the axis is removed, but when the testa is removed, it increases. The testa also inhibits α-amylase formation and starch mobilization in pea and mung bean cotyledons. In the case of the latter it has been suggested that the testa acts by preventing the loss of a diffusible inhibitor that, in some way, is also nullified when the axis is present.

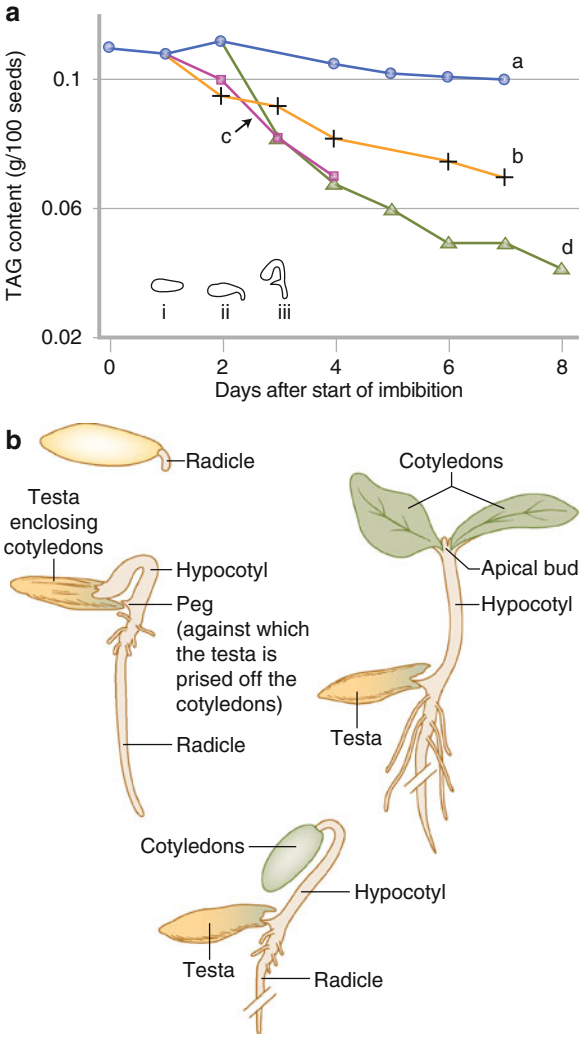


Fig. 5.32 (**a**) Triacylglycerol (TAG) mobilization in cucumber cotyledons. Total TAG content was extracted and measured at daily intervals from cotyledons of seeds treated in the following ways: (a) Radicle/hypocotyl axis removed from the dry seed; testa still around the cotyledons. (b) As (a), but surrounding testa and membrane also removed. (c) Testa removed from freshly imbibed seed; radicle/hypocotyl axis intact. (d) Fully intact seed; axis present and testa intact from the start of imbibition. The appearance of the intact seed/seedling over the first 2.5 days is shown at the bottom left of the diagram: Stage i, dry seed; stage ii, radicle emergence; stage iii, testa displacement. The testa becomes displaced at day 2; TAG breakdown in the cotyledons then begins. Adapted from Slack et al. (1977). (**b**) Larger diagram to show the displacement of the testa in the cucumber seedling due to it becoming hinged on the peg protruding from the hypocotyl that elongates to bring the cotyledons above the ground (epigeal seedling growth)

There are few reports on the influence of ABA on reserve mobilization. In most examples its effect is indirect, in that the hormone slows down or inhibits germination or seedling growth, thus reducing the synthesis and activity of hydrolytic enzymes. In Arabidopsis seeds, when ABA is applied at concentrations that inhibit germination, there is also a considerable reduction in TAG mobilization in the embryo, by some 50–65%, although there is still limited expression of the genes that encode β-oxidation and glyoxylate cycle enzymes. This is attributed to the expression of the gene for the transcription factor ABI4 (ABSCISIC ACID INSENSITIVE 4), a component of the ABA response signalling network, making the embryo sensitive to the hormone. The endosperm, although only one-cell thick, and not regarded as an important storage tissue, does contain TAGs, but the *ABI4* gene is not expressed therein, rendering it insensitive to ABA; hence, TAG mobilization is not impaired. Likewise, in the *abi4* mutant, which lacks the gene for the transcription factor, mobilization in the embryo is not diminished by ABA. TAG hydrolysis in the endosperm of tobacco seed also appears to be ABA insensitive. To what extent endogenous ABA controls reserve mobilization during and following germination in these seeds remains to be determined, however.

5.10.2.1 Mode of Regulation by the Axis

So how does the axis control the mobilization of reserves in the storage tissue? Two possibilities have been considered to explain this: (1) Specific regulatory substances move from the axis to the storage organs or tissues where enzyme production occurs, i.e., a hormonal mechanism. This explanation invokes a system similar to the one in cereal grains, where the embryo regulates enzyme production in the aleurone layer through the action of gibberellins that it secretes (Sect. 5.5.3), and possibly in lettuce (Sect. 5.10.1). (2) The axis is a sink, drawing off the products of reserve mobilization from the cotyledons or endosperm, which would otherwise arrest continued enzymatic activity by feedback inhibition. Evidence for these two options is discussed below:

(1) Hormonal control by the axis. Most of the support for the possibility that hormones control the development of activity of the mobilizing enzymes comes from testing the effects of adding growth regulators to isolated storage tissues following their dissection from the seed. This approach attempts to answer the question, can these chemicals replace the influence of the axis? In many cases, applied hormones induce greater breakdown of reserves in isolated tissue and/or increase the activity of enzymes concerned with mobilization. Cytokinins, for example, cause increases in amylolytic and proteolytic activity of isolated chickpea cotyledons as well as mobilization of carbohydrate and protein (Fig. 5.33), and in the activity of certain proteolytic enzymes and protein hydrolysis in excised squash cotyledons. They also enhance the activities of isocitrate lyase (in the glyoxylate cycle; Sect. 5.7) in watermelon and sunflower cotyledons. Activities of enzymes of the glyoxylate cycle in hazel cotyledons, for β-oxidation of fatty acids and for hydrolysis of stored protein reserves in castor bean endosperm, are increased by GA, which also promotes α-amylase activity in excised pea cotyledons. Auxin is

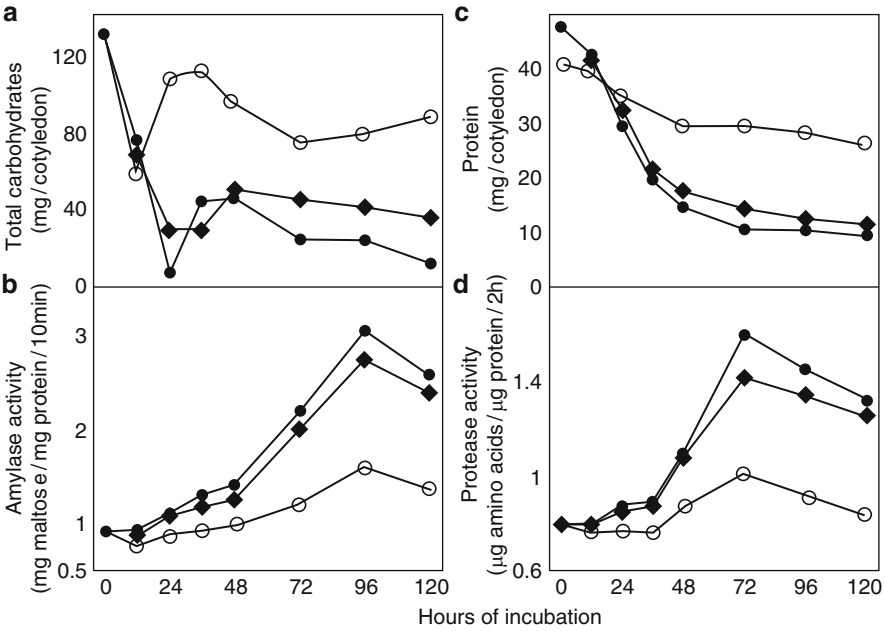


Fig. 5.33 Effect of cytokinin on amylolytic and proteolytic activity in cotyledons of chickpea. Activities are shown of cotyledons in the intact seed (●), of excised cotyledons on water (o), and of excised cotyledons that were treated so as to restore the normal concentration of endogenous, native cytokinins (♦). (**a**) Total carbohydrate. (**b**) Amylase activity. (**c**) Protein content. (**d**) Proteolytic activity. Based on Muñoz et al. (1990)

also effective in the latter, as it is in inducing the mobilization of xyloglucan-hydrolyzing enzymes in the excised cotyledons of *Hymenaea courbaril* seedlings. Most frequently, however, the effects of applied growth regulators are relatively small (and may be indirect: see below) even though sometimes comparable with the action of the axis (e.g., Fig. 5.33). Nowhere do they match the strong induction of enzyme production that is achieved by GA in cereal aleurone layer cells (Sect. 5.5.3).

Other approaches include the use of hormone-synthesis or -transport inhibitors in intact seeds or embryos, or attempts to correlate hormone content with hydrolytic activity. There are some instances, also, where diffusates or extracts of embryos stimulate enzyme activity in isolated storage tissue, e.g., fructose-1,6-diphosphatase in castor bean endosperm and isocitrate lyase in megagametophytic tissue of Ponderosa pine. But in no case has it been shown convincingly that a known hormone, e.g., a cytokinin, auxin, or gibberellin, moves from the embryo or axis to the storage tissue in the intact seed and exerts a unique control in regulating mobilizing activity; this is in marked contrast to the situation in the cereal aleurone layer.

(2) *The axis as a sink.* Many enzymes are inhibited by the products of the reactions they catalyze. The effect can involve repression of enzyme synthesis or inhibition of the activity of already existing enzyme proteins, or both. Such feedback inhibition

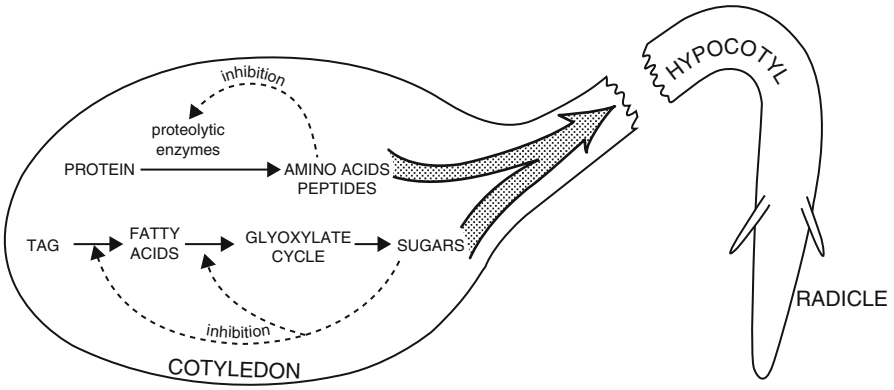


Fig. 5.34 An illustration of a potential regulatory mechanism for the mobilization of storage reserves in cucumber cotyledons in the absence of the axis. When the growing axis (sink) is removed there is a build-up of hydrolytic products in the cotyledons (source): amino acids and peptides from storage proteins, and sugars from triacylglycerols (TAG) following gluconeogenesis. These may slow down or inhibit activity of some key enzymes in the catabolic pathways, either directly or as a result of lowered transcription of their genes. The reduced mobilization of the reserves could be due to one or more of: feedback (substrate-level) inhibition of specific enzymes in the catabolic pathway, perturbation of the signalling pathways that are the fine control for cellular and metabolic interactions, a change in osmotic potential that decreases enzyme synthesis, or due to an imbalance of the C:N ratio. From data of Davies and Chapman (1980) and Slack et al. (1977)

is important in the regulation of activity of the enzymes for reserve mobilization. Growth of the axis requires the products of reserve breakdown, to where they are transported; hence, they do not accumulate in the storage tissues. The continual withdrawal of these products into the axis could account for the rise and maintenance of activity of the mobilizing enzymes; i.e., the axis regulates enzyme activity simply by virtue of its action as a sink.

This mechanism seems, in several cases, to account for the beneficial effect of the axis on mobilization. In cucumber, for example, although removal of the axis does not hinder development of several lipolytic enzymes in the cotyledons, TAG breakdown itself is much curtailed; hence, the activity of the enzymes apparently stops in the absence of the axis. Reducing sugars and sucrose accumulate in the excised cotyledons as lipolytic activity slows down. Moreover, addition of sucrose to isolated cotyledons leads to an even greater inhibition of lipolysis. The fact that TAG breakdown proceeds in isolated cotyledons when the testa is removed might be thought to argue against regulation by a sink, since the normal sink—the growing axis—is missing. However, such isolated cotyledons enlarge, making additional cell wall material (e.g., cellulose) as they do so, presumably from the sugars resulting from TAG catabolism; in addition, synthesis of starch occurs. So even though the axial sink is absent, two other sinks, cellulose and starch synthesis, serve to drain off the products of TAG mobilization and, by preventing their accumulation, permit the activity of the lipolytic enzymes to continue. The activity of extracted proteolytic enzymes from the cotyledons is similarly unaffected by removal of the axis, but

within the cotyledons protein hydrolysis itself is minimal. Accumulated amino acids, especially leucine and phenylalanine, as well as the dipeptide tryptophylphenylalanine, inhibit the activity of an aminopeptidase in the cotyledons, thus preventing the completion of protein degradation.

Thus, in the absence of a sink for the products of reserve mobilization, normally the growing axis, hydrolytic activity and processing of the catabolites of the polymeric reserves is prevented, as illustrated in Fig. 5.34 for cucumber seeds. A temporary sink may replace the axis as the cells of the cotyledons expand, resulting in some breakdown of storage reserves; this may account for the positive effect of applied hormones that enhance cell wall growth and extensibility in isolated cotyledons.

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Chapter 6
Dormancy and the Control of Germination

Abstract Dormancy is the temporary failure of a seed to complete germination under favorable conditions. It allows for the dispersal of seeds in space and time. There are several types of dormancy, which include physical, mechanical, or chemical inhibition by the covering layers of the embryo, the inability to germinate because of an undifferentiated or immature embryo, and the repression of germination by metabolic restraints. The breaking of dormancy is governed by environmental cues, including temperature, light, nitrate, and some smoke components. This allows seedling establishment during suitable conditions to maximize survival. The breaking of physiological dormancy and the induction of germination are regulated via hormone signaling pathways and mainly through the GA- (gibberellin) and ABA-(abscisic acid) biosynthetic and catabolic pathways. The ABA–GA balance appears to be a central regulatory feature that integrates multiple interactions among environmental cues.

Keywords Abscisic acid • Dormancy • Dormancy classification • Gibberellin • Germination • Hormone signaling • Light • Nitrate • Nitric oxide • Phytochrome

Whether or not a viable seed germinates and the time at which it does so depend on a number of factors, including those present in the seed's environment. First, the conditions to support germination must be right. Water must be available, oxygen may have to be present since the seed must respire, and inhibitory chemicals should be absent. Furthermore, the temperature must be suitable and so also, in many cases, must the light quality and quantity. But in many instances all these conditions may be satisfied and nevertheless the seed fails to germinate. The reason is that there exists within the seed (or dispersal unit) itself some block(s) that must be removed or overcome before germination can be completed: such a seed is said to be dormant. To be released from dormancy, a seed must thus experience certain environmental factors for minimal lengths of time, the perception of which induces metabolic and structural changes within the seed that favor germination. Based on

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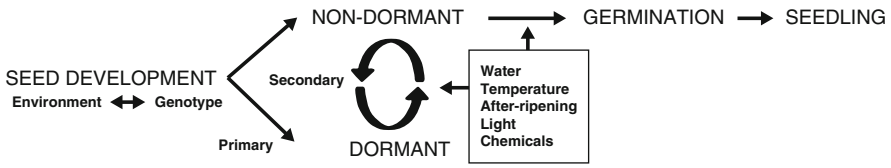

Fig. 6.1 Relationships between seed dormancy and germination. Seeds can be dormant (primary dormancy) or nondormant at the end of seed development depending upon both genotype and maternal environment. Dormancy can be alleviated by various environmental factors. Alternatively, nondormant seeds can be induced into dormancy (secondary dormancy) by some of the same factors. Seeds may cycle seasonally between dormant and nondormant. Nondormant seeds can progress to germination, again influenced by some of the same environmental factors

the above considerations, seed *dormancy* can now be defined as a temporary failure of a (viable) seed to complete germination under favorable conditions.

Dormancy is a trait that has been acquired by many species during evolution by selection for the ability to survive in unfavorable environments, such as heat, cold and drought. The origin of dormancy is possibly related to climatic changes that have occurred during the Earth's history. The number of plant species with seed dormancy tends to increase with the geographical distance from the equator, i.e., as seasonal variation in precipitation and temperature increases. Therefore, a wide range of blocks to germination has evolved as adaptations to the diversity of climates and habitats in which they operate. The relationships between dormancy and germination and the points at which controls exist are shown in Fig. 6.1.

6.1 Dormancy: Its Biological Role

The function of a seed is to establish a new plant but it can do this only once, because the completion of germination is essentially irreversible. Dormancy provides a strategy for seeds to spread germination in time in order to reduce the risk of premature death in an unfavorable environment. This strategy occurs in three ways:

(1) Seeds are dispersed from the same parent plant with different degrees of dormancy, a phenomenon known as polymorphism, heteromorphy, or heteroblasty. Frequently, the variation in dormancy is reflected in the appearance of the seeds or dispersal units—color, size, and thickness of coat. This may also be a reflection of different levels of maturity of the seeds, for at a given moment they may be at different stages of development on the parent plant and, hence, at different levels of dormancy since this is acquired during development. For example, in many members of the Poaceae grass family caryopses from different spikelets, or from the florets within, have different levels of dormancy. If a developing caryopsis is removed, it will influence the dormancy of those remaining. The green seeds produced by *Salsola volkensii* have virtually no dormancy, but the non-green seeds

have dormancy. The dispersal unit (fruit) of *Xanthium* spp. contains two seeds of which the upper one is deeply dormant whereas the lower one has a very shallow dormancy. In *Chenopodium album* four types of seeds can be found—brown or black ones each with reticulate or smooth coats; of these, the smooth, black seeds have the deepest dormancy. Thus, differences such as these, in both appearance and dormancy, may be displayed by seeds from the same plant or from different plants (e.g., *C. album*). In the former case, correlative effects are operating, that is plant parts are influencing each other to produce the variation, whereas in the second, both environmental and genetic causes can be traced. When there are polymorphic seeds, germination is spread temporally, with new seedlings emerging at irregular intervals, thus reducing competition and spreading environmental risks, increasing the likelihood that some individuals will survive. Such a temporal distribution clearly can have advantages with regard to the continuation and spread of the species.

(2) Dormancy also results in the distribution of germination in time through the dependence of dormancy breakage on environmental factors which in turn have their own time distribution. For example, seeds are commonly released from dormancy by being chilled, sometimes for several weeks or months at 1–5°C. Since such temperatures occur only during the winter, seeds that rely on this means of dormancy breakage must await the passage of this cold season before they can germinate. The advantage of this strategy is that the young seedling emerges in the spring and establishes itself over the favorable succeeding months; emergence before winter would entail the risk of succumbing to the severe conditions of that season.

(3) Seed dormancy can also lead to a distribution of germination in space—another aspect of its biological importance. Dormant seeds may be dispersed over long distances by wind, water, and animals; these dispersal types are called anemochory, hydrochory, and zoochory, respectively (Sect. 7.1).

6.2 Categories of Dormancy

The completion of germination (radicle protrusion) is the net result of the opposing forces of expansion by the embryo versus the restraints of the surrounding tissues. In the case of *embryo dormancy* the properties of the embryo are of principal importance. In *coat-imposed dormancy* the properties of the covering tissues are determinative: these include mechanical, chemical and permeability features, all of which may interfere with or suppress the successful completion of germination by the embryo. Thus, in the case of coat-imposed dormancy, removal of the tissues surrounding the embryo (e.g., endosperm, pericarp, or extrafloral organs) is sufficient for successful completion of germination. In the case of embryo dormancy, removal of the coat does not permit such embryos to germinate normally, and so the block to germination is, in a sense, more profound than in seeds with coat-imposed dormancy. Embryo dormancy is common in woody species, especially in the Rosaceae, but is sometimes found in herbaceous plants such as some grasses (e.g., wild oats). Examples of the two categories of dormancy are given in Table 6.1.

Table 6.1 Some species having coat-imposed or embryo dormancy

Coat-imposed dormancy[a]	Embryo dormancy
Acer pseudoplatanus (pericarp, testa)	*Acer saccharum*
Arabidopsis thaliana (endosperm)	*Avena fatua*—some strains
Avena fatua—some strains (palea, lemma, pericarp)	*Corylus avellana*
Betula pubescens (pericarp)	*Fraxinus americana*
Hordeum spp. (palea, mainly pericarp)	*Hordeum spp.*
Lactuca sativa (endosperm)	*Prunus persica*
Lepidium sativum (endosperm)	*Pyrus communis*
Peltandra virginica (pericarp)	*Pyrus malus*
Phaseolus lunatus (testa)	*Sorbus aucuparia*
Pyrus malus—some cvs. (endospermal membrane)	*Syringa reflexa*
Sinapis arvensis (testa)	*Taxus baccata*
Syringa spp. (endosperm)	
Xanthium pennsylvanicum (testa)	

[a] Tissues responsible are in parentheses

Very often both types of dormancy exist simultaneously or successively. In apple seeds, for example, embryo dormancy predominates, but a contribution is made by the endosperm and testa, and their removal reduces the amount of dormancy-breaking treatment (chilling) that is required (Sect. 6.6.3). Mature sycamore dispersal units (actually fruits, not seeds) possess only coat-imposed dormancy, yet just before the end of their maturation on the plant they have embryo dormancy. And in the grasses *Aristada contorta* and *Bouteloua curtipendula*, dormancy of the seed in the newly dispersed units is so deep that removal of the covering hull has no effect, whereas some months later this treatment promotes their germination. The later dormancy is therefore coat imposed.

Different types of seed dormancy also can be distinguished on the basis of the timing of the induction of dormancy rather than the location or mechanism of dormancy. Seeds that are shed from the parent plant in a dormant state display *primary dormancy*. Seeds in the soil may (gradually) acquire *secondary dormancy* (Fig. 6.1) if the conditions for germination are unfavorable or if seed germination is inhibited by other means, for example osmotic stress. Secondary dormancy imposed on imbibed light-requiring seeds maintained in the dark is termed *skotodormancy*, and that imposed by high temperatures is *thermodormancy* or *thermoinhibition*.

Seeds of several species display more complex patterns in which the parts of the embryonic axis differ in their depth of dormancy. In the so-called *epicotyl dormancy* (e.g., in *Paeonia* spp. and *Lilium* spp.), radicle emergence occurs readily but the epicotyl fails to grow. In *Trillium* spp. and *Caulophyllum thalictroides*, the radicle has some dormancy but it is less deep than that of the epicotyl because the two organs differ in the duration of the chilling treatment needed to break dormancy; such cases are said to exhibit *double dormancy*.

6.3 Mechanisms of Dormancy

Embryo growth potential (mainly associated with the radicle) must increase to allow radicle extension growth and protrusion through the covering layers. The restraint of the covering layers (pericarp, testa, and/or endosperm) must be weakened; in particular, weakening of the micropylar endosperm covering the radicle is of utmost importance (Sect. 4.6.1). The combination of radicle extension growth and weakening of any surrounding tissues are the key processes for dormancy removal and seed germination in most species and share known molecular mechanisms of which several are evolutionarily conserved. Thus, in both embryo and coat-imposed dormancies, the embryo is unable to overcome the constraints imposed on it, in the former case by factors within the embryo itself and in the latter by the enclosing tissues. Whole seed dormancy, therefore, is the result of one or more blocks to germination (in either or both embryo and coat). Removal of these blocks is always governed by environmental cues, including chilling, after-ripening, light, nitrate, and permissive temperatures.

6.3.1 Blocks to Germination Within the Embryo

Among the possible blocks to germination within the embryo are: (1) undifferentiated embryo, (2) immature embryo, (3) chemical inhibitors, and (4) physiological constraints. These are each discussed below.

6.3.1.1 Undifferentiated Embryo

In a number of species, seeds do not follow the general pattern of development. In the genera of some plant families, such as the Orchidaceae and Orobanchaceae, seeds may contain undifferentiated embryos. Orchid seeds have no endosperm and the small embryo is enclosed only by a seed coat that is composed of a thin layer of cell walls (Fig. 6.2a). Orchid seeds are exceptionally small, usually 0.1–0.2 mm wide and 0.2–1 mm long. The embryos may consist of less than 100 cells. They do not contain sufficient food reserves for growth, which necessitates the utilization of exogenous sources of nutrition, e.g., from higher plants (parasitic) or from mycoheterotrophs (fungal associations). Evidently, developmental arrest in these seeds occurs at a very early stage of embryonic histodifferentiation. Thus, these embryos will have to complete their developmental phase before they can start the germination program. Seeds with undifferentiated embryos are not dormant in the strict sense and are therefore not commonly included in current dormancy classifications.

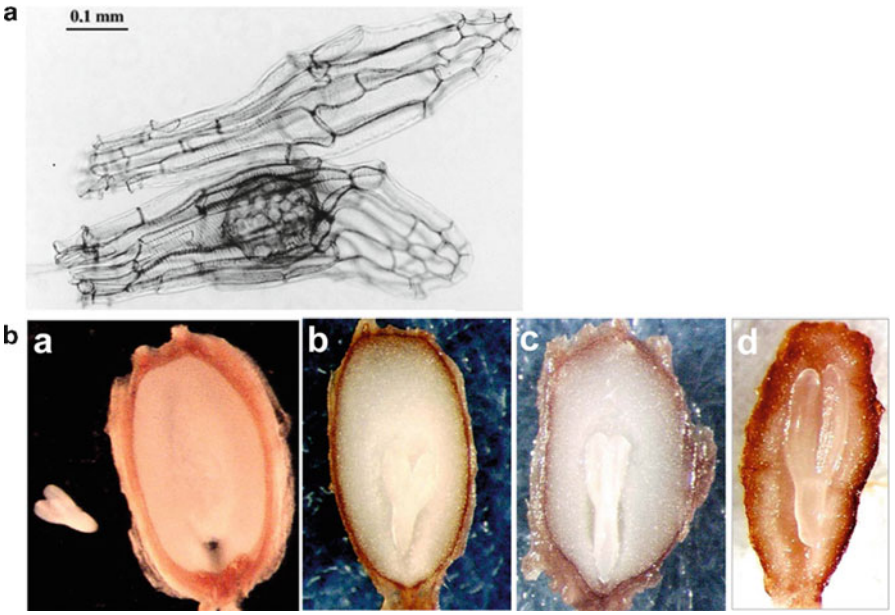

Fig. 6.2 (**a**) Underdeveloped embryo in seed of the orchid *Anacaptis palustris*. Note the empty seed at the top, and the small embryo in the lower viable one. From Cafasso et al. (2005). With permission of Oxford Univ. Press. (**b**) Completion of development of the immature carrot embryo following imbibition of the dry seed dispersed from the mother plant. The initially small embryo (dissected in a) grows into a cavity in the endosperm formed by the dissolution of its cell walls by the hemicellulase, endo-β-mannanase. (a–d) Seeds imbibed for 12, 18, 30, and 40 h, respectively. Completion of germination, noted as emergence of the radicle from the seed, occurs from 2 to 4 days after the start of imbibition, although under field conditions embryo development and germination will be much slower. After Homrichhausen et al. (2003). With permission of Cambridge Univ. Press

6.3.1.2 Immature Embryo

Seeds of other species do complete their morphogenetic phase and have a fully differentiated embryo, but do not appear to enter the maturation phase, i.e., they do not expand and accumulate food reserves. Seeds of this type usually contain relatively large amounts of endosperm tissue, often entirely embedding the small embryo. These embryos have to grow inside the dispersed seed prior to germination, sometimes by cell division, as well as by cell expansion, depending on their extent of development at the time of shedding. This has been studied particularly well in seeds from coffee, celery, carrot (Fig. 6.2b), and *Annona crassiflora*. In these species the thick cell walls of the surrounding endosperm are enzymically digested in conjunction with growth of the embryo after maturation and dispersal. This growth is facilitated by utilization of the energy-rich carbohydrate degradation products of the endosperm cell walls and, at the same time, the provision of space for expansion of the embryo. Radicle protrusion does not occur until the embryo has attained a predefined length and the micropylar endosperm has been sufficiently degraded.

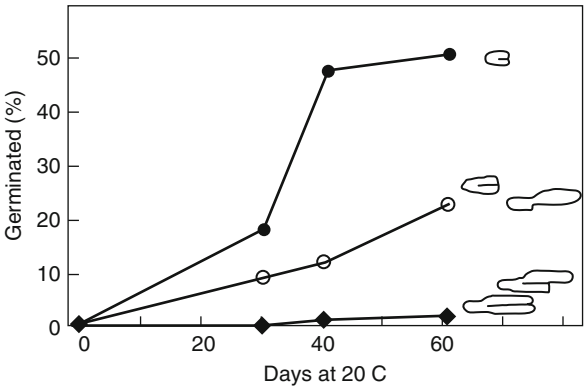

Fig. 6.3 Effect of cotyledon removal on embryo dormancy in apple. Portions of cotyledons(s) were removed from isolated, dormant embryos as indicated. The treated embryos were placed on moist cotton wool and their germination recorded. After Thevenot and Côme (1973)

6.3.1.3 Chemical Inhibitors

Only few cases of dormancy caused by chemical inhibitors have been examined in detail, but in those that have, two factors appear to be involved: (1) the cotyledons and (2) germination inhibitors. Amputation of the cotyledons often allows the embryonic axis of the dormant embryo to germinate and grow. In this way, dormancy is partially or completely broken in hazel and the spindle tree by excising one cotyledon, and in ash by cutting off two. Embryo dormancy in barley can be relieved by removal of the scutellum (which is a modified cotyledon), whereas dormancy of apple embryos is progressively reduced as increasing amounts of cotyledonary tissue are cut off (Fig. 6.3). These results suggest the presence of inhibitory substances in the cotyledons that are transported to the radicle where growth is inhibited. There is good evidence that in apple cotyledons the inhibitor is abscisic acid (ABA). The cotyledons of apple contain bound forms of ABA from which free ABA is gradually released and transported to the radicle, whose extension is thus inhibited. Observations on sunflower embryos suggest that in this species the continuous synthesis of ABA (and not its release from bound forms) is required for the maintenance of embryo dormancy. Embryos lose this dormancy when treated with fluridone, an inhibitor of ABA synthesis; they are then released from inhibition and commence radicle growth. The axes themselves are dormant and when excised they too respond positively to the fluridone treatment. In Arabidopsis, higher ABA contents of seeds clearly correlate with the presence of dormancy. Interestingly, ABA decreases during the first 24 h following imbibition in both dormant and in nondormant seeds, but after that ABA increases only in the dormant seeds (Fig. 6.4a). In seeds of *Nicotiana plumbaginifolia* ABA synthesis can be inhibited by knocking out the gene that encodes for zeaxanthin epoxidase, an enzyme involved in the synthesis of ABA (*see* Fig. 6.13a, step: zeaxanthin epoxidation to violaxanthin). The result is a substantial reduction in the lag time for the commencement and completion of germination compared to the wild

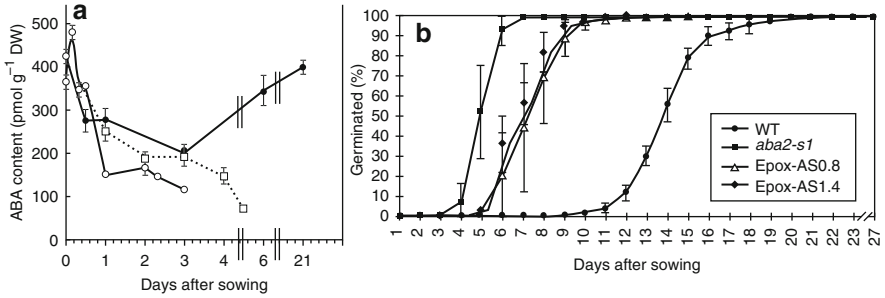

Fig. 6.4 (**a**) ABA content of *Arabidopsis thaliana* seeds during imbibition of dormant (●) and after-ripened nondormant (○) seeds and seeds whose dormancy has been broken by cold stratification (□). From Ali-Rachedi et al. (2004). (**b**) Germination of seeds from *Nicotiana plumbaginifolia*, comparing wild type (WT), an ABA-deficient mutant (*aba2-s1*), and two transgenic lines with impaired ABA biosynthesis (Epox-AS0.8 and Epox-AS1.4) as a result of mutations in the gene encoding zeaxanthin epoxidase (Fig. 6.13). From Frey et al. (1999)

type (Fig. 6.4b). This also illustrates that dormancy can be evident as delayed germination even when all seeds are eventually able to complete germination.

It seems likely, therefore, that embryos are held in a state of dormancy by ABA, either generated by the cotyledons, or synthesized within the axis, or possibly both. Dormant embryos of many species contain ABA and detailed studies have confirmed its pivotal role in the regulation of dormancy (Sect. 6.6.1.1).

6.3.1.4 Regulatory and Metabolic Constraints

The term "physiological dormancy" is often used to denote a reversible type of dormancy that is located in the embryo. This type of dormancy is widespread and central to the phenomenon of seasonal dormancy cycling of seeds in the soil, which is crucial to the establishment and survival of plant communities (Sect. 7.3). There is good evidence that physiological dormancy is maintained by active suppression of several germination-related cellular activities. ABA plays an important role in this suppression, whereas GA may counteract its effect. A GA–ABA balance appears to be decisive for the loss or maintenance of dormancy (Sect. 6.6.1).

6.3.2 Blocks to Germination by the Covering Layers

The following are possible effects on germination of the tissues enclosing the embryo: (1) interference with water uptake, (2) interference with gas exchange, (3) prevention of the exit of inhibitors from the embryo, and (4) mechanical restraint. Whereas physiological dormancy is a reversible block to germination, i.e., it can be lost and reinduced as secondary dormancy, coat-imposed dormancy is irreversible.

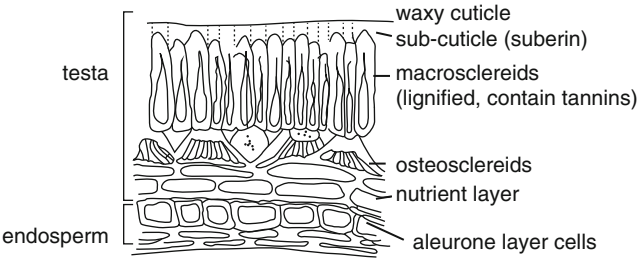

Fig. 6.5 A section of the sclerified seed coat of *Melilotus alba*. After Hamly (1932)

Embryo coverings include seed and fruit tissues that may be dead or alive. Their contribution to the level of dormancy may be of a physical, chemical, or mechanical nature, or combinations of these. Many seeds contain an endosperm that functions as the major source of stored reserves, e.g., cereals and endospermic legumes (Sect. 1.2.2). In others, especially where it is not the main storage tissue, it may contribute considerably to the expression of dormancy and germination. The seeds of fewer species contain a perisperm, a diploid endosperm-like tissue of maternal origin.

6.3.2.1 Interference with Water Uptake

The seed coat, derived from the integuments, and/or surrounding fruit tissues may prevent germination. The term "physical dormancy" is often used to denote absence of germination as the result of seed coat impermeability to water. This is a common effect, especially in seeds of the Leguminosae, but it is also found in at least 15 other families, including the Cannaceae, Convolvulaceae, Chenopodiaceae, and Malvaceae. Many species have seeds with extremely hard coats which, by preventing the entry of water, may delay germination for many years. For example, about 20% of soaked *Robinia pseudoacacia* seeds remained ungerminated for 2 years because insufficient water reached the embryo, and some of the seeds may remain in this state for 20 years! It could be argued that these are not really cases of dormancy in the strict sense, because the embryos simply do not have sufficient water, one of the basic requirements for germination; nevertheless, hard-coated seeds, in which water and oxygen entry are the factors limiting germination, are generally considered under the heading of dormancy.

Seed coat impermeability is usually caused by the presence of one or more layers of palisade cells in the testa. These palisade layers are composed of sclereid cells with thick lignified secondary cell walls, as in *Melilotus alba* (Fig. 6.5). Fruit tissues may also contain sclereid cells. Apart from lignin in the secondary cell walls, a number of other components are water repellent, including cutin, quinones, suberin, waxes, callose, phenolics, and hydrophobic (lipid-like) substances. All of these may contribute to varying extents; sometimes one of them plays a major role in waterproofing of the coat, e.g., the waxy cuticle in some species of Leguminosae.

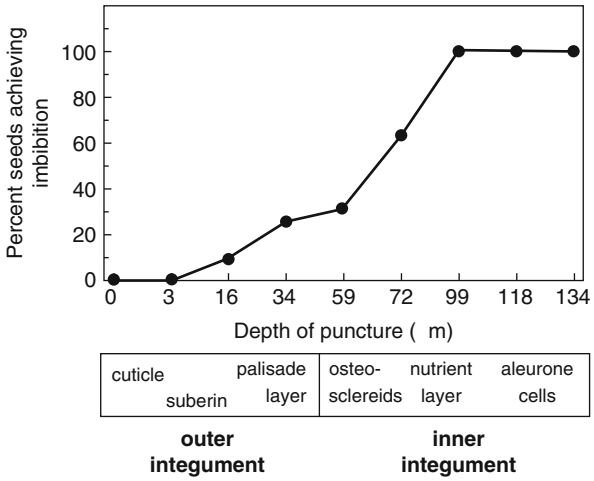

Fig. 6.6 The effect of puncturing on seed coat permeability to water. The testae of *Coronilla varia* seeds were punctured to different depths (names of the layers are noted below the graph, increasing in depth from *left* to *right*) with a fine needle and afterward placed on moist filter paper for imbibition. Adapted from McKee et al. (1977)

However, the main barrier to water uptake is offered by the osteosclereids, e.g., in coats of the vetch *Coronilla varia*. Only when this layer is punctured are most seeds capable of imbibing water (Fig. 6.6).

Some seeds contain mucilage layers that function as a water "gauge." Too much water will make the layer impermeable to oxygen, e.g., in *Blepharis*, and too little water will only hydrate the mucilage layer to a certain extent; hence there will be insufficient water reaching the embryo to support its germination.

The testa of many seeds contains specialized structures that regulate the uptake of water. These are generally derived from tissues that close the natural openings in the seed or fruit coat, such as the micropyle, hilum, and chalazal area (Sect. 1.2.3). For example, a chalazal cap is known in members of the Malvaceae and Cistaceae, a strophiole (between hilum and chalaza) in the Papilionoideae and Mimosoideae, and an operculum (derived from the micropyle) in the Musaceae. The development of seed coat impermeability is controlled genetically and also by the relative humidity of the air during the maturation phase of seed development.

6.3.2.2 Interference with Gas Exchange

The initiation of respiration is one of the early events during imbibition; this entails uptake of oxygen and release of carbon dioxide (Sect. 4.4). Any disturbance in gaseous exchange may thus lead to an inhibition of germination or maintenance of dormancy. The tissues surrounding the embryo might limit the capacity for gaseous exchange by the embryo in two ways: entry of oxygen may be impeded or escape of

carbon dioxide may be hindered. The possibility that the seed coat imposes dormancy by affecting gaseous exchange was suggested from the fact that in many cases the inhibitory action of the tissues surrounding the fully imbibed embryo is much reduced simply by scratching or puncturing them. Thus, a pinprick through the endosperm, near the radicle, of the lettuce seed or through the pericarp over the embryo of the intact, dormant wheat grain can cause some germination. Numerous cases of such effects are known and it seems unlikely that such moderate "surgical" operations can interfere appreciably with the mechanical resistance of the coat. However, at the same time, it also seems likely that such pinpricks would appreciably increase oxygen diffusion through the covering tissues because also puncturing with a needle at the opposite end from the radicle may induce germination. Stronger evidence that germination of the intact dispersal unit is prevented by insufficient oxygen is that dormancy is frequently overcome by oxygen-enriched atmospheres. These observations suggest that in some species the embryo in the intact, dormant dispersal unit fails to germinate because of the restrictions imposed by the enclosing tissues, including oxygen uptake. Removal, abrasion, or puncturing of these tissues may give the embryo access to oxygen, allowing germination to proceed to completion.

In several species permeability of the seed coat to oxygen is less than that of water. In *Sinapis arvensis*, for example, it is lower by a factor of about 10^4 and in *Xanthium pennsylvanicum* by about 10^2. The reasons for these differences are not clear in all instances, but one possibility is that there is resistance to the entry of oxygen offered by the layer of mucilage around many seeds (*S. arvensis*, for example). Another way in which the coats act is by consuming oxygen themselves. This is probably due to the enzymatic oxidation of various chemical constituents, an occurrence that is known in the testa of apple and of other seeds, where various phenolic compounds (e.g., phloridzin and chlorogenic acid) are implicated. The dormancy-imposing hull of rice and the glumellae of barley also chemically consume oxygen. As dormancy slowly diminishes during dry storage of the grains, the oxygen-consuming capacity of the hull decreases: this correlation supports the possibility that the hull imposes dormancy because it deprives the embryo of oxygen. Also correlated with the extent of dormancy in different cultivars of rice is the activity in the hull of peroxidases, enzymes that may form part of an oxygen-consuming complex. In barley grains, 40–50% of the total oxygen uptake is accounted for by the activity of the glumellae, which impose dormancy, yet this does not reduce the energy charge (essentially, ATP production) of the embryo. Hence, sufficient oxygen appears to enter it to satisfy the demand in ATP generation. Embryos of several species are satisfied by extremely low partial pressures of oxygen. For example, isolated embryos of birch, *Sinapis arvensis* and wheat can germinate even in nitrogen atmospheres! Thus, while seed coats can limit oxygen diffusion to the enclosed embryo, they do not seem to impose dormancy simply by restricting the amount of oxygen available for respiration. Oxygen microsensors have been developed that are small enough to be embedded in individual seeds; their use could help resolve the actual oxygen partial pressure inside of dormant seeds.

Nevertheless, high partial pressures of oxygen and facilitated access to air (by pricking or scratching the seed coats) still cause some intact, dormant seeds to

germinate. If the oxygen is not needed to support respiration, what is it for? Inhibitors have been invoked, in some cases, to answer this. In *Xanthium*, for example, growth inhibitors are present in the embryo, which, in the intact seed, are oxidized under high oxygen concentrations but not in air; hence, the effect of exposing intact seeds to elevated oxygen is to inactivate these inhibitors. The inhibitors can also diffuse out of the isolated embryo when it is set on a moist substratum, but they do not cross the seed coat of an intact seed. Thus, there is a twofold effect of the intact coat: primarily, it causes the retention of inhibitor, and secondarily, it can act as a barrier to oxygen, preventing the entry of sufficient oxygen from the surrounding air to support the oxidation of the inhibitor.

6.3.2.3 Prevention of Exit of Inhibitors from the Embryo

A range of chemical compounds derived from seeds or dispersal units, including phenolic acids, tannins, and coumarins, may inhibit germination. Such chemicals have been extracted from all seed and fruit parts. However, the specific role of these compounds in the inhibition of germination is not certain, for in most cases a direct relationship between content and physiological action is lacking. On the other hand, germination may be accelerated by extensive rinsing of seeds containing inhibitory chemicals with water. It is possible that many of these inhibitory substances are primarily present to avoid predation or microbial infections.

A detailed study of the seed coat pigmentation of Arabidopsis has revealed the existence of multiple ways of influencing dormancy and germination, as well as longevity, by modification of seed coat properties. From mutagenesis experiments, a series of mutants were obtained with aberrant seed coat properties, mainly in coloration, which included the so-called *transparent testa* (*tt*) mutants. Permeability of the seed coats of these was tested in tetrazolium salt, a compound that turns bright red in living tissue. Wild-type seeds displayed both dormancy and impermeability to the tetrazolium salt. In general, improved germination of the *tt* mutants was associated with a greater permeability to the tetrazolium compound. Most of the *tt* mutants displayed a reduced dormancy, perhaps due to a greater porosity of the coats to either an endogenous inhibitor (e.g., leakage of ABA) or to an exogenous stimulant of germination (e.g., uptake of oxygen) or, because of their thinner structure, the testae are less resistant to expansion of the embryo. The enhanced seed coat permeability is likely related to a reduction or absence of proanthocyanidins (condensed tannins) and the concomitant shrinkage of the testa cells.

6.3.2.4 Mechanical Restraint

Tissues surrounding the embryo will almost always impose a certain mechanical restraint to expansion of the embryo. Removal or partial removal of these tissues will often lead to normal embryo growth, indicating that the block to completion of germination is entirely located in the embryo coverings, the endosperm, perisperm,

and/or testa. The embryo may overcome the mechanical restraint by the generation of sufficient thrust but, alternatively, the restraint may be weakened by activity of the micropylar endosperm cells, controlled by the embryo, but with no apparent changes in embryonic growth potential. This has been demonstrated for a number of species in which the endosperm is digested by hydrolytic enzymes, e.g., tomato, *Datura ferox,* tobacco, and coffee (Sect. 4.6.1).

6.4 Embryonic Inadequacy: The Causes

6.4.1 Energy Metabolism of Dormant Seeds

There is a general but incorrect impression that energy metabolism is repressed in the dormant state and, hence, respiration and accumulation of ATP and NAD/NADH are likely being affected. The approach frequently taken is to compare the metabolism of dormant seeds with that of after-ripened, i.e., nondormant, ones. To ensure that the comparison is meaningful, it cannot be made between a dormant seed and one that has germinated, so only metabolism occurring in the early times after imbibition, well before radicle emergence from the nondormant seed, can be considered as relevant. Unfortunately, few studies have taken this into account.

Beginning with respiration, it seems that dormant seeds display a similar respiratory activity to nondormant material. For example, dormant and after-ripened seeds of *Xanthium* and wild oat show equivalent oxygen consumptions up to the time of emergence of the radicle from the germinable seed. In seeds of *Sisymbrium officinale,* oxygen uptake and carbon dioxide evolution are associated neither with the breaking of primary dormancy nor with the breaking or induction of secondary dormancy. Nevertheless, oxygen uptake does decrease with the induction of secondary dormancy when respiration is measured at temperatures above 20°C. The respiratory quotient RQ (O_2 uptake/CO_2 evolution) remains constant between 0.55 and 0.7, which is indicative of mostly normal respiratory pathways of oil-containing seeds, without much oxygen consumption by, for example, phenol oxidation in the testa. And hand in hand with the equal oxygen utilization by dormant and nondormant seeds, their ATP contents are comparable. However, there is evidence that it is not the absolute amount of ATP in the seeds that correlates with the dormant vs. nondormant states, but its distribution within the embryo. In tomato, there is no significant difference in ATP content between dormant and nondormant seeds, but when its distribution is visualized within them (Fig. 6.7), the ratio of ATP concentration in the radicle–cotyledons correlates with the dormant state: it is 1.1 in dormant seeds (embryos) and up to 1.7 in the nondormant ones, i.e., there is more present in the radicle of the nondormant seed.

Secondary dormant seeds held in an imbibed state for prolonged periods exhibit a progressive decline in respiration, perhaps to limit the use of their reserves which eventually will be required during and following germination. When lettuce seeds

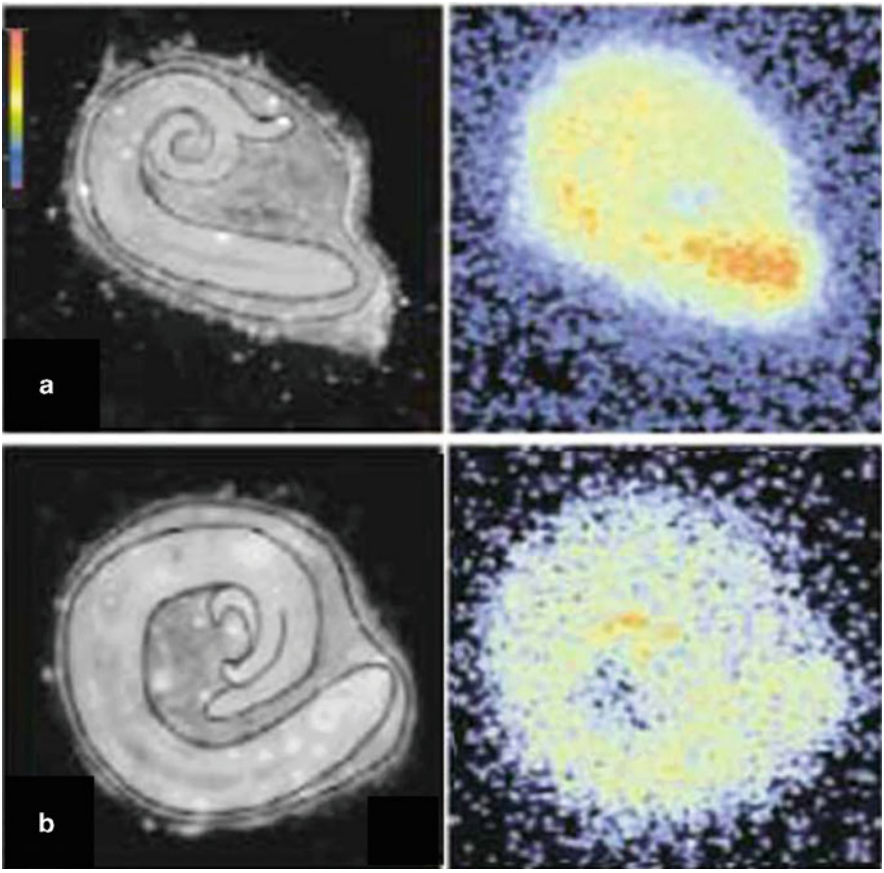

Fig. 6.7 ATP distribution in cryosections of (**a**) ungerminated 24-h-imbibed nondormant and (**b**) 5-day-imbibed dormant tomato seeds. Low (*blue*) to high (*orange*) ATP concentrations are evident in the seed, with the amount of ATP being greatest in the radicle of the nondormant embryo. Images on the *left* show positioning of embryo within the seed. Scale of color from high (*red*) to low (*blue–purple)* ATP concentrations is shown to the *left* in (**a**). From Spoelstra et al. (2002). With permission of Cambridge Univ. Press

are released from secondary dormancy there is an increase in respiration during germination, but to a considerably lower extent compared to seeds germinating following the breaking of primary dormancy.

Several other aspects of energy metabolism have received attention with respect to their possible participation in dormancy. Quantitative and qualitative differences have been recorded in some hexose and triose phosphates between dormant and nondormant seeds. Higher concentrations of fructose-2,6-bisphosphate are achieved in nondormant than in dormant grains of oats during the first few hours of imbibition.

This is a consequence of the higher amounts of phosphoenolpyruvate and glycerol-3-phosphate in dormant grains, two compounds that inhibit phosphofructokinase, the enzyme phosphorylating fructose-6-phosphate to fructose-2,6-bisphosphate. One important action of fructose-2,6-bisphosphate is to regulate gluconeogenesis through its inhibitory effect on fructose-1,6-bisphosphatase, but the possible significance of this in respect of dormancy is obscure. Changes in the concentration of this hexose phosphate have been reported only in one other species (rice) so it is not known if the phenomenon is widespread.

6.4.2 Genetic Aspects of Dormancy

Although environmental factors are important (Sect. 6.5), the entry into dormancy is also under genetic control; heritable variation of this trait has been demonstrated in many species. A wide range of dormancy is encountered in natural accessions of wild oat, for example, from types that have a short-lived dormancy to those whose dormancy is very prolonged. A vast range of accessions with various levels of dormancy is also known in Arabidopsis, which are associated with their geographical distribution. Hybridization studies with dormant and nondormant pure lines have revealed that seed dormancy may be controlled by both the maternal and paternal parent genotypes. Crossing of accessions with a large parental disparity in dormancy and the subsequent derivation of recombinant inbred lines (RIL) have provided the tools to study the genetic aspects of the natural variation in dormancy. One gene discovered in such studies is *DELAY OF GERMINATION 1* (*DOG1*), which appears to correlate very closely with the onset and depth of dormancy in Arabidopsis. *DOG1* encodes a protein of currently unknown function, but which is likely a transcription factor. Similarly, a genetic analysis of a RIL population of lettuce, combining lines with low (cv. UC96US23) and high (cv. Salinas) susceptibility to thermodormancy, revealed that the *LsNCED4* gene is largely responsible for the observed variation. This gene encodes the enzyme 9-*cis*-epoxycarotenoid dioxygenase, which is involved in ABA synthesis (Sect. 6.6.1.1). The sensitivity of *LsNCED4* expression to elevated temperature, resulting in higher ABA and lower germination, may thus determine the upper temperature limit for lettuce seed germination (Fig. 6.8). Similarly, Arabidopsis seeds germinate well at 22°C but become dormant at high temperatures (e.g., 34°C) due to an increase in *NCED9* expression and ABA synthesis. In contrast, seeds of the *nced9* mutant, which lacks this gene, are resistant to thermoinhibition.

In this context, it is important to note that dormancy can depend on an interaction between coat and embryo, and therefore three genetically distinct components are involved: (1) the diploid embryo with maternal and paternal genes, (2) the diploid testa, pericarp, and hull of maternal constitution only, and (3) the triploid endosperm bearing two sets of maternal genes. It may well be, then, that the genetics of coat-imposed dormancy are rather complex.

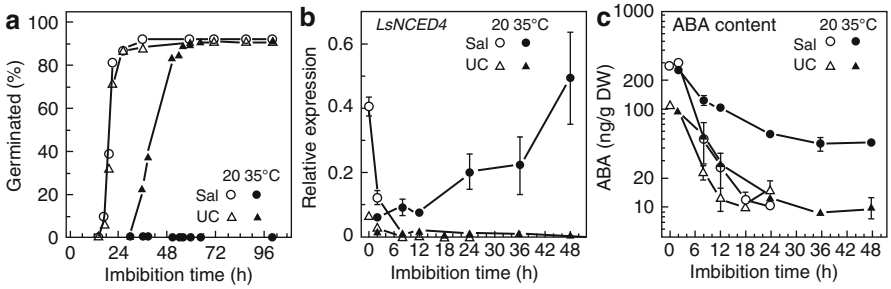

Fig. 6.8 (**a**) Germination time courses of *Lactuca sativa* cv. Salinas (Sal, *circles*) and *L. serriola* cv. UC96US23 (UC, *triangles*) seeds at 20°C (*open symbols*) and 35°C (*closed symbols*) in the light. (**b**) Relative expression (mRNA content) of *LsNCED4* in seeds of cv. Salinas and UC96US23 seeds imbibed at 20 and 35°C in the light. (**c**) ABA contents of cvs. Salinas and UC96US23 seeds during imbibition and germination at 20°C and 35°C in the light. ABA values are plotted on a logarithmic scale. Error bars indicate ± SE ($n = 3$). After Argyris et al. (2008). Copyright American Society of Plant Biologists

6.5 The Environment in Dormancy Inception

In addition to genetic factors, the environment has a profound influence on the acquisition of dormancy during seed development. The effect of environmental factors largely explains why dormancy varies with provenance. Species commonly studied by seed physiology researchers, such as Arabidopsis, are notorious in this respect, and one cannot assume that a particular batch will have any dormancy at all: this depends on where it was grown and what environmental factors were operative at the time of its development and maturation. What are these factors that can have such a profound effect on the development of dormancy? Some of the major ones are discussed below.

(1) Soil. This edaphic influence (i.e., that of soil characteristics rather than of the climate) can play a role in the establishment of dormancy. A clear example is the uptake and distribution of nitrate, an important regulator of dormancy and germination (Sect. 7.2.4). The nitrate content of mature seeds depends on the amount of this anion taken up by the mother plant and allocated to the seeds. This, in turn, depends, among other things, on soil moisture and nitrate content, the nitrate reductase activity in the roots of the mother plant, and activity of a seed-specific nitrate transporter, such as the *Arabidopsis thaliana* NITRATE TRANSPORTER2.1 (AtNRT2.1). The amount of nitrate accumulated in dry seeds is directly related to the amount of nitrate in the soil during their development and determines the extent of their germination (Fig. 6.9a, b). There is very little information about the possible influence of other nutrients on dormancy acquisition, such as phosphate, cations such as sodium and potassium, or micronutrients such as iron, zinc, and copper.

(2) Temperature. Much more is known about the effects of temperature on the acquisition of dormancy during seed development. In general, its occurrence is

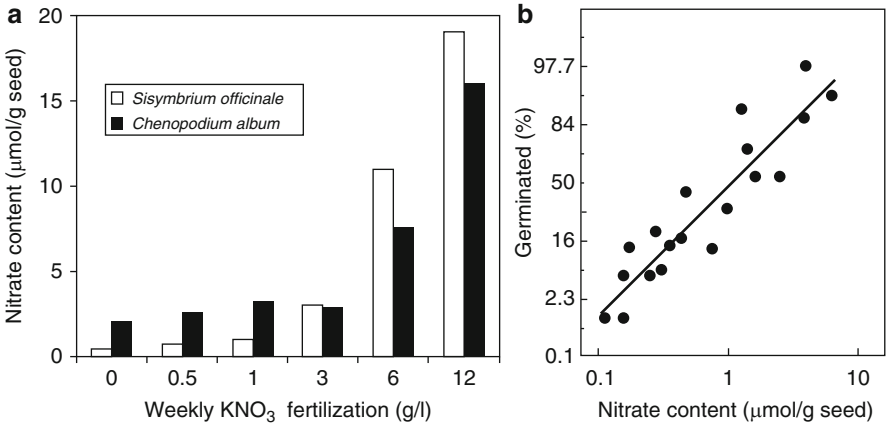

Fig. 6.9 (**a**) Effect of potassium nitrate fertilization of *Chenopodium album* and *Sisymbrium officinale* plants on the nitrate content of produced seeds. The plants were cultivated in plots in an open field. (**b**) Nitrate content and germination of *Sisymbrium officinale* seeds. Seeds were taken from 20 lots, derived from plants grown in liquid culture or in the field, treated with red light and germinated at 24°C. Seed nitrate content is plotted on a log scale and germination percentage on a probit scale. From Hilhorst and Karssen (2000). Courtesy of CAB International

promoted by relatively low temperatures, as in *Rosa* spp., in grasses such as wild oats, and the cereals, wheat, and barley. In line with this, Arabidopsis seeds developing under cool or warm temperatures display high and low dormancy, respectively (Fig. 6.10a). A global gene expression (transcriptomic) analysis of the dry seeds has identified a low-temperature-regulated set of genes, including *DOG1, GA2ox6*, and *NCED4* (Fig. 6.10b), that are strongly associated with dormancy. *GA2ox6* encodes the enzyme GA-2-oxidase, which is a component of the inactivation pathway of gibberellins, whereas DOG1 and NCED4 are involved in the acquisition of dormancy and ABA biosynthesis, respectively (Sect. 6.6.1.1). In contrast, the lower level of dormancy of seeds matured at higher temperature is associated with elevated expression of *CYP707A2* encoding cytochrome P450 707A2, which is a principal component of ABA catabolism (Fig. 6.10b). It is clear that, at least in Arabidopsis, the control of dormancy by the maternal environment is mediated by a GA–ABA balance (Sect. 6.6.1.4). However, there are species in which seed dormancy is induced by elevated temperatures. *Syringa vulgaris*, for example, can be made to produce dormant seeds by holding the mother plant at relatively high temperatures (18–24°C) during the last week of seed maturation, a treatment that appears to make the endosperm (the tissue imposing dormancy) tougher.

(3) Light. Light quantity, its daily distribution, and spectral quality can all have a profound influence on the development of dormancy. Photoperiodic effects on the inception of seed dormancy are well known in several species, and *Chenopodium* spp. provide good examples. *C. album* (Fig. 6.11) has deeply dormant seeds when the fecund plants are held under long days, but nondormant seeds under short days. Not only is the dormancy pattern affected by day length but also the structure of the

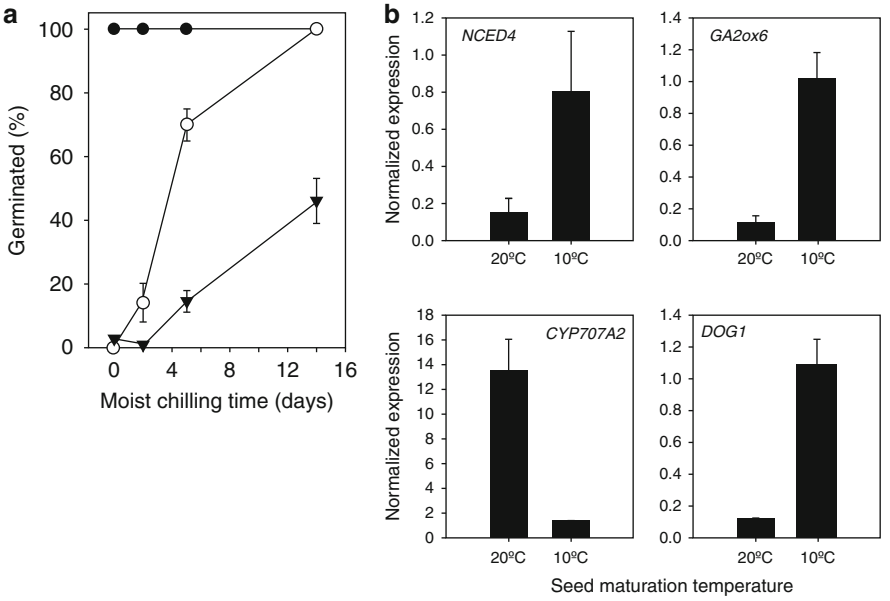

Fig. 6.10 (**a**) Germination of freshly harvested wild-type Arabidopsis Columbia-0 seeds matured at (▲) 10°C, (○) 15°C, and (●) 20°C in response to moist chilling. (**b**) Gene expression changes in Arabidopsis seeds developed at 10 or 20°C. Low seed maturation temperatures induced *GA2ox6, DOG1*, and *NCED4* expression, whereas warm seed maturation temperatures induce high *CYP707A2* expression, the transcripts of which were present in mature dry seeds. From Kendall et al. (2011). Copyright American Society of Plant Biologists

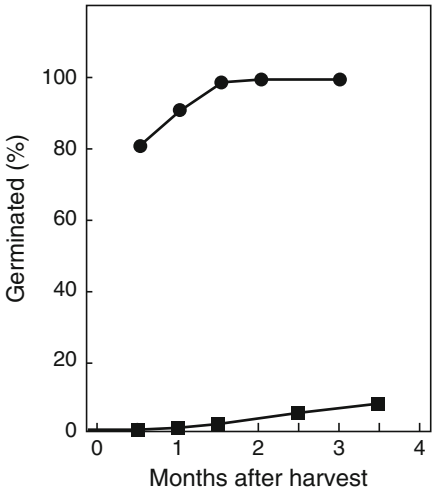

Fig. 6.11 Germination of *Chenopodium album* seeds that developed under different photoperiods. Plants with developing seeds were held under short days or long days. After harvesting and being maintained in the dry state, germination of the seeds was tested at intervals of several months. Seeds developing and maturing under short days (●) have a high germination percentage whereas those from long-day-treated plants (■) have a dormancy that lasts for at least 3.5 months. All germination tests were carried out in darkness. Adapted from Karssen (1970)

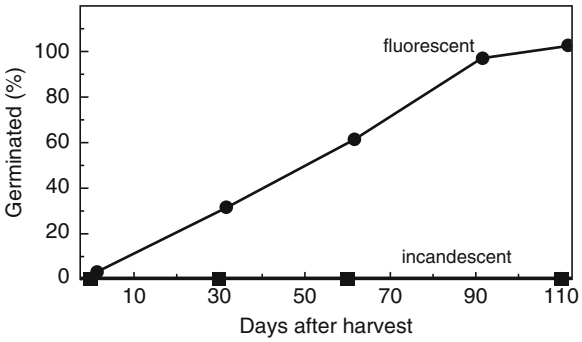

Fig. 6.12 Light quality during seed maturation and its influence on *Arabidopsis thaliana* dormancy. Seed-bearing plants were kept in white incandescent or white fluorescent light during seed maturation. Germination was subsequently tested in darkness. Seeds maturing in white fluorescent light (●) have less dormancy than those maturing in incandescent light, which are deeply dormant for more than 100 days after harvest (■). Adapted from Hayes and Klein (1974)

seeds, for those maturing in long days are smaller and thicker coated than those in short days. Dormancy induced by photoperiod is not always associated with coat thickness, however, since a short-term dormancy of seeds with thin coats is brought about by long photoperiods given for just a few days after the end of flowering. An effect of photoperiodic conditions on coat structure is also seen in seeds of several other species. Seeds of Arabidopsis matured under short-day conditions are in general less dormant than those ripened under long days. However, there is a strong dependency on accession type and temperature for the expression of dormancy. An interaction of flowering time and dormancy appears to be pivotal for germination timing and, hence, dispersal and survival of the species (Sect. 7.4.2). Light, quite apart from photoperiod, has an important role in dormancy induction in several species. Seeds of Arabidopsis maturing in white fluorescent light lose their dormancy with time after harvesting, whereas those which have experienced incandescent light during maturation remain deeply dormant for at least several months (Fig. 6.12); similar results have been reported for lettuce. This is because white fluorescent light is relatively rich in the red wavelengths whereas incandescent light has a relatively high component of far-red light. Under the former illumination conditions, more of the active Pfr form of phytochrome (Sect. 6.6.5) accumulates in seeds than under the latter type of light. Seeds with a high Pfr content can often germinate in darkness because they have already exceeded the required threshold of Pfr for germination, while those with a low Pfr concentration remain dormant. Treatment with far-red light alone, of course, has the same effect: cucumber seeds are made dormant when fruits are irradiated with far-red light. It appears that this kind of phenomenon also occurs in nature, where the source of far-red light is light filtered through green tissues (Sect. 7.2.3.1). Chlorophyll absorbs red light (peak ca. 660 nm) but not wavelengths longer than about 710 nm; hence the transmitted light, being rich in the far-red component, serves to lower the amount of Pfr.

6.6 The Release from Dormancy

Because seeds can germinate only once, those of many species have evolved sophisticated means to sense the environment and determine the right moment to proceed towards the completion germination. Flowering time and dormancy are the principal cues that determine germination timing. Thus, in many instances there are seasonal components involved in the ultimate "decision" of a seed to germinate, which will be discussed in Chap. 7. Here the different factors by which dormancy is terminated are detailed while making a distinction between their long-term and short-term efficacies. For example, dry after-ripening and moist chilling are often associated with seasonal length (i.e., the temporal environment), whereas light and chemicals may be active instantaneously and appear to be indicators of the prevailing (or spatial) environment.

For simplicity, the different factors are discussed separately but it must be appreciated that in nature a seed is not subject to the influence of just one factor but to several simultaneously. For example, the release of *Arabidopsis* seeds from primary dormancy requires the removal of several "blocks" to germination, including after-ripening, chilling, and exposure to nitrate and light, often in a certain order. Thus, the termination of dormancy in the field is not likely to be the prerogative of just one factor in the seed's environment but will be influenced by several, and in some circumstances seeds of the same species might have their dormancy ended by different cues.

6.6.1 Perception, Signaling, and Role of Hormones with Respect to Dormancy and Germination

Before the diverse environmental factors affecting release from dormancy are discussed, this section focuses on the function and quantitative changes in the expression of genes associated with the metabolism of the major seed hormones which suppress or promote germination. Hormones play a vital role in dormancy maintenance or release in response to the environment, so consideration is given here to their synthesis and catabolism, and to the mechanisms by which cells perceive their presence and respond appropriately. Although there are other dormancy-breaking chemicals, GA and ABA in particular have a profound influence on the dormancy and germination of seeds. Whether or not the latter is completed is effected during Phase II of germination (Fig. 4.1) by hormone biosynthetic and catabolic enzymes, whose abundance is controlled primarily at the level of transcription. Also important is perception of the hormones by receptors and subsequent signal transduction pathways to influence gene expression. Therefore, identifying hormone metabolism genes and analyzing their expression patterns are critical to understanding the control of dormancy and germination. Posttranslational modifications of signaling proteins by phosphorylation or dephosphorylation, and ubiquitination, which affects their stability, play critical roles. Cold, light, and other effectors, such as smoke and

soil nitrate, affect dormancy and germination, at least in part, through hormone metabolism and signal transduction. Cross talk between environmental signals and hormonal regulation provides a complex integrated network through which seeds can adjust their germination potential to match ecological opportunities.

6.6.1.1 Regulation by ABA

According to the hormone balance theory, the relative actions of ABA (inhibitory) and GA (promotive) are the primary determinants of seed dormancy and germination. The relative activities of ABA and GA are in turn a result of the amounts of each present and the sensitivity of the target cells or tissues to them, dependent on their respective perception and signaling pathways. The amounts of these hormones in seeds are regulated by their rates of synthesis and deactivation. ABA is produced via the carotenoid pathway, and β-carotene is an important upstream substrate in its synthesis (Fig. 6.13a). This is converted to other forms of C40 carotenoids, such as zeaxanthin and violaxanthin, which are eventually processed into xanthoxin, a C15 precursor of ABA, via oxidative cleavage. While active ABA is produced from ABA aldehyde by ABA aldehyde oxidase (AAO), the rate-limiting (most critical) step in ABA synthesis is the conversion of 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin to xanthoxin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Fig. 6.13a). Therefore, the rate of ABA production, which is associated with the induction and maintenance of seed dormancy, is considered to be regulated primarily by NCED.

The *NCED* gene was first identified during characterization of the maize mutant *viviparous 14* (*vp14*) (Sect. 2.4.2) and is now known to be present in many other species. In addition, multiple *NCED* family members are present within a species, providing opportunities for temporal and tissue-specific expression. For example, because ABA is involved in both seed germination and in responses to water stress, those functions often involve different *NCED* gene family members. Even in the seed, *NCED6* is expressed specifically in the endosperm while *NCED9* is expressed in the endosperm/testa and in the peripheral cells of the embryos of developing Arabidopsis seeds, suggesting the involvement of both of these regions in the induction and maintenance of seed dormancy. When *NCED6* gene expression is experimentally induced in imbibed nondormant seeds, germination is suppressed. This indicates that the carotenoid and ABA biosynthesis pathways are operating in nondormant seeds, and that the substrates for NCED are being synthesized even in seeds that are capable of completing germination. This ready availability of substrate for ABA synthesis is probably important in allowing seeds to respond quickly to produce the inhibitor when there are environmental changes, and also in the induction of secondary dormancy under adverse conditions (e.g., by high temperatures; Sect. 6.4.2). Thus, NCED can be regarded as a “rate-limiting” enzyme for ABA synthesis in seeds and is important in the control of their germination.

Continuous ABA synthesis is apparently necessary for the suppression of germination because carotenoid biosynthesis inhibitors such as fluridone and norflurazon promote the germination of dormant seeds. The carotenoid biosynthesis pathway is

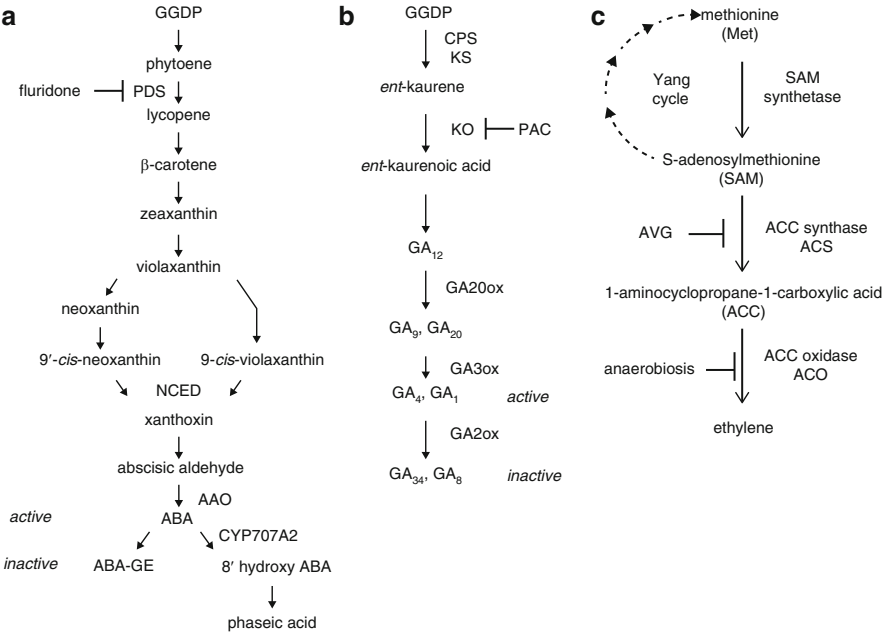

Fig. 6.13 Hormones involved in the regulation of germination and dormancy. (**a**) Abscisic acid (ABA) is derived from geranyl-geranyl diphosphate (GGDP) through the carotenoid pathway. The conversion of 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin to xanthoxin is the rate-limiting (most critical) reaction, which is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED). The conversion of abscisic aldehyde to active ABA is catalyzed by ABA aldehyde oxidase (AAO). ABA is then deactivated by CYP707A2, an 8′-hydroxylase, or by conjugation with glucose to form ABA glucose ester (ABA-GE). ABA biosynthesis can be blocked by fluridone, an herbicide that inhibits phytoene desaturase (PDS), an enzyme that functions in the upstream, carotenoid biosynthesis pathway. (**b**) Gibberellin (GA) biosynthesis is initiated by the conversion of GGDP to *ent*-kaurene by coparyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS); kaurene is then converted to *ent*-kaurenoic acid by kaurene oxidase (KO). The rate-limiting reaction in the GA biosynthesis pathway is the last step of the conversion of an inactive to an active form, for example, GA_9 to GA_4 in Arabidopsis seeds and GA_{20} to GA_1 in lettuce seeds, both of which are catalyzed by GA-3-oxidase (GA3ox). GA-2-oxidase (GA2ox) catalyzes the deactivation of active GAs to the inactive forms GA_{34} and GA_8. Paclobutrazol (PAC) inhibits KO, and hence GA biosynthesis. (**c**) Ethylene is derived from S-adenosylmethionine (SAM), which is converted to 1-aminocyclopropane carboxylate (ACC) by ACC synthase (ACS). ACC, in turn, is converted to ethylene (and CO_2 and cyanide) by ACC oxidase (ACO). Methionine is regenerated for reuse in the ethylene pathway via the Yang cycle and SAM synthetase. ACS can be inhibited by aminoethoxyvinyl glycine (AVG), and ACO can be inhibited by anaerobiosis. The structures of ABA, GA and ethylene are shown in Fig. 2.7. Only representative intermediates in the pathways are shown. A single arrow does not necessarily represent a single step in the pathway. (⊣) indicates the sites of action of inhibitors typically used in seed biology experiments. Modified from Yamaguchi et al. (2007)

upstream of that for ABA synthesis (Fig. 6.13a); therefore, blockage of the former hinders the latter and as a consequence alleviates seed dormancy.

The ABA content of seeds is controlled not only by its synthesis but also by its deactivation. One of the many cytochrome P450s, CYP707A2, is an ABA-8′-

hydroxylase and deactivates ABA, reducing its content in seeds and releasing seed dormancy. *CYP707A2* is expressed during early germination of nondormant seeds of Arabidopsis, for example, while those of the *cyp707a2* mutant that are defective in ABA deactivation exhibit hyperdormancy. Thus CYP707A2 is a key regulatory enzyme for ABA deactivation and dormancy release, although ABA can also be deactivated through its conjugation to sugar (e.g., ABA-glucose esters) by ABA glucosyltransferase.

Developing and imbibed mature seeds respond promptly to changes in ABA concentration because they contain the components for hormone perception and response. The hormone is initially perceived by binding to the ABA receptor PYRABACTIN RESISTANCE1 (PYR1), followed by a sequence of downstream events involving multiple proteins and their modification (Fig. 6.14). In the absence of ABA, protein phosphatase 2C (PP2C) binds to and represses Sucrose non-fermenting-Related Protein Kinase 2 (SnRK2), a positive regulator of ABA responses. The ABA-receptor protein changes its conformation upon binding to the hormone and then binds PP2C, releasing SnRK2 from its inhibition (Fig. 6.14). SnRK2 then phosphorylates downstream target proteins, such as ABRE BINDING FACTOR (ABF), which then induce ABA responses, including inhibition of seed germination and dormancy. Seeds of mutants lacking the ABA receptor (e.g., *pyr1*), PP2C (e.g., *abi1*, *abi2*), SnRK2 (*snrk2.2/snrk2.3* double mutants), or ABF (e.g., *abi5*) exhibit ABA-insensitive germination phenotypes, i.e., they complete germination even in the presence of the inhibitor.

These basic mechanisms of ABA metabolism are conserved among species, and are common to seeds of monocots and dicots. In barley, the expression of *HvNCED* (*Hordeum vulgare NCED*) maintains a high ABA content in grains and imposes dormancy. In contrast, the expression of *HvABA8′OH-1* (encoding the ABA-deactivating ABA-8′-hydroxylase), an ortholog of the Arabidopsis *CYP707A2* gene, is associated with dormancy release. This gene is expressed mainly in the coleorhiza, which surrounds the radicle tip, and is probably associated with weakening of this covering tissue in the absence of ABA (Sect. 4.6.1).

6.6.1.2 Regulation by GA

GA is involved in many aspects of plant development in addition to germination, including vegetative growth, flowering, and pollen production. Typical symptoms of GA-deficient mutants are dwarfism and reduced or completely suppressed germination. There are many forms of GA molecules in plants, the majority of which are inactive and are intermediates in the GA biosynthetic pathway. Here, only key steps in the pathway that are critical to an understanding of seed dormancy and germination are considered.

The GA biosynthetic pathway shares a common precursor, geranyl-geranyl diphosphate (GGDP), with that for ABA (Fig. 6.13b). GGDP is converted to *ent*-kaurene by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). The *ga1* mutant in Arabidopsis is defective in CPS and synthesis of GA is inhibited; the seeds are absolutely dependent on an exogenous supply of the hormone for germination.

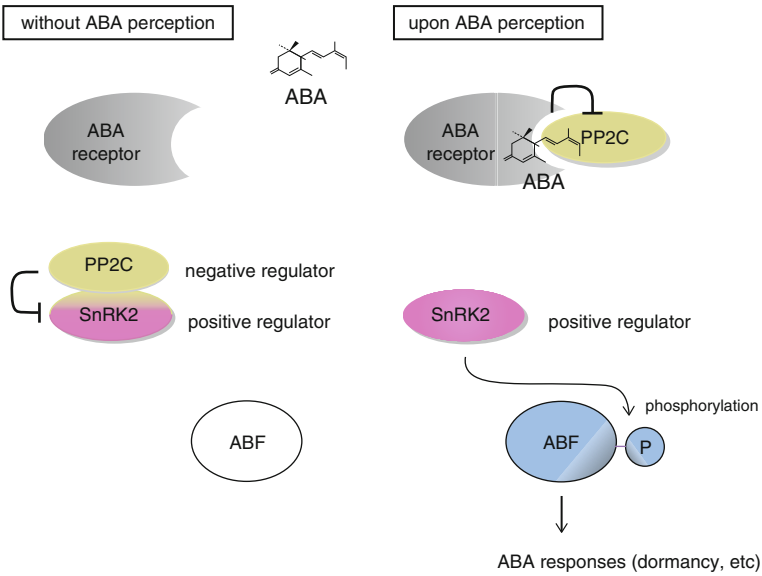

Fig. 6.14 ABA perception and signaling in the control of seed germination. In the absence of ABA, protein phosphatase 2C (PP2C), a negative regulator of ABA responses, binds to and suppresses protein kinase (SnRK2: Sucrose Non-fermenting-Related Protein Kinase 2), a positive regulator of ABA responses. Upon ABA perception, ABA receptors such as PYRABACTIN RESISTANCE 1 (PYR1, also called RCAR: regulatory components of ABA receptor) change their conformation, forming a pocket to which PP2C binds. In this way, PP2C function is suppressed, which releases and activates SnRK2. The downstream factors such as ABRE BINDING FACTOR (ABF) are then phosphorylated (P) and activated by SnRK2, which triggers ABA responses including germination suppression and dormancy. Based on Park et al. (2009)

Notably, the *ga1* embryo is able to grow when excised from its surrounding structures, indicating that the lack of CPS does not affect seed viability, but rather the ability of the embryo to overcome coat-imposed dormancy (Sect. 4.6.1). The tomato *gib-1* mutant has a similar defect and its seeds also require exogenous GA to complete germination. However, the germinated seeds produce dwarfed seedlings. GA-deficient mutants of other species, such as rice, still are able to complete germination without the addition of GA. The reason for this is unknown, but other factors such as the properties of the covering tissues, or ABA metabolism and signaling, might play a role.

ent-Kaurene is converted to *ent*-kaurenoic acid by *ent*-kaurene oxidase (KO) (Fig. 6.13b). Plant growth retardants, such as paclobutrazol (PAC), uniconazole-P, tetcyclacis, and ancymidol, inhibit KO and when applied to seeds cause a decline in GA content and prevent germination. However, caution is required in the interpretation of data from experiments using these inhibitors because they not only influence a specific enzyme involved in GA synthesis but can affect others, such as those involved in ABA deactivation.

ent-Kaurenoic acid is converted to several inactive forms of GA before GA_{12} is converted to GA_9 by GA-20-oxidase (GA20ox). The last step in the pathway

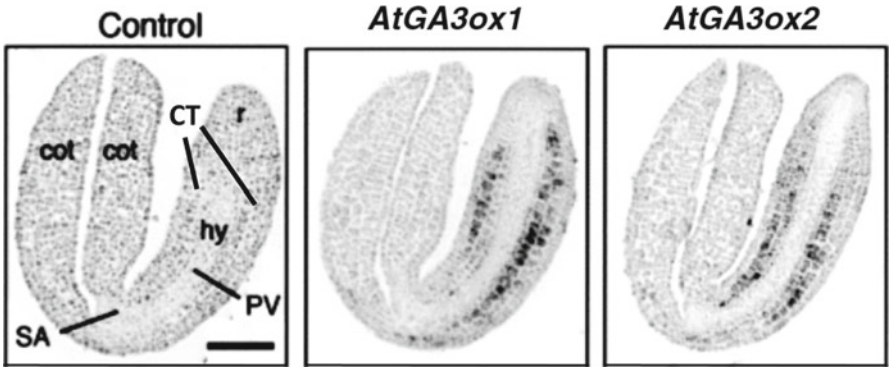

Fig. 6.15 Expression of the GA biosynthetic genes (*AtGA3ox1* and *AtGA3ox2*) in the radicle/hypocotyl region of Arabidopsis embryonic axes. The photographs are of longitudinal sections of the embryos, with the dark signals indicating gene expression. The localization of mRNAs was detected by hybridization with antisense RNA probes and visualized with a colorimetric enzyme reaction. No signal was detected in Control sections (hybridized with a sense *GA3ox1* probe), whereas strong signals were detected in the cortical cells of the hypocotyl using the antisense probes for both genes. cot, cotyledon; CT, cortical tissue; hy, hypocotyl; PV, provasculature; r, radicle; SA, shoot apical meristem. Scale bar = 100 μm. Modified from Yamaguchi et al. (2007). Courtesy of Wiley-Blackwell

(GA_9 to GA_4 in Arabidopsis; GA_{20} to GA_1 in lettuce) is the rate-limiting one, which is catalyzed by GA-3-oxidase (GA3ox) (Fig. 6.13b). There are two major genes for GA3ox in Arabidopsis seeds; *ga3ox1* or *ga3ox2* single mutant seeds can still complete germination while those of the *ga3ox1 ga3ox2* double mutant cannot. This indicates that these two genes play overlapping (redundant) roles in the rate-limiting step of GA synthesis, with one substituting for the other when it is mutated. When both are mutated, however, GA synthesis is prevented. Genes encoding *GA3ox* are expressed in the embryonic axis and are most likely associated with the generation of embryo growth potential (Sect. 4.6.1).

It is noteworthy that in Arabidopsis two key genes for enzymes of the GA biosynthesis pathway (*AtGA3ox1* and *AtGA3ox2*) are expressed specifically in cells in the hypocotyl transition zone immediately behind the radicle that will expand during germination (Figs. 4.19, 6.15), which is suggestive of an intimate association between GA synthesis and cell expansion.

There are more than 130 different structures of GA molecules in plants, the majority of which are precursors and/or inactive forms, including GA_9. Only a small number, such as GA_4 and GA_1, are active, and vary among species. For example, GA_4 and GA_1 are the active endogenous GAs in Arabidopsis and lettuce, respectively, although many seeds respond positively to application of the commercially available GA_3.

When active GA is produced excessively or is not required, it is converted into an inactive form (GA_4 to GA_{34} or GA_1 to GA_8; Fig. 6.13b). The major enzyme catalyzing these reactions is GA-2-oxidase (GA2ox), which reduces the GA content in

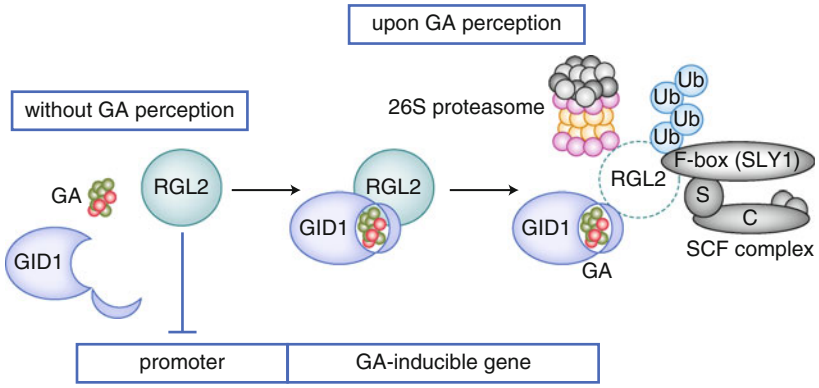

Fig. 6.16 GA perception and signaling for seed germination control. RGA-LIKE2 (RGL2) suppresses expression of GA-inducible genes and germination in the absence of GA. GA binds to the GA receptor GA-INSENSITIVE DWARF (GID1), which triggers its interaction with RGL2. This then triggers ubiquitination (Ub) of RGL2 by SLEEPY1 (SLY1), an E3 ubiquitin ligase component of the SCF (Skp, Cullin, and F-box) complex. Ubiquitinated RGL2 is recognized by the 26S proteasome and is degraded or inactivated. In this way, the repression of GA-inducible genes and of seed germination by RGL2 is removed. Based on Seo et al. (2009)

seeds and negatively affects germination. GA deactivation by GA2ox plays an important role in the regulation of seed germination by light (Sect. 6.6.5). GA can also be deactivated by GA methyltransferase, although this enzyme functions mainly during seed development.

Identification of GA metabolism genes and analysis of their expression have contributed to an understanding of the mechanisms underlying seed responses to environmental signals. For example, high temperatures inhibit germination of Arabidopsis seeds not only through the promotion of *NCED* expression and ABA synthesis but also through the suppression of expression of *GA20ox* and *GA3ox,* and hence of GA synthesis.

As with ABA signaling, the GA signal transduction pathway involves multiple protein components and their modification. GA-inducible genes that are most likely required for germination completion are suppressed in the absence of this hormone by RGA-LIKE2 (RGL2), a seed-germination-repressor protein. RGL2 and its homologs also are called “DELLA” proteins due to a characteristic amino acid sequence that they contain. GA binds to its receptor GA-INSENSITIVE DWARF (GID1), which then interacts with RGL2 (Fig. 6.16). RGL2 is then recognized by SLEEPY1 (SLY1), an E3 ubiquitin ligase that is a component of the SCF (Skp, Cullin, and F-box) complex, which attaches ubiquitin peptides to RGL2. The ubiquitinated RGL2 is recognized by the 26S proteasome complex and is degraded or inactivated. This removal of repression (i.e., de-repression) is central to dormancy release and germination induction. Similar posttranslational modifications play an important role also in the perception and transduction of light signals during dormancy release (Sect. 6.6.5).

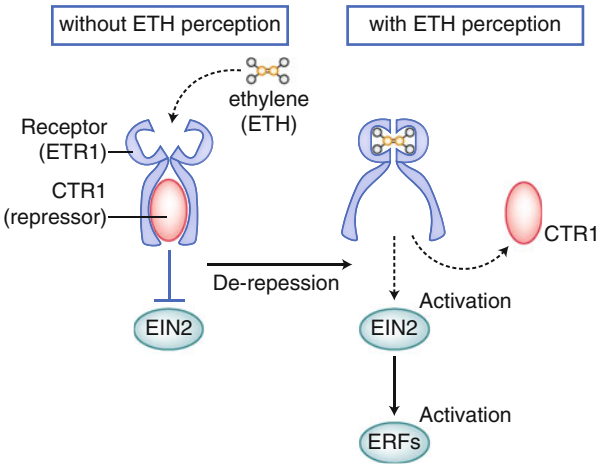

Fig. 6.17 Ethylene perception and signaling pathway for seed germination control. ETH receptor protein (ETR1, also called an ERS) dimers interact with the CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) protein that represses the positive regulator EIN2 (*left*). When ETH binds to the receptor protein (*right*), conformational changes result in the release and deactivation of CTR1, freeing EIN2 from repression. This results, through a number of intermediate kinases and proteins, in production of ETHYLENE RESPONSE FACTOR proteins (ERFs) that upregulate transcription of genes associated with germination

6.6.1.3 Regulation by Ethylene and Brassinosteroids

While ABA and GA are the primary inhibitory and promotive hormones in regulating seed dormancy and germination, other hormones and compounds also play roles in these processes, often through their interactions with the ABA/GA biosynthetic and regulatory pathways. Ethylene (ETH), in particular, often exhibits a promotive effect on germination. Ethylene (C_2H_4) is a gaseous hormone that is synthesized from S-adenosylmethionine (SAM) in only two enzymatic steps. The enzyme ACC synthase (ACS) converts SAM to the unusual cyclic amino acid 1-aminocyclopropane carboxylate (ACC), which is then converted to ETH in the presence of oxygen by ACC oxidase (ACO) (Fig. 6.13c). Expression of genes encoding ACS and ACO and release of ETH often increase during germination in parallel with expression of *GA20ox* and *GA3ox*. Provision of ETH (or ACC) can improve the germination of some seeds, particularly under stressful conditions, while mutations in ACO have been shown to reduce germination capacity.

As for ABA and GA, the signal transduction pathway for ethylene also involves specific receptors that remove downstream inhibitors and thus de-repress germination. In the absence of ETH, the ETH receptor proteins (ETR or ERS) interact with a kinase protein (CONSTITUTIVE TRIPLE RESPONSE 1, CTR1) that represses ETHYLENE INSENSITIVE 2 (EIN2), a positive regulator of downstream signaling processes (Fig. 6.17). When ETH binds to the receptor proteins, this repression of

EIN2 by CTR1 is removed, triggering a signaling cascade that results in production of ETHYLENE RESPONSE FACTOR proteins (ERFs) that upregulate transcription of genes associated with germination. A number of genes expressed late during germination, just prior to radicle emergence, including for pathogenesis- and wounding-related proteins such β-1,3-glucanase and chitinase, are regulated by ERFs. Mutations in the ETH signaling pathway were isolated in screens to restore ABA sensitivity in ABA-insensitive mutants, suggesting that ETH acts by reducing the sensitivity of germination to inhibition by ABA.

Brassinosteroids (BRs) are another group of plant hormones that can promote germination (Fig. 2.7). BRs are involved in various aspects of plant growth and development, including flowering time, plant architecture, seed yield, and stress tolerance. BR-insensitive and BR-deficient mutants are more sensitive to inhibition of germination by ABA, and BR treatment rescues germination of seeds having defects in GA biosynthetic enzymes, as can mutations in ABA biosynthetic enzymes. Thus, like ETH, BRs apparently promote germination at least in part by reducing the sensitivity of seeds to inhibition by ABA.

6.6.1.4 ABA–GA Balance and Hormonal Cross Talk in the Regulation of Dormancy

The inhibitory and promotive effects of ABA and GA on seed dormancy and germination have been known for several decades. Central to our current understanding of how they interact is the fact that there is mutual regulation or "cross talk" between these two hormones, i.e., ABA regulates GA metabolism and signal transduction while GA reciprocally affects ABA metabolism and signal transduction.

The expression of *GA3ox* and *GA20ox*, GA synthesis genes, is elevated in seeds of the Arabidopsis ABA-deficient mutant *aba2-2*, suggesting that GA synthesis is normally suppressed by ABA in the wild type. Furthermore, the expression of *GA2ox*, which results in GA deactivation, is reduced in these mutant seeds, which suggests that GA deactivation is promoted by ABA in wild-type seeds (Fig. 6.18). These results indicate that there is double regulation of GA metabolism by ABA, i.e., suppression of its synthesis and promotion of its deactivation, both of which result in less GA in the seeds and lower completion of germination.

On the other hand, GA can also influence ABA metabolism and signal transduction (Fig. 6.18). RGL2, a key DELLA component of the GA signal transduction pathway and a germination repressor, stimulates ABA biosynthesis and the activity of ABI5, one of the ABFs in the ABA signaling pathway (Fig. 6.14). Since RGL2 is subjected to degradation or inactivation upon GA perception (Fig. 6.16), the hormone causes a decrease in RGL2 activity and hence also in ABA synthesis and signaling, both of which have a positive effect on germination. The multiple layers of regulation involved in the ABA–GA balance in seeds seem to function in such a way that each signal is amplified rapidly (i.e., GA triggers an increase in GA content and response by eliminating ABA production and signal transduction, or ABA increases its synthesis and signal transduction by causing a decline in GA). This rapid

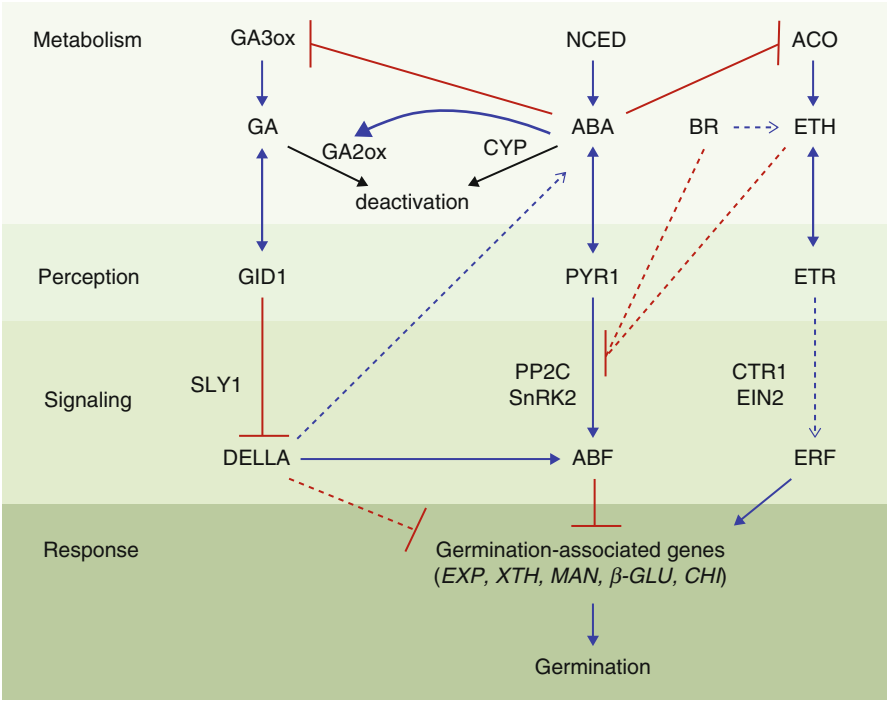

Fig. 6.18 Interactions and cross talk between GA, ABA, ETH, and BR metabolism, perception, signaling, and response pathways. Examples of regulated proteins are shown to indicate the respective pathways for hormone production (GA3ox, NCED, ACO) or deactivation (GA2ox, CYP [CYP707A]). Receptors for GA (GID1), ABA (PYR1), and ETH (ETR) interact with the respective hormones and lead to de-repression of downstream signaling pathways involving the indicated intermediates. These pathways result in either the removal (in the case of DELLAs [e.g., RGL2]) or activation (ABFs [e.g., ABI5] or ERFs [e.g., ERF1]) of transcriptional regulators of germination-related genes (e.g., *EXP*, expansins; *XET*, xyloglucan endotransglycosylases; *MAN*, endo-β-mannanase; *β-GLU*, β-1,3-glucanase; *CHI*, chitinase). *Blue arrows* indicate promotion of the indicated interaction and *red bars* indicate repression. *Dashed lines* indicate where the interaction is known, but the specific mechanisms involved are not

amplification of hormonal signals may be important for seeds to respond to environmental cues promptly and thus to commence or suspend germination. Altering the ABA–GA balance in seeds is also an important part of light signal transduction for germination control (Sect. 6.6.5).

Ethylene and brassinosteroids also influence the ABA signaling pathway. Both hormones act by as yet unclear mechanisms to reduce the ability of ABA to inhibit germination, apparently by acting downstream of ABA synthesis in its signal transduction pathway (Fig. 6.18). In turn, ABA negatively regulates the transcription of *ACO* genes, reducing ethylene synthesis. Although not confirmed specifically in seeds, BR can also elevate ETH biosynthesis, suggesting that BR may also act indirectly through enhancing ETH action.

The picture that emerges is one of extensive cross talk and interaction among the primary hormones regulating germination. Rather than thinking of these as independent pathways, they more likely constitute a network of reciprocally interacting regulatory factors that are constantly adjusting the relative strengths of the different inputs to the downstream transcriptional regulators. These master integrators (e.g., DELLAs, ABFs, ERFs) can then shift transcriptional patterns between dormancy or germination modes in response to internal or external cues (Sects. 4.5.1, 7.3.2).

6.6.2 After-Ripening

Seeds of many species require variable periods of dry after-ripening or moist chilling ("cold stratification") to relieve their dormancy, varying from as little as a few weeks (e.g., barley) to as long as 60 months (e.g., *Rumex crispus*). Under natural conditions dry after-ripening may occur in winter annuals in which dormancy is broken by high summer temperatures in order to make the seeds germinable in the fall, whereas moist chilling is effective in many summer annuals to break dormancy during the cold winter months. However, this distinction is not absolute. For example, dormancy in an Arabidopsis accession from the Cape Verde Islands (Cvi), a winter annual, can also be broken by a short period of cold stratification. The rate of after-ripening and decrease of dormancy can vary, depending on environmental conditions during seed maturation, seed storage and germination conditions.

After-ripening occurs in dry dormant seeds. Seeds are considered "dry" when they have less than ~20% water content (dry weight basis; <~−20 MPa) (Sect. 8.4.1). In this range (hydration level II), seeds contain both strongly (<~4%) and weakly bound (4–20%) water but there is no free water available for enzymatic catalysis and other biochemical events (Figs. 2.29, 8.9). The efficacy of after-ripening depends on the environmental conditions—moisture, temperature, and oxygen. Since it generally occurs in seeds below a certain water content, it may be prevented at higher seed water contents. Indeed, at intermediate moisture contents (>20–40%, or >−20 MPa to −8 MPa) not only might after-ripening fail but the seed may also lose viability (Sect. 8.2.2), and at higher water contents dormancy is maintained or secondary dormancy may be induced (Sect. 7.3). On the other hand, if seeds become too dry (e.g., <4% water content or <~−300 MPa), after-ripening is delayed or prevented (Sect. 7.2.1.3).

Besides moisture, the rate of after-ripening depends on the temperature. Hydrotime- and hydrothermal-time models can be used to study its progress and predict its duration (Sect. 7.2.1.2). This is particularly useful to monitor and accelerate the loss of dormancy in agriculturally important species, such as barley for malting. Also, these models provide information about how physiological and environmental factors may interact to control the termination of dormancy. After-ripening is delayed when oxygen tensions are low and accelerated when they are high; however, this has been determined experimentally using concentrations of oxygen from near zero to 100%, a range that dry seeds in nature will never encounter.

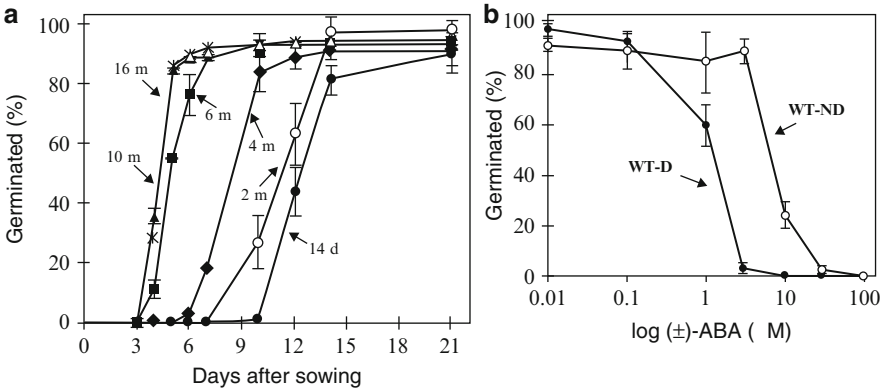

Fig. 6.19 (**a**) Effect of the duration of dry storage on the time course of germination of seeds of *Nicotiana plumbaginifolia*. Mature seeds were after-ripened for 14 days, 2, 4, 6, 10, and 16 months, reducing the lag time of germination with time of treatment. (**b**) Effect of ABA on the germination of 14-day (WT-D) and 12-month AR (WT-ND) seeds of *N. plumbaginifolia,* showing that long-time after-ripened seeds are less sensitive to the inhibitor. From Grappin et al. (2000)

Our understanding of what occurs in dry seeds during after-ripening is very limited (Sect. 8.5). The obvious constraint that restricts the study of dry organisms is that any biochemical or molecular technique that requires the use of aqueous solutions can initiate a rapid response of preformed enzymes, leading to an alteration in their activity and/or contents of substrates and products. Also it is not always clear how dry a seed is, and this may be variable between experiments. Therefore, the interpretation of results from different laboratories, often using different seeds, seed lots and experimental approaches, should be approached with caution. Nevertheless, there are some convincing indications of changes that occur in dry seeds that may be associated with the release of dormancy.

Seeds of several species display a decrease in ABA content during after-ripening. A small decrease of approximately 10% occurs during 6 months of after-ripening of deeply dormant Arabidopsis Cvi seeds. Nevertheless, dormancy is completely broken and this suggests that the sensitivity of the seed to ABA also plays a role. Seeds of *Nicotiana plumbaginifolia* after-ripen in approximately 10 months (Fig. 6.19a), during which their ABA content may drop to ~40% of the initial amount, with a concomitant reduction in the lag time of germination and a tenfold decrease in sensitivity to ABA upon subsequent imbibition (Fig. 6.19b). As ABA is a potent inhibitor of seed germination, these changes in its content and sensitivity are related to the increased germination potential of after-ripened seeds. When after-ripening results in an increase in germination of *Sisymbrium officinale* seeds on water, they also exhibit a greater sensitivity to GA (Fig. 6.20), allowing for a more rapid completion of germination, perhaps due to an increase in germination potential following this treatment.

The molecular mechanisms that decrease the dormancy status during after-ripening are not well understood but they apparently involve various chemical changes in the

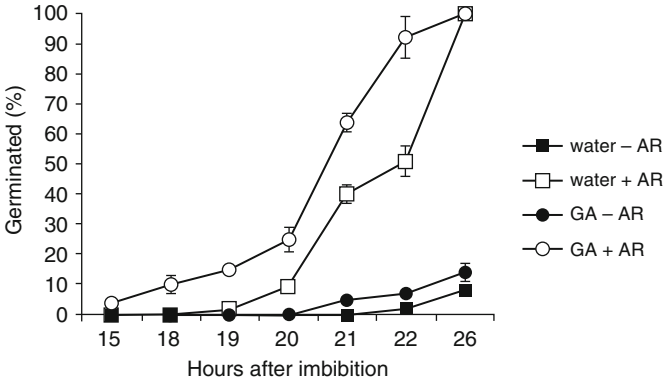

Fig. 6.20 The sensitivity to exogenous GA_{4+7} increases with after-ripening of *Sisymbrium officinale* seeds. Germination was tested in water (■, □) and 100 μM of GA_{4+7} (●, ○) of non-after-ripened (*closed symbols*) or 6-month-after-ripened (*open symbols*) seeds. From data in Iglesias-Fernández and Matilla (2009)

dry seed. The presence of stored (residual) mRNAs in mature dry seeds is seemingly universal in flowering plant species. Their function is debatable (Sect. 4.5.1) but they do participate in protein synthesis early during germination. As after-ripening proceeds in seeds of several species, including tobacco, barley, and Arabidopsis, there are changes in the transcript pool concomitant with their release from dormancy, as well as in the spectrum of proteins identified on 2D gels (e.g., Fig. 4.17). Some transcriptomic studies have identified a few mRNAs that appear to increase during after-ripening, but in general, a decrease in abundance seems to be the most frequent occurrence. In Arabidopsis, the mRNAs of 30 dormancy-associated genes, including *DOG1* (Sect. 6.4.2), decrease in seeds during after-ripening. In dry seeds of Arabidopsis, also several proteins appear to accumulate during after-ripening, but the mobility of existing proteins on 2D gels could have been altered by chemical modifications (e.g., glycosylation or oxidation). While the majority of transcripts and proteins do not change in amount during after-ripening, those that do may be related to loss of dormancy. There are plausible alternative reasons for the variations in the mRNA and protein pools during after-ripening, other than due to changes in transcription and translation; these are elucidated in Sect. 8.5.

6.6.3 Low Temperatures (Chilling)

A high proportion of species—probably the majority of nontropical ones—can be released from dormancy when, in the hydrated condition, they experience relatively low temperatures, generally in the range 1–10°C, but in some cases as high as 15°C. The importance of this kind of control in nature is obvious: dormancy of the hydrated seed is slowly broken over the winter (in temperate regions). This is presumably a

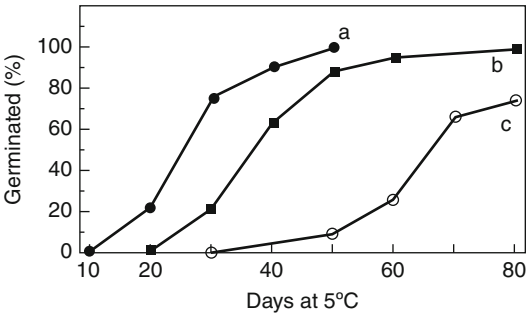

Fig. 6.21 Termination of apple seed dormancy by chilling. Imbibed seeds were kept at 5°C and periodically removed for testing. The percentage germination of the following was determined for (**a**) isolated embryos, (**b**) seeds with testa removed but endosperm intact, and (**c**) intact, whole seeds. Note that embryo dormancy is terminated completely by 40–50 days of chilling, endosperm-imposed dormancy by 60 days of chilling, and whole-seed dormancy partially by more than 80 days of chilling. After Visser (1956)

means of preventing germination during frequently occurring short spells of elevated temperatures until after the winter has passed (Sect. 7.3). Chilling of seeds to break dormancy is a long-standing practice in horticulture and forestry and is generally referred to as "stratification," because the seeds are sometimes arranged in layers (i.e., stratified) in moist substrata.

Chilling is effective in seeds with embryo, coat-imposed, primary and secondary dormancy. In apple seeds, for example, it is possible to show how the different components of dormancy are differentially broken by chilling. Chilling for 50 days suffices to remove embryo dormancy; the presence of the endosperm increases the required time to about 60 days, whereas the whole seed needs even longer (Fig. 6.21). Evidently, this is a reflection of the gradual increase in germination potential of the embryo which becomes great enough to overcome, first, the resistance of the endosperm alone and eventually of both enclosing tissues. In general, woody species of the temperate regions require fairly extensive treatment times, sometimes as much as 180 days (*Crataegus mollis*), but usually 60–90 days are satisfactory. On the other hand, dormancy in some herbaceous species may be broken by just a few days of low temperature (e.g., 7 and 14 days, respectively, in *Poa annua* and *Delphinium ambiguum*) and by just 12 h in wheat!

Recorded optimum temperatures for chilling are generally in the region of 5°C, but this figure may be misleading, as exemplified by *Rumex obtusifolius* (Fig. 6.22). The seeds are released from dormancy almost as effectively by 1.5, 10, and 15°C within an initial 2-week treatment period. When the treatment time is prolonged, secondary dormancy sets in, predominantly at 10 and 15°C. Thus, in this case 1.5°C is the most suitable temperature for dormancy relief (Fig. 6.22).

Early studies showed that chilling increases the seed's sensitivity to environmental factors, such as light and nitrate, and also to applied GAs. Cold treatment of crab apple, hazel, and Arabidopsis seeds results in a higher abundance of bioactive GAs than

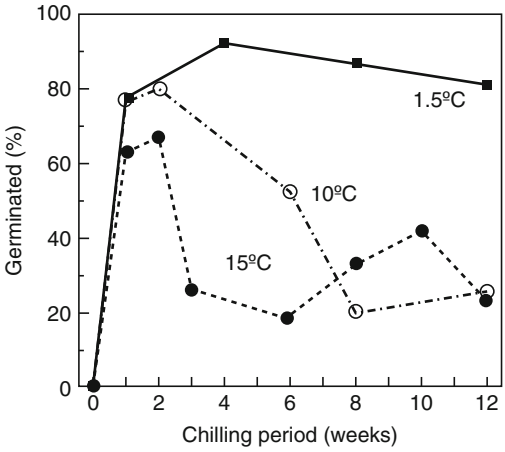

Fig. 6.22 Temperature–time relationships for the termination of dormancy of *Rumex obtusifolius* seeds. Imbibed seeds were held in the light at three temperatures for up to 12 weeks. They were then transferred to 25°C for 4 weeks, after which the percentage of germinated seeds was determined. After Totterdell and Roberts (1979)

Table 6.2 Decline in ABA content of *Taxus mairei* seeds due to cold stratification and a warm/cold combined treatment

		ABA content (pg/seed)		
Treatment	Period (months)	Testa	Gametophyte + embryo	Total
Untreated control	0	6,696 ± 2,931	2,192 ± 933	8,888
Stratified at 5°C	12	367 ± 92	169 ± 65	536
25/15°C	6	198 ± 37	194 ± 84	392

The warm/cold combined treatment was 25/15°C (12 h light/12 h dark). ± Standard error of 3 replicates. Based on data in Chien et al. (1998)

in untreated samples. Cold appears to induce genes that encode enzymes of the GA-biosynthetic pathway. *GA3ox1,* encoding the enzyme that converts inactive GA_9 to active GA_4 (Fig. 6.13b), is specifically upregulated by cold in Arabidopsis seeds. Interestingly, this gene is also upregulated by red light (Sect. 6.6.5.4).

There are also reports of a decrease in ABA content as a result of combined alternating temperatures and chilling as well as due to cold stratification, as in *Taxus mairei* seeds (Table 6.2), but more often ABA content does not significantly decrease during the chilling treatment but only afterwards, when the nondormant seeds display a rapid decline in ABA content, which does not occur in the non-chilled seeds. The decrease in ABA content is not caused only by its simple leakage from the seed but also by ABA catabolism. Furthermore, although dormancy release of yellow cedar seeds by a combined warm and cold treatment results in a decrease of ABA content of the embryo by ~50%, this alone is not sufficient for dormancy release; a concurrent decrease in sensitivity to ABA is also required. In beech tree seeds, expression of an ABA-inducible protein phosphatase type-2C (*PP2C*) gene increases during the first weeks of cold stratification. Overexpression of the beechnut *PP2C* gene in Arabidopsis results in less dormant seeds with a markedly reduced ABA sensitivity. This supports the role of PP2C protein as a negative regulator of ABA signaling (Fig. 6.14) and suggests that phosphorylation/dephosphorylation is involved in seed dormancy release during chilling.

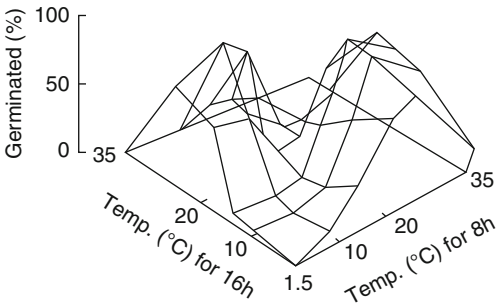

Fig. 6.23 Breaking dormancy in *Rumex obtusifolius* by alternating temperatures. Imbibed seeds were held in darkness for 28 days at different temperature combinations: 16 h at one temperature followed by 8 h at another. Germinated seeds were counted after 28 days. Note that high germination percentages (i.e., termination of dormancy) occur in the following temperature and time combinations: (**a**) 16 h at 25–35°C, 8 h at 1.5–15°C; (**b**) 8 h at 25–35°C, 16 h at 1.5–20°C. After Totterdell and Roberts (1980)

6.6.4 Other Effects of Temperature on Dormancy

In the field, dormant seeds are commonly subjected to fluctuating temperatures, for example, low night temperatures and high daytime temperatures. Such temperature fluctuations, or temperature alternations, are frequently effective in dormancy breakage, in cases such as *Bidens tripartitus,* tobacco, and *Rumex* spp., which all have coat-imposed dormancy. While alternating temperatures break dormancy of *Rumex obtusifolius* seeds, the seeds remain dormant at constant temperatures, as is shown by the valley running across the diagonal of Fig. 6.23. Dormancy is broken when different temperatures are combined, with the maximum effect at the greatest temperature differentials (i.e., amplitudes). So to be effective, the temperature alternation must have a certain minimum amplitude, and in some species this need be only a few degrees. In addition, the temperatures of the pair must be above and below certain values, the duration of exposure to each is important, and the number of cycles of fluctuating temperatures can be decisive. This illustrates some of the exacting environmental requirements that seeds can sense in relation to the breakage or maintenance of dormancy.

In a few species, relatively high temperatures can break, or assist in breaking, dormancy. Seeds of *Hyacinthoides non-scripta* require several weeks at 26–31°C followed by a germination phase at 11°C. Several species that are chilling sensitive need a period at relatively high temperature before the cold. *Fraxinus* spp., for example, require a few weeks at about 20°C prior to chilling at 1–7°C. Softening of the seed coat of woody species might occur at the higher temperature. In contrast, seeds of *Annona crassiflora* cannot be germinated in the lab under any favorable temperature regime. In the soil seed bank they require several months of below-average temperatures (<15°C) to break physiological dormancy and allow the embryo to grow to maturity when the field temperature rises and the rainy season

Table 6.3 Variations in the illumination conditions required for the breaking of seed dormancy

Illumination conditions	Examples
Seconds or minutes	*Agrostis tenuis*
	Arabidopsis thaliana
	Chenopodium album
	Lactuca sativa cv. Grand Rapids
	Lepidium sativum
	Nicotiana tabacum
Several hours (or intermittent)	*Hyptis suaveolens*
	Lythrum salicaria
Days (or intermittent)	*Epilobium cephalostigma*
	Kalanchoë blossfeldiana
Long days	*Begonia evansiana*
	Betula pubescens (at 15°C)
	Chenopodium botrys (at 30°C)
Short days	*Chenopodium botrys* (>20°C)
	Tsuga canadensis
	Betula pubescens (>15°C)

commences. Numerous examples of the various temperature regimes that break dormancy can be found in the literature, a reflection of how species and their seeds are adapted to a range of climatic conditions.

6.6.5 Light

Light is an extremely important factor for releasing seeds from dormancy. Almost all light-requiring seeds have coat-imposed dormancy. Seeds of many species are affected by exposure to white light for just a few minutes or seconds (e.g., lettuce) or even milliseconds, whereas others require intermittent illumination for sometimes prolonged periods of time (e.g., *Kalanchoë blossfeldiana*). Photoperiodic effects also exist, so that some species require exposure to long days and others to short days (Table 6.3). The light requirement frequently depends on the temperature. Grand Rapids lettuce seeds, for example, generally are dormant in darkness only above about 23°C, so below this value they germinate without illumination. Seeds of some species of *Betula*, on the other hand, are dormant in darkness at lower temperatures (e.g., 15°C) but not at 25–30°C. Sensitivity to light in many species is enhanced by chilling and various temperature alternations and temperature shifts also interact with light.

6.6.5.1 Phytochrome: Action Spectra

In nature, white light (i.e., sunlight) breaks dormancy, although wavelengths in the orange/red region of the spectrum are the most effective. In 1954, a detailed action

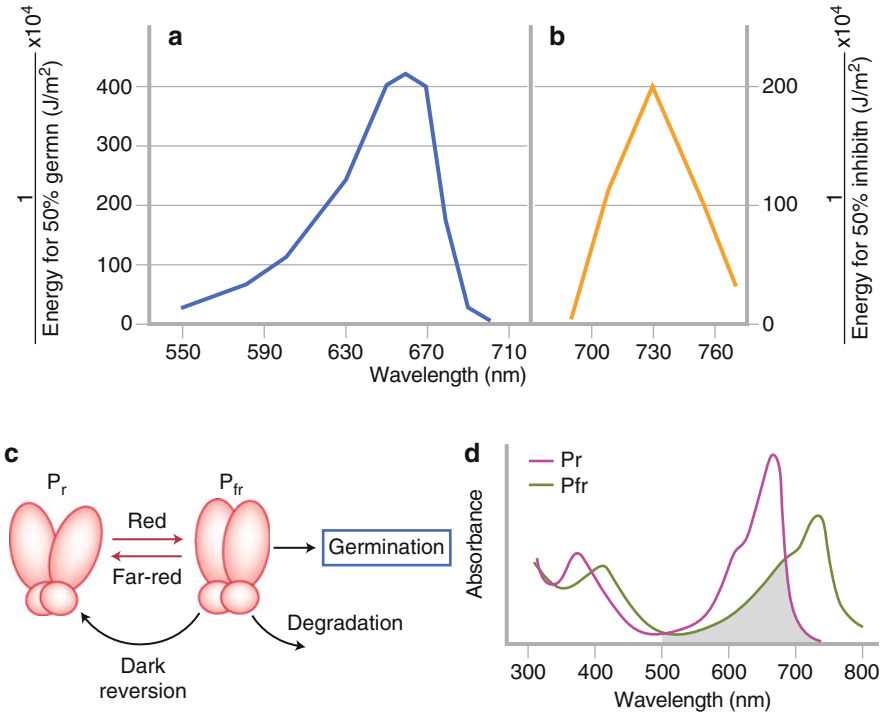

Fig. 6.24 (**a**, **b**) Action spectra showing the wavelengths of light that affect germination and dormancy of lettuce seeds, cv. Grand Rapids. In (**a**) seeds were imbibed in darkness for 16 h before irradiation with the shown wavelengths of light and then returned to darkness and the percentage of seeds germinated later counted. The energy of each wavelength required to achieve 50% germinated seeds (i.e., break dormancy of 50% of the seeds) was determined. In (**b**) the energies of different wavelengths required to reverse the effects of red light and to maintain dormancy are shown. (**c**) Pictorial model of a phytochrome dimer molecule and the interconversion of the inactive Pr and the active Pfr forms by red and far-red light. The lower part of the molecule has kinase activity. (**d**) Absorption spectra for the two forms of phytochrome (Pr and Pfr). The shaded area shows the wavelengths that both forms of the pigment absorb. **a**, **b**, and **d** based on Borthwick et al. (1954)

spectrum for the breaking of dormancy in the Grand Rapids cultivar of lettuce, obtained by Borthwick, Hendricks, and their colleagues, revealed that the major promotive activity is at 660 nm (Fig. 6.24a). Prior to this, inhibitory parts of the spectrum were also known, with an especially potent waveband in the far-red, i.e., wavelengths longer than about 700 nm; the action spectrum shows that 730 nm is the wavelength of maximum activity (Fig. 6.24b). At about the same time as the action spectrum was discovered, Borthwick, Hendricks, Parker, E. H. Toole and V.K. Toole showed that red (R) and far-red (FR) light are mutually antagonistic. This was done by exposing lettuce seeds to a sequence of red and far-red irradiations: only when the last exposure in the sequence was to red light was dormancy terminated (Table 6.4).

Table 6.4 Phytochrome photoreversibility and the breaking of lettuce seed dormancy[a]

Irradiation sequence	Germinated (%)
None (darkness)	4
R	98
FR	3
R, FR	2
R, FR, R	97
R, FR, R, FR	0
R, FR, R, FR, R	95

[a]Seeds of the Grand Rapids cultivar of lettuce were imbibed in darkness and then exposed to red light (640–680 nm) (R) for 1.5 min and far-red light (>710 nm) (FR) for 4 min in the sequence shown. After irradiation, they were returned to darkness for 24 h before germinated seeds were counted

This established the fact of photoreversibility; i.e., the two wavelengths 660 nm and 730 nm are able to reverse each other's effect. To act, light must be absorbed by molecules of a pigment, and the one participating in the breaking of dormancy, as well as in other photoresponses, came to be called phytochrome. This pigment, therefore, exists in two forms (Fig. 6.24c). One is present in unirradiated, dormant seeds; its absorption spectrum shows that it absorbs red light (peak at 660 nm) and is therefore designated as Pr (Fig. 6.24d). This form of phytochrome obviously cannot break dormancy (if it could, the seeds would not be dormant!), but when activated by 660-nm light, it is changed into an active, dormancy-breaking form. But this active form absorbs FR light (730 nm) (Fig. 6.24d); hence it is designated as Pfr, and is then reverted to Pr at this wavelength.

Ignoring for the moment the dark-reversion path, it is easy to see how the reversibility of dormancy breakage works: if Pfr is left in the seed at the end of the radiation sequence, dormancy is terminated, but if Pr is left, dormancy is retained. Note that photoreversion only stops the breaking of dormancy if Pfr has been given insufficient time in which to act. If dosage with far-red light is delayed for a few hours, Pfr can then operate, and even if photoreversion occurs later, dormancy has been terminated and a point-of-no-return (i.e., the "escape time") has been passed.

The energies needed to carry out these photoconversions are relatively small. A saturating dose of R light in lettuce seeds is about 10 $J\,m^{-2}$, an amount given by about 0.2 s of direct, summer sunlight. Up to saturation value, the effect is directly proportional to the total amount of energy, irrespective of how the energy is delivered, i.e., by a lower fluence rate (irradiance) for a longer time, or by a higher fluence rate (irradiance) for a shorter time. As long as the products, fluence rate x time, are equal, the same effect (e.g., percentage of seeds breaking dormancy) is achieved. This is to say that the phytochrome system shows reciprocity. It is intriguing to note that this reciprocity is analogous to the concept of thermal time, where temperature and time requirements are inversely related (Sect. 7.2.2.2), just as photon fluence and time of exposure are for phytochrome action. In a more complex response type, e.g., where repeated doses of R light are required, reciprocity is shown for each exposure, and not for the total amount of light involved. The dosage of FR light needed

for photoreversion is higher; in lettuce, for example, approximately 600 $J\,m^{-2}$ of light at 730 nm causes about 50% reversion of the effect of saturating R light. In the laboratory, higher doses of FR are usually secured by higher fluence rates and/or longer irradiation times.

The scheme for pigment photoconversion (Fig. 6.24c) also contains a dark-reversion component. This was discovered when lettuce seeds that were transferred to a relatively high temperature in the dark for a few hours immediately after exposure to R light failed to germinate; they then required a second dose of R light. Thus, the high temperature caused a slow loss of active phytochrome by a reversion of Pfr to Pr, so-called dark reversion. Loss of Pfr might also occur by its destruction; Pfr is labile and has a half-life of about 1.25 h.

Discussion so far has centered on the photoconversions of phytochrome by R and FR light. In nature, seeds are exposed to mixtures of wavelengths such as those which exist in sunlight. Conversions of phytochrome can occur at any wavelength or mixtures thereof, in both directions, to generate a certain proportion of Pfr, as there is some absorption by both forms across the visible spectrum (Fig. 6.24d). It is the proportion of Pfr established under a given condition that determines dormancy breakage, as explained in the next section.

6.6.5.2 Phytochrome: Photoequilibria

The properties of phytochrome so far discussed were all derived simply from the experimental work on the physiology of light action in seed dormancy. Complete confirmation of these points came when phytochrome was isolated from plants, not from seeds but from dark-grown (i.e., non-green) tissues such as oat or rye coleoptiles. Phytochrome is a blue chromoprotein, the chromophore being an open-chain tetrapyrrole not unlike the phycocyanins of the blue–green algae. In solution, it shows photoreversibility with R and FR light, and the absorption spectra of the two forms can be determined (Fig. 6.24d). Peak absorption of Pr is at 660 nm and of Pfr is at 730 nm, just as predicted from the experiments with lettuce seeds! An important point to note is the overlap in absorbances of Pr and Pfr. Because both forms absorb over the spectrum from about 300 nm to about 730 nm (Fig. 6.24d), irradiation with monochromatic light in this range sets up an equilibrium mixture of Pr and Pfr, the photoequilibrium or photostationary state of Pfr/Ptotal or ϕ (phi). At 660 nm, for example, Pr is photoconverted to Pfr, but Pfr also absorbs at this wavelength and hence some Pfr molecules are phototransformed back to Pr. A mixture of approximately 80% Pfr and 20% Pr is thus established (Pfr/Ptot, ϕ=~0.8). Even at 730 nm, where Pfr absorbs most strongly, there is also some absorption by Pr; here the ϕ value is 0.02, i.e., 2% Pfr. Inspection of the absorption spectrum shows that no waveband region can produce 100% Pfr; on the other hand, almost 100% Pr can be achieved by irradiation at 740–800 nm. Consequently, irradiation with mixed wavelengths also establishes a certain ϕ value. In midday sunlight, ϕ is about 0.55, in white incandescent light it is about 0.45, and in white fluorescent light about 0.65. The effectiveness of phytochrome in terminating dormancy is determined by the ϕ value generated in

Table 6.5 Photoequilibrium values of phytochrome (Pfr/Ptot ϕ) required for dormancy-breakage[a]

Species	Pfr/Ptot (ϕ)
Amaranthus retroflexus	0.001
Amaranthus caudatus	0.02
Wittrockia superba	0.02
Sinapis arvensis	0.05
Cucumis sativus	0.1–0.15
Chenopodium album	0.3
Lactuca sativa	0.59

[a]Values given are sufficient to break dormancy in most seeds of the population

the seeds, the required value depending on the species (Table 6.5). Lettuce is barely satisfied by the ϕ value brought about by sunlight, and *Wittrockia superba* can be stimulated to germinate even by broad-band far-red light ($\phi > 0.02$).

The variation in ϕ values required for the breaking of dormancy makes clear that different species have different Pfr thresholds. This has significant ecological implications in the fact that light-requiring seeds can use phytochrome to detect different light qualities when the proportions of R and FR light change. This happens to some extent according to the time of day, but more importantly it occurs when light is transmitted through green leaves, whose chlorophyll absorbs R light but allows FR light to pass. Thus, a seed under a leaf canopy is in light rich in FR, which sets up a ϕ value too low to satisfy most (but not all) light-requiring seeds. In a sense, phytochrome is the device used by the seed to detect where it is, especially in relation to burial in the soil or to neighboring plants. Phytochrome is also involved in the photoinhibition of germination, when light of rather high fluence rates given for relatively long periods of time prevents even nondormant seeds from germinating. This is returned to with respect to the ecophysiology of dormancy in Sect. 7.2.3.

6.6.5.3 Phytochrome: Multiple Forms

The formation of Pfr alone does not break dormancy. Rather, Pfr engages signaling pathways that ultimately lead to the termination of dormancy and the completion of germination by the seed. These signaling pathways appear to be rather complex (Sect. 6.6.1) and show extensive cross interactions with other environmental factors. First of all there are different kinds of phytochromes. Two that have received the most attention are phytochrome A and phytochrome B, which are encoded by different genes, *PHYA* and *PHYB* respectively. PHYA protein accumulates in dark-grown seedlings while PHYB, a light-stable form, is found in non-etiolated, green plants; it is now clear that the two types have different roles in photomorphogenesis. In addition to phytochromes A and B, three more phytochrome genes, *PHYC*, *PHYD*, and *PHYE,* are present in Arabidopsis which encode phytochromes C through E. A powerful experimental approach to classifying the roles of the discrete

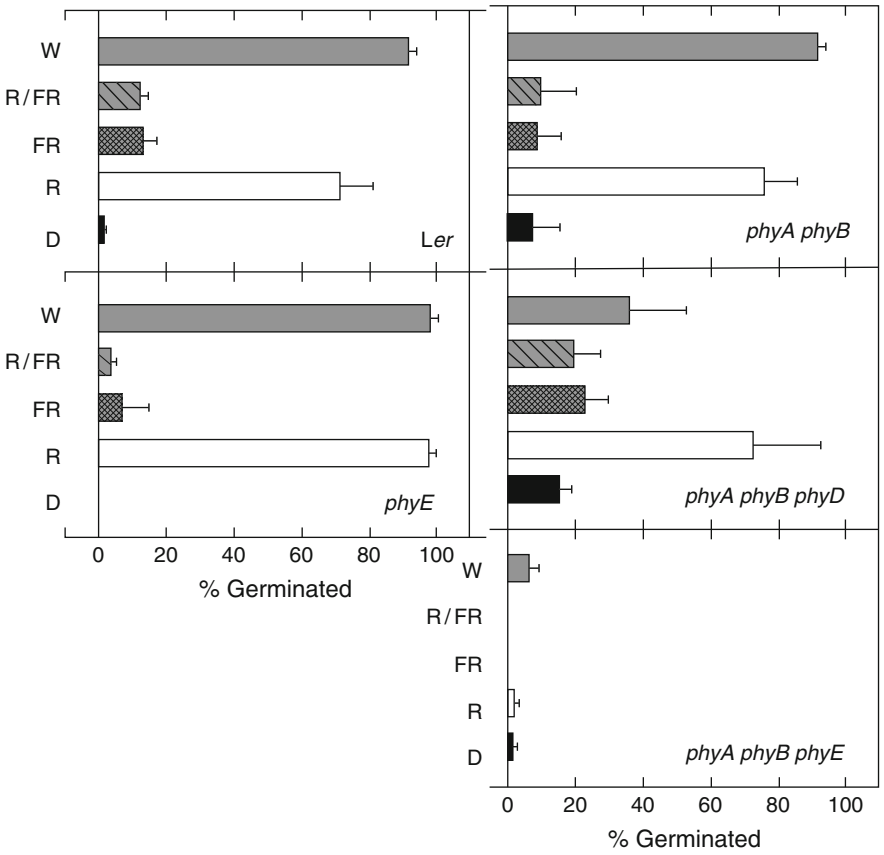

Fig. 6.25 Induction of germination by light pulses of wild-type (L*er* ecotype: Landsberg *erecta*) and single (*phyE*), double (*phyA phyB*) and triple phytochrome loss-of-function mutant (*phyA* and *B* with *phyD* or *E*) seeds of *Arabidopsis thaliana*. Imbibed seeds were incubated for 24 h at 4°C in the dark, followed by continuous white light (W) or hourly light pulses of the indicated light quality at 25°C for 3 days, or continuously in the dark (D). R, *red*; FR, *far-red*; R/FR, R pulse followed by FR pulse. From Hennig et al. (2002). Copyright American Society of Plant Biologists

phytochromes has been the generation of mutations in the apoproteins of each of the phytochromes and by subsequently crossing individuals with single mutations to create plants containing multiple mutations with progressively fewer functional phytochromes. The results of these analyses show that there are substantial redundancies among the functions for each type of phytochrome. For example, mutants lacking PHYE only, both PHYA and PHYB, or PHYA, PHYB and PHYD still respond in a very similar way to white, R and FR light as the wild type L*er* (accession Landsberg *erecta*), but those that lack PHYA, PHYB, and PHYE do not respond to light anymore (Fig. 6.25). Thus, there is redundancy of function, but absence of PHYA, B and E together leaves the seeds blind to light. In addition, the different phytochromes respond differently to temperature, implying that the breaking or

maintenance of dormancy at a given temperature may depend on one or more specific phytochromes. More studies are required to establish the exact roles of the phytochromes in the control of dormancy and regulation in different environments. As mentioned above, the existence of several types of phytochromes and their overlapping functions considerably increases the complexity of phytochrome signaling in concert with the other factors that affect dormancy, e.g., temperature.

6.6.5.4 Phytochrome: Downstream Signaling

From physiological experiments it has long been known that the action of light on seeds, through phytochrome, may lead to the synthesis of GAs that then engage a signaling pathway to effect the completion of germination. Now, gene expression analysis has shown that phytochromes (Pfr) increase the amount of bioactive GAs in seeds by activating the transcription of *GA-3-oxidase* genes (*GA3ox1* and *GA3ox2*), which are also positively regulated by cold (Sect. 6.6.3), and repressing the transcription of a *GA-2-oxidase* gene (*GA2ox2*) that is involved in the degradation of active GAs (Fig. 6.13b).

The regulation of ABA metabolism genes by light is mediated through phytochrome-interacting factors (PIFs). A protein called PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5), a light-labile basic helix-loop-helix transcription factor, inhibits seed germination by repressing the above-mentioned *GA3ox1* and *GA3ox2* genes and upregulating *GA2ox2* (Fig. 6.26). Furthermore, PIL5 causes an increase in ABA by activating genes whose expression are required for its synthesis, and repressing a gene required for ABA catabolism. When *PIL5* is knocked out in Arabidopsis, *pil5* mutant seeds become resistant to FR inhibition while seeds overexpressing *PIL5* are incapable of responding to R and do not germinate in response to the light stimulus. Therefore, PIL5 is probably a critical component of phytochrome responses and a negative regulator of seed germination.

Finally, PIL5 also increases the expression of *DELLA* genes (e.g., *GA-INSENSITIVE* [*GAI*] and *REPRESSOR OF GA1-3* [*RGA*]) that results in the repression of GA action. Light perceived by phytochromes represses this inhibition due to a reduction in the amount of PIL5 protein, which in turn results in a decrease in the transcription of *DELLA* directly, in addition to the indirect effects of reduced PIL5 on ABA and GA metabolism, resulting in the breakage of dormancy (Fig. 6.26). Thus, PIL5 modulates both ABA and GA metabolism through the stabilization of DELLA (e.g., RGL2, Fig. 6.16) and plays a critical role at the intersection of light and hormone signaling.

6.6.6 Dormancy Release of Seeds with Impermeable Coats

Many seeds with impermeable coats contain specialized structures that regulate the uptake of water (Sects. 1.2.3, 6.3.2.1). They are generally derived from tissues that close the natural openings in the seed or fruit coat, such as the micropyle, hilum, and

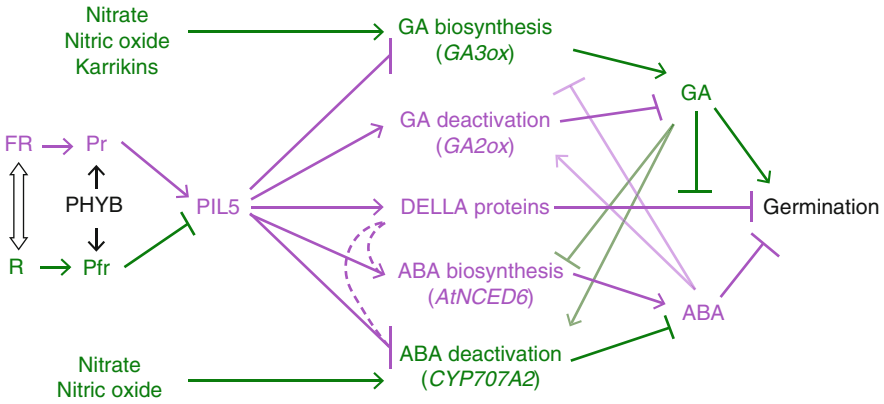

Fig. 6.26 Proposed molecular events leading to light-, nitrate-, nitric oxide-, or karrikin-induced seed germination in *Arabidopsis thaliana*. In the dark or presence of Pr, PIL5 activates the expression of DELLA genes (*RGL2*, *GAI, RGA*) by binding directly to their promoters through G-box elements. PIL5 also represses genes of the GA biosynthetic and ABA deactivation pathways as well, promoting expression of those responsible for GA deactivation and ABA biosynthesis. This results in decreased GA and increased ABA. The decrease in GA stabilizes DELLA proteins, leading to their increase and the suppression of GA responses and of subsequent germination. These events are highlighted in purple. Upon light irradiation, activated phytochromes (Pfr) induce PIL5 degradation, leading to decreased DELLA proteins, less ABA biosynthesis and increased GA biosynthesis. DELLA proteins also decrease due to the increased bioactive GA. Nitrate and nitric oxide also reduce ABA content through enhancement of *CYP707A2* expression, while also promoting *GA3ox* expression. Karrikins, components of smoke, promote expression of genes encoding GA biosynthetic enzymes. As a result of the increased GA synthesis and action, and the decrease in ABA synthesis, the seeds are able to complete germination. These events are highlighted in green. The reciprocal regulatory circuit between GA and ABA biosynthesis and degradation is shown by the lighter *purple* and *green lines*. Modified from Oh et al. (2007) and Martin et al. (2010)

chalazal area. It appears that the breaking of dormancy of seeds with impermeable coats proceeds mostly through these specialized structures. Under natural conditions physical dormancy is often released by exposure to extremes in temperature and temperature fluctuations. Detailed studies of species such as *Stylosanthes humilis*, *S. hamata*, and *Heliocarpus donnell-smithii* suggest that (extreme) temperature fluctuations rather than absolute temperature are decisive for the unplugging of seed openings. The required day–night temperature amplitudes may range from 15 to 40°C. It is likely that the perception of large temperature fluctuations represents a gap-detecting mechanism. The best known condition of exposure of seeds to extreme heat is fire, which is a natural component of many ecosystems. The regeneration of many plant species after a fire is essentially due to the breaking of dormancy of seeds in the soil. Evidently, seeds cannot withstand fire temperatures of over 600°C but a steep temperature gradient in the soil top layer results in more moderate temperatures a few centimeters beneath. Many seeds lose physical dormancy when exposed to temperatures between 50 and 100°C for a limited time. A spectacular

response is seen in seeds such as *Albizzia lophantha,* in which the strophiolar plug is audibly ejected from the seed as high temperature is reached, leaving a strophiolar crater through which water can enter. Such effects of high temperature are thought to be important in pyric species whose seedlings emerge as a consequence of forest fires. Burnt vegetation leaves gaps and open spaces which creates greater day–night temperature fluctuations. Apart from unplugging of the seed coat openings, exposure to dry heat also causes cracks, often starting from the opening. It is likely that this type of damage not only increases the permeability of the seed coat but also decreases its mechanical restraint.

However, depending on the moisture content of the seed and the relative humidity of the air, imbibition of water may also occur across the whole seed coat. In many species, seed coat impermeability increases as seed moisture content decreases. Seed coats become fully impermeable in a range of moisture contents from 2 to ~20%. It has been suggested frequently that microbial action can mediate in the opening of impermeable seed coats. However, there is no conclusive evidence to support this suggestion. Similarly, there are few data available to support the possibility of dormancy relief by passage through an animal's digestive tract, although it has been documented for the passage of seeds of wild tomatoes through Galapagos tortoises! Although studies have shown that such passage through an animal enhances germination, it is not known how this is accomplished. Within the animal, dormancy may be broken by either mechanical scarification or by acid in the digestive tract. Also, passage through the animal may indirectly break the dormancy, because its waste material is usually dropped on the surface, thereby exposing seeds to higher (fluctuating) temperatures, as well as elevated temperatures as a result of fermentation of the fecal material and perhaps some of its chemical constituents.

6.6.7 Breaking of Dormancy by Chemicals

A selected list of chemicals that can break dormancy is given in Table 6.6. Only a few of these are likely to be encountered by seeds in their natural environment, but they are nevertheless of great interest because they may help provide an understanding of the mechanism of dormancy breakage. The possible action of many of these substances has led to hypotheses concerning the mechanisms and regulation of dormancy breakage. Only a few, if any, of these hypotheses have stood the test of time. A short account of two of them follows:

(1) The membrane hypothesis of dormancy breakage. This hypothesis was built on the observation that anesthetics, such as ethyl ether, chloroform, acetone, ethanol, propanol, and other alcohols, can break dormancy of some seeds, e.g., *Panicum* spp., *Digitaria* spp., grasses, and also lettuce. Anesthetics affect cells by entering membranes, thus altering the relationships among membrane components by decreasing their packing density. Consequently, these components, including membrane-associated proteins such as receptors, may become more accessible to substances that bind to them. It was thought that these receptors could bind such dormancy-breaking factors

Table 6.6 Some chemicals that break seed dormancy

Class of compound	Species
Respiratory inhibitors	
Cyanide	*Lactuca sativa*
Azide	*Hordeum distichum*
Iodoacetate	*Hordeum distichum*
Dinitrophenol	*Lactuca sativa*
Sulfhydryl compounds	
Dithiothreitol	*Hordeum distichum*
2-Mercaptoethanol	*Hordeum distichum*
Oxidants	
Hypochlorite	*Avena fatua*
Oxygen	*Xanthium pennsylvanicum*
Nitrogenous compounds	
Nitrate	*Lactuca sativa*
Nitrite	*Hordeum distichum*
Thiourea	*Lactuca sativa*
Growth regulators	
Gibberellins	*Lactuca sativa*
Cytokinins	*Lactuca sativa*
Ethylene	*Chenopodium album*
Various	
Ethanol	*Panicum capillare*
Methylene blue	*Hordeum distichum*
Ethyl ether	*Panicum capillare*
Fusicoccin	*Lactuca sativa*

as phytochrome and nitrate. Furthermore, membranes are highly sensitive to changes in temperature, an important factor in the regulation of dormancy, and it is possible that anesthetics mimic these. The germination of red rice grains can only be stimulated by those alcohols that are metabolized by the enzyme alcohol dehydrogenase. Thus, besides exerting possible anesthetic effects, alcohols may affect germination and dormancy through (unknown) metabolic modifications.

(2) The pentose phosphate pathway hypothesis. The hypothesis that the pentose phosphate pathway (PPP) could play a unique role in dormancy breakage arose largely from studies of the effect of certain chemicals on dormancy. Dormancy of seeds of several species, including lettuce, rice, and barley, is broken by application of inhibitors of respiration, including those that inhibit terminal oxidation and the tricaboxylic acid cycle in the mitochondria (e.g., cyanide and malonate) and some that inhibit glycolysis (e.g., fluoride). Electron acceptors such as nitrate, nitrite, and methylene blue can also break dormancy, as can high oxygen concentrations. Explanations for the effects of these substances could include that respiratory inhibitors block the consumption of oxygen by conventional respiration and thus it becomes available for other processes, or alternatively, that oxygen could be made more available by elevating its external concentration. The PPP has been suggested to be the important "other" process and it may require the oxygen for the oxidation of reduced NADP ($NADPH + H^+$), which this pathway generates. Oxidation of

NADPH can also be brought about directly by the electron acceptors methylene blue, nitrate, and nitrite, in which case oxygen is not needed. Although the special significance and function of the PPP has never been explained, the hypothesis has been thoroughly investigated. The results, however, are mixed and it has been suggested that the techniques that were used can give misleading results. Thus, evidence for the participation of the PPP in dormancy breakage is, at best, equivocal.

However, some chemicals do play an important natural role in the breaking of dormancy and their involvement has been investigated in several species. These chemicals are nitrate, nitroxides (NO_x) and active smoke components, the butenolides.

6.6.7.1 Breaking of Dormancy by Nitrate

There is little doubt that nitrate is an indicator of soil fertility for seeds in the soil seed bank; this and its other ecological roles are discussed in Sect. 7.2.4. Nitrate has long been known to break the dormancy of seeds of a variety of species, often in combination with light. Some, such as *Sisymbrium officinale*, show an absolute requirement for both of these factors; only the combination is successful in the breaking of dormancy. The mechanism of action of nitrate has often been associated with the pentose phosphate pathway (previous Section), but there is no conclusive evidence for this. Moreover, in *S. officinale* seeds nitrate remains effective even when nitrate reductase activity is completely inhibited. Therefore, the nitrate signal for the breaking of dormancy may be nitrate itself in its unreduced form.

In the deeply dormant Cvi accession of Arabidopsis, nitrate can substitute for the long period (7–12 months) of dry storage or several days of cold stratification required to break dormancy. There is good evidence that nitrate signaling is targeted at the ABA and GA signaling pathways. Nitrate, either from endogenous pools or when applied, decreases ABA content in seeds of Arabidopsis (Fig. 6.27a, b). Moreover, it stimulates the expression of the *CYP707A2* (Fig. 6.27c) gene that encodes for the cytochrome P450 protein that catalyzes the degradation of ABA (Fig. 6.13a). Interestingly, transcript profiling of imbibed seeds treated with or without nitrate revealed that the latter results in an mRNA profile very similar to that of stratified or after-ripened seeds, which corroborates the observation that nitrate can substitute for these. This role of nitrate in the breakage of dormancy supports the model displayed in Fig. 6.26.

6.6.7.2 Breaking of Dormancy by Nitric Oxide

Nitric oxide (NO) is a very reactive gaseous free radical that is a ubiquitous and potent signaling molecule in plants and animals. Among many other plant processes, NO is involved in the regulation of dormancy and germination in many species. It is produced in plant cells from nitrite and arginine. Nitrite is the first product of the reduction of nitrate by nitrate reductase, and is then further reduced, both enzymatically and nonenzymatically, to NO. Since NO is a highly reactive gaseous substance that partitions into hydrophobic and hydrophilic environments, it is challenging to

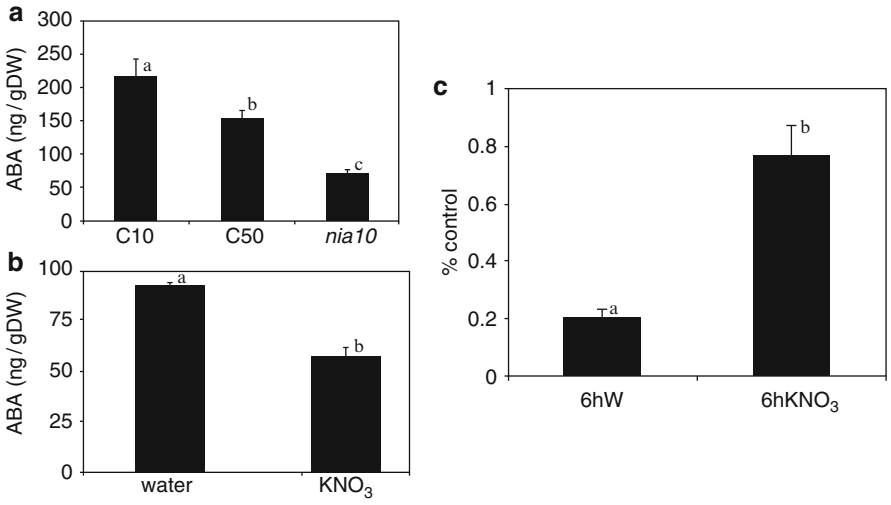

Fig. 6.27 ABA contents of nitrate-treated seeds of *Arabidopsis thaliana* (Col-0). (**a**) ABA contents of mature seeds that were produced on plants grown on 10 mM (C10) or 50 mM (C50) nitrate and seeds from the *nia1nia2* nitrate reductase mutant grown on 10 mM nitrate (*nia10*). In the *nia1nia2* mutant much less nitrate is converted to nitrite and, hence, a larger nitrate pool is available to the seed. (**b**) ABA contents of seeds treated with exogenous nitrate. Freshly harvested seeds from plants grown on 10 mM of nitrate were imbibed for 20 h in water or 10 mM nitrate. (**c**) Differences in *CYP707A2* transcript content (relative to a gene expression standard) of C10 seeds imbibed for 6 h in water (W, control) or nitrate. a, b and c indicate significant statistical differences between treatments. After Matakiadis et al. (2009). Copyright American Society of Plant Biologists

use in experiments; instead, chemicals are used that function as NO-donors in vivo. These are, for example, sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). As controls, NO scavengers, such as (carboxy)2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) are utilized. Application of SNP and SNAP to light-requiring lettuce seeds results in the breaking of dormancy, which is effectively counteracted by PTIO, providing evidence that NO can bypass the light-requirement for germination (Table 6.7).

Arabidopsis mutants lacking the *AtNOS1* gene and which are thus deficient in endogenous NO have increased dormancy and lower seed germination and seedling establishment rates than wild-type seeds due to enhanced ABA inhibitory action. These effects can be reversed by application of NO to the mutant seeds. There is good evidence that NO enhances the degradation of ABA through activation of the *CYP707A2* gene and the biosynthesis of GAs through enhancing the activity of the *GA3ox* genes (Fig. 6.13a, b) and thus contributes to the breaking of dormancy.

6.6.7.3 Breaking of Dormancy by Smoke

Smoke, as well as charcoal extracts, has long been known to break seed dormancy in many species. Smoke is an effective stimulant that breaks dormancy in some

Table 6.7 Nitric oxide stimulation of light-dependent germination of lettuce seeds in the dark

Treatment	% Germinated
100 μM SNP	98.2 ± 2.4
10 μM SNP	50.1 ± 4.3
100 μM SNP + 100 μM PTIO	3.6 ± 1.8
100 μM SNAP	96.7 ± 2.3
100 μM SNAP + 100 μM PTIO	4.4 ± 2.1

Germination of lettuce seeds in the dark on water at 26°C is negligible. Means ± standard error of 3 replicates. SNP (sodium nitroprusside) and SNAP (*S*-nitroso-*N*-acetylpenicillamine) are donors of NO in vivo, whereas PTIO ([carboxy] 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide) is a scavenger of NO. After Beligni and Lamattina (2000)

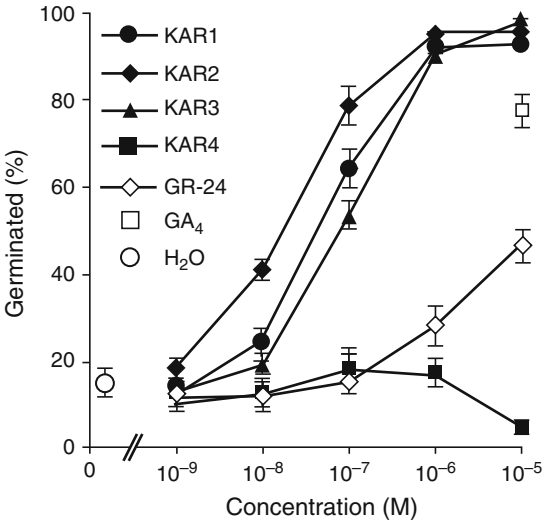

Fig. 6.28 Karrikins (KAR) break *Arabidopsis thaliana* seed dormancy. Germination of dormant *Ler* seeds after 7 days from the start of imbibition on water, 1 nM to 10 μM KAR1, KAR2, KAR3, or KAR4, GR-24 (a synthetic strigolactone), or GA_4. After Nelson et al. (2009). Copyright American Society of Plant Biologists

1,200 species of more than 80 genera worldwide. Fire offers an important opportunity for the breaking of dormancy, germination, and subsequent seedling establishment by altering key environmental factors such as light, space, and nutrients. The flush of new growth immediately after the fire event indicates a massive breaking of dormancy of seeds in the soil seed bank. Adding to what was discussed earlier (Sect. 6.6.6), heat is not the only factor required for this type of response since the application of cold smoke is also effective. In other words, this effect is not physical, as in the cracking of hard seed coats, but is of a chemical nature.

One of the active chemicals in smoke is karrikinolide, previously referred to as "butenolide," (3-methyl-2H-furo[2,3-c]pyran-2-one), a member of the class of karrikins. There are several active karrikins, denoted KAR1 through KAR4. These have been compared with strigolactones that bear some structural resemblance. Strigolactones, including the synthetic compound GR-24, are effective in breaking dormancy of parasitic weed seeds (Sect. 7.2.6). Most of the karrikins are similarly effective in breaking dormancy of Arabidopsis seeds and even can be more effective than GAs (Fig. 6.28)! It must be noted, however, that the effectiveness of karrikin

action depends on the seed ecotype. As to their mechanism of action, they induce an increase of expression of the two principal genes for GA-3-oxidase, *GA3ox1* and *GA3ox2* (Fig. 6.13b), but do not affect any of the key genes related to ABA synthesis or catabolism (Fig. 6.26). Interestingly, cyanohydrins have been identified also as a group of active chemicals in smoke, stimulating germination. Cyanide, which is slowly released from cyanohydrin, has long been known to break dormancy and stimulate germination by virtue of its capacity to inhibit respiration (Sect. 6.6.7 and Table 6.6).

As this and the previous two sections suggest, many dormancy responses induced by environmental factors ultimately affect ABA and/or GA signaling, often via the control of the same genes and proteins involved in ABA and GA synthesis and catabolism (Fig. 6.26).

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Chapter 7
Environmental Regulation of Dormancy and Germination

Abstract The seed stage of the higher plant life cycle allows for the survival of individual species, as well as populations, over long periods of time. Emergence of seedlings from the soil seed bank depends on a range of environmental factors of which water and temperature are the most important. The existence of germination response thresholds for (soil) water and temperature has allowed for the development of hydrothermal time models to predict germination and emergence. Additional environmental cues that determine germination are light (through phytochrome), nitrate, and smoke components. These factors are pivotal in sensing competitors and vegetation gaps. Seeds in the soil seed bank undergo dormancy cycling in synchrony with the seasons. In this way precise timing of emergence in the correct season and location are ensured. The survival of a population of annual species predominantly depends on flowering time and dormancy.

Keywords Dormancy cycling • Germination • Hydrothermal time model • Seedling establishment • Secondary dormancy • Seed dispersal • Soil seed bank

The biological importance of dormancy must be seen in relation to the ecology of germination—when and where a seed germinates. Interactions between seasonal dormancy-breaking agents, such as chilling and after-ripening, and the sensitivity of seeds in the soil to such dormancy-relieving factors as light and nitrate, in combination with sufficient amounts of oxygen and water, are responsible for determining whether a seed will germinate in a particular situation and season. The germination and dormancy mechanisms are therefore of great adaptive importance in ensuring that seedling emergence occurs at the most advantageous time and place. This chapter will consider some examples to illustrate the ecological significance of the control processes.

J.D. Bewley et al., *Seeds: Physiology of Development, Germination and Dormancy, 3rd Edition*, DOI 10.1007/978-1-4614-4693-4_7,

7.1 Seed Dispersal and Burial

After seeds become detached from the parent plant they may travel short to long distances before they are buried in the soil and are subjected to the ambient environment for variable periods of time before they germinate and emerge, if at all. Even then, seeds may relocate because of soil disturbance, for example by flooding, predation, and cultivation. Seeds, or rather "dispersules" that may include fruits or other structures along with the seed, may have morphological adaptations to aid to their dispersal. These are usually modifications of the seed/fruit coat, such as "wings" and "hairs" (Fig. 7.1).

Seeds may be dispersed in several ways. Gravity is a simple dispersal mechanism, e.g., of apples, coconuts, and other larger fruits. They may become detached because of their weight and then roll away. Wind dispersal has two primary forms: seeds can float on the breeze or they can flutter to the ground. A well-known example is the dandelion, which has a feathery pappus attached to the seeds that allows them to float on the wind for dispersal over long distances. Maples have winged seeds (samara) that flutter to the ground over much shorter distances.

Many aquatic and some terrestrial species use hydrochory, or seed dispersal through water; examples are water lily, palm, and mangrove seeds. Some species disperse their seeds using autochory, which is the physical and sometimes explosive discharge of seeds from the fruit, as in Impatiens and some legumes.

Seeds can also be dispersed in several ways by animals. They can be transported on the outside of vertebrate animals, which is a process known as epizoochory. Epizoochorous seeds can have a variety of morphological adaptations, including an assortment of hooks, spines and barbs to attach to the skin or hair of animals. (As a side note, the tiny hooks on burdock fruits were the inspiration for the development of Velcro™ fasteners.) Many representatives of this mode of dispersal can be found in the Apiaceae and Asteraceae families. Seeds may also be dispersed via ingestion by vertebrate animals or endozoochory, which is the dispersal mechanism for many tree species. Finally, seeds can be dispersed by ants (myrmecochory), which is a dispersal mechanism of many shrubs and herbs. Myrmecochorous seeds have a lipid-rich attachment (elaiosome), which is a food source for ants. Ants carry these seeds into their colonies, feed the elaiosome to their larvae and discard the still viable seed in an underground chamber, thus adding it to the soil seed bank.

7.1.1 The Soil Seed Bank

After dispersal, a great quantity of seeds ends up on or in the soil, forming the soil seed bank. Germination may then take place instantly or may be delayed for an indeterminate period of time. Buried seed densities are highly variable. Lowest densities are found in tropical and temperate woodlands and alpine and arctic areas. Counts of over 100,000 seeds m^{-2} have been reported in wetlands. The highest density recorded for any species is 488,708 seeds m^{-2} for *Spergula marina*, a species with very tiny seeds (seed weight 0.05 mg). However, buried seed densities can be extremely patchy.

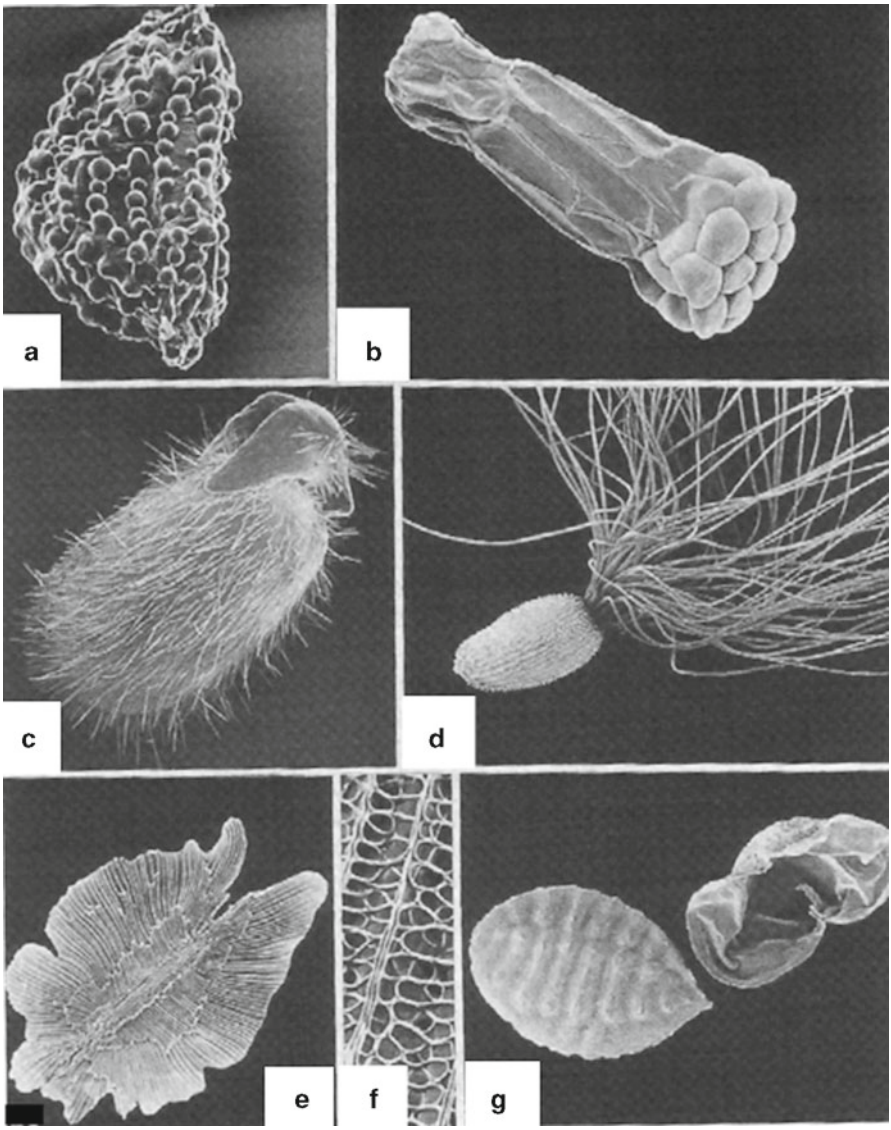

Fig. 7.1 Scanning electron micrographs of seeds showing different dispersal types. (**a**) *Voyria spruceana*, dust seed. (**b**) *Begonia glabra*, balloon seed with inflated seed ends. (**c**) *Polygala vulgaris*, hairy seed with elaiasome. (**d**) *Epilobium hirsutum*, seed with hair tuft. (**e**, **f**) *Paulownia tomentosa*, winged seed and detail of wing. (**g**) *Oxalis europeae*, dispersed seed with reversed outer part of outer integument. From Boesewinkel and Bouman (1995). Photograph courtesy of F. Bouman, Univ. Amsterdam

The distinction has been drawn between transient and persistent seed banks, the former consisting of seeds that mostly are viable in the soil for no more than 1 year, and the latter having a significant proportion of the seeds remaining viable for many years. The different types of seed banks exhibit varying seasonal acquisition of

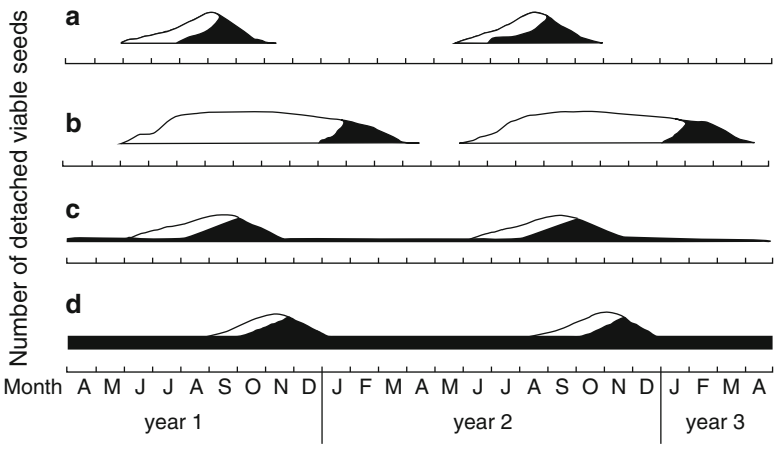

Fig. 7.2 Seed banks common in soils of temperate regions. Shaded areas indicate seeds that can germinate immediately when tested in the laboratory (i.e., nondormant seeds) and unshaded areas represent viable seeds that are dormant. (**a**) Seeds of annual and perennial grasses of dry or disturbed habitats (e.g., *Lolium perenne*). (**b**) Seeds that germinate in early spring, colonizing gaps in the vegetation. They include annual and perennial herbs, trees, and shrubs (e.g., *Impatiens glandulifera, Acer pseudoplatanus*). (**c**) Seeds of winter annuals, which germinate mainly in the fall. A small, persistent seed bank of nondormant seeds is maintained (e.g., *Erophila verna*). (**d**) Seeds of annual and perennial herbs and shrubs, which have large, persistent seed banks (e.g., *Stellaria media*). After Grime (1979)

germinability (or loss of dormancy), which gives each group of plants its characteristic emergence pattern (Fig. 7.2). These seasonal patterns are largely controlled by the seeds' responses to prevailing environmental factors, such as moisture, temperature, light and various chemicals in conjunction with seasonal environmental factors (e.g., chilling, after-ripening) that sensitize the seeds to the environment. The emergence of seedlings depends on more factors than those required for the breaking of dormancy and the induction of germination. During their prolonged stay in the soil, seeds and young seedlings may be victim to predation by animals or degradation by microorganisms. Also, successful seedling emergence depends on the depth at which germination occurs, as well as on soil properties such as physical impedance or crusting.

7.2 Environmental Control of Germination

The interactions of seed dormancy mechanisms with both the accumulated and current environmental conditions determine whether and what fraction of seeds in a seed bank will germinate at a given time (Fig. 7.3). The weather and soil physical characteristics establish the microclimate perceived by the seed. Depending upon whether the seeds are dry or wet, different mechanisms may be involved in alleviating dormancy, e.g., after-ripening or chilling. Once dormancy has been alleviated by the seed's response to cues from the microenvironment, sensitivity to the current environment, particularly to water, temperature, light, and nutrients, determine the rate

and extent of germination of nondormant seeds. Following germination, seedlings may fail to emerge or survive due to physical factors (soil crusting, burial depth) or to predation or disease. All of these factors constitute an interacting network of environmental signals and biological responses determining the entry and release of seeds from the soil seed bank (Fig. 7.3). This section considers these various factors and their interactions in relation to seed dormancy and germination in an ecological context.

7.2.1 Water

The most essential environmental factor required for seed germination is water. Seeds imbibe water from their surroundings, and the ψ of the soil water determines the maximum ψ that the seed can attain (Sect. 4.3). In dry environments, seeds in the seed bank may persist primarily in the dry state, then imbibe quickly and achieve germination when water is available following rainfall. Flushes of seedlings emerge following rain episodes, but otherwise germination is limited by lack of water. In humid regions, seeds in the seed bank persist largely in a hydrated state, and dormancy mechanisms rather than water availability prevent untimely germination.

7.2.1.1 Hydrotime Model of Germination

Water availability (i.e., water potential or ψ) affects both the rate of germination and the fraction of seeds in the population that will germinate. If the ψ is too low (e.g., at −1.2 MPa), germination is prevented (Fig. 4.10), as was described for the case of seed priming (Sect. 4.7). At higher ψ, both the speed and percentage of germination increase in a characteristic manner (Fig. 7.4a). The water relations of germination have been described by the hydrotime model, which is a unifying approach to describe the patterns of germination that occur in response to ψ of the seed's environment. It is based on the concept that the time to completion of germination (here called germination for simplicity) is proportional to the magnitude of the difference between the seed ψ and the water potential threshold for radicle emergence (termed "base water potential" or ψ_b). The ψ_b is the minimum ψ at which germination will occur for a given seed, and the time from the start of imbibition to completion of germination varies in proportion to the extent that the seed ψ exceeds this threshold ψ_b. In addition, individual seeds in a population vary in their ψ_b values; seeds with the lowest (most negative) ψ_b values germinate quickest in a population, followed in order by seeds with increasing ψ_b values. The distribution of ψ_b values in a seed population is generally normal or Gaussian, and can be defined by the median ($\psi_b(50)$) and standard deviation ($\sigma_{\psi b}$) (Fig. 7.4b), but other distributions are possible depending upon the characteristics of the seed population. Seed banks, for example, may be composed of seeds from different years of production that have experienced different after-ripening or dormancy-breaking regimes, resulting in multiple subpopulations with different dormancy characteristics; the relative proportions of

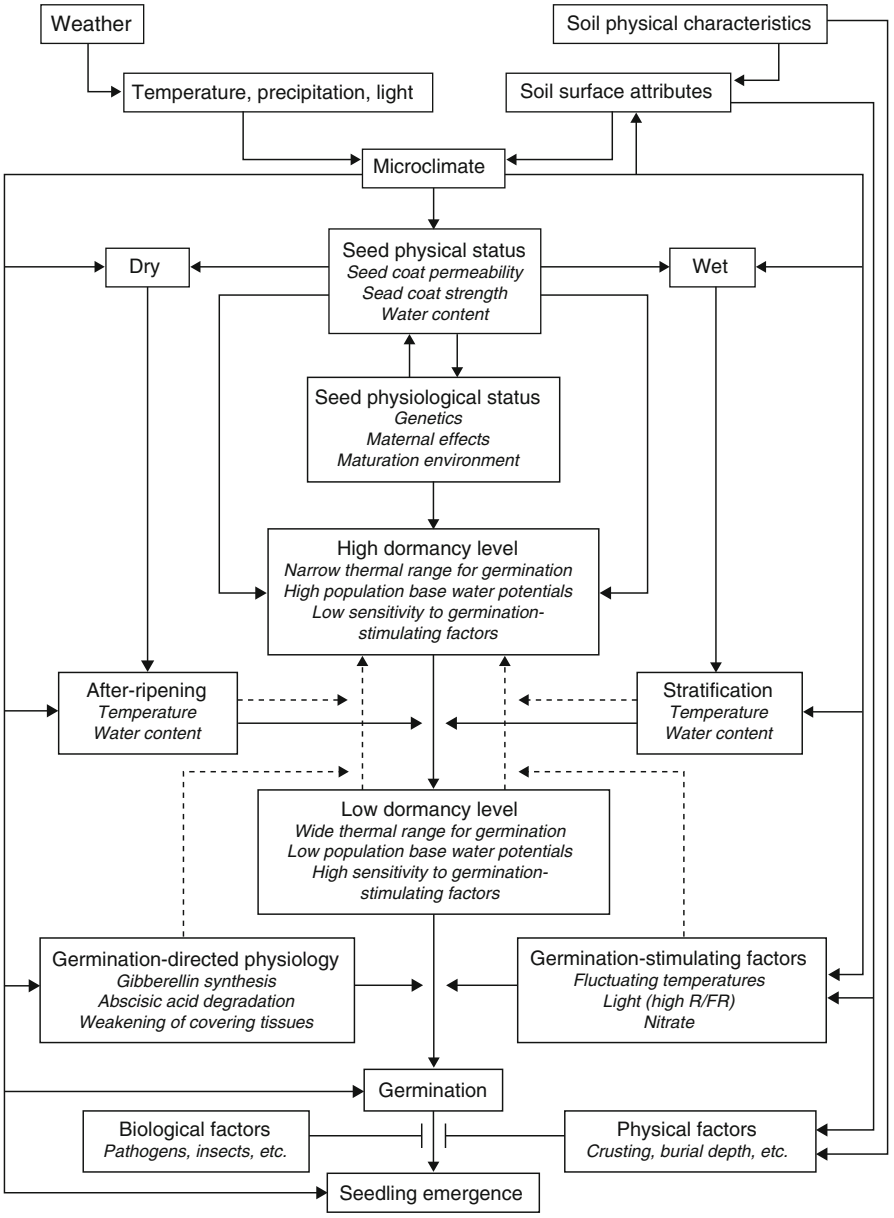

Fig. 7.3 Physical and biological factors associated with environmental control of seed dormancy and germination. Weather, soil physical characteristics and soil surface attributes (e.g., presence or absence of litter layer, topography) determine the seed zone microclimate. Microclimate controls seed dormancy status and germination primarily through moisture content and temperature. Physical attributes of the seed, such as seed coat permeability, can mitigate these microclimate effects. The physiological state of the seed, including its genetic background and maternal and environmental effects during development and maturation, influences the initial dormancy level.

different subpopulations in the total population can skew the distribution toward higher or lower ψ_b values.

The following equation describes the hydrotime model:

$$\theta_H = (\psi - \psi_b(g))t_g \tag{7.1}$$

where θ_H is the hydrotime constant (MPa-days or MPa-hours), ψ is the seed water potential, $\psi_b(g)$ is the base or threshold water potential defined for a specific germination fraction g, and t_g is the time to radicle emergence of fraction g of the population. The hydrotime model proposes that θ_H is a constant for a given seed population, implying that t_g must increase proportionately as the difference between ψ and $\psi_b(g)$ is reduced and approaches zero. This is illustrated for the 16th, 50th, and 84th germination fractions in Fig. 7.4c. These fractions are chosen because they represent the median (50%) and one standard deviation below and above the median in the $\psi_b(g)$ distribution (Fig. 7.4b). In a normal distribution, one standard deviation on either side of the median encompasses from 16 to 84% of the entire population. The times to germination of these fractions of the seed population in relation to ψ illustrate the threshold nature of the hydrotime model, with all seeds having similar times to germination when ψ is high, but as ψ decreases, the times to germination sharply increase. Due to the differences in their threshold ($\psi_b(g)$) values (Fig. 7.4b), the ψ at which germination times increase sharply varies among the different fractions. This corresponds to the skewness in the germination time courses (Fig. 7.4a), where the slower seeds to germinate are affected more by reduced ψ. In this way, the hydrotime model simultaneously accounts for both the timing and the final germination percentages of seed populations in response to changes in ψ.

Fig. 7.3 (continued) Dormancy-breaking processes occur in dry seeds (after-ripening) and in imbibed seeds (e.g., chilling or stratification). A high dormancy level (whether primary or secondary) is associated with a narrow thermal range permissive for germination, relatively high (more positive) population base water potentials, and a low sensitivity to germination-stimulating factors. Conversely, a low dormancy level is associated with a wider temperature range permissive for germination, lower (more negative) population base water potentials and increased sensitivity to germination-stimulating factors. Seeds can shift back and forth between low and high dormancy states through the imposition and release of secondary dormancy in response to environmental cues. Even for seeds in a low dormancy state, germination may still be dependent upon stimulation by factors such as light, nitrate or fluctuating temperatures. After germination has occurred, additional biotic and abiotic stresses can reduce the number of seedlings that successfully emerge from the soil. Interactions between environmental factors and seed dormancy status are shown via arrows; bars indicate repressive interactions. Dashed arrows indicate that when environmental conditions are not conducive to dormancy-breakage or germination, seeds can revert to a higher dormancy level (secondary dormancy). From Allen et al. (2007). Courtesy of Wiley

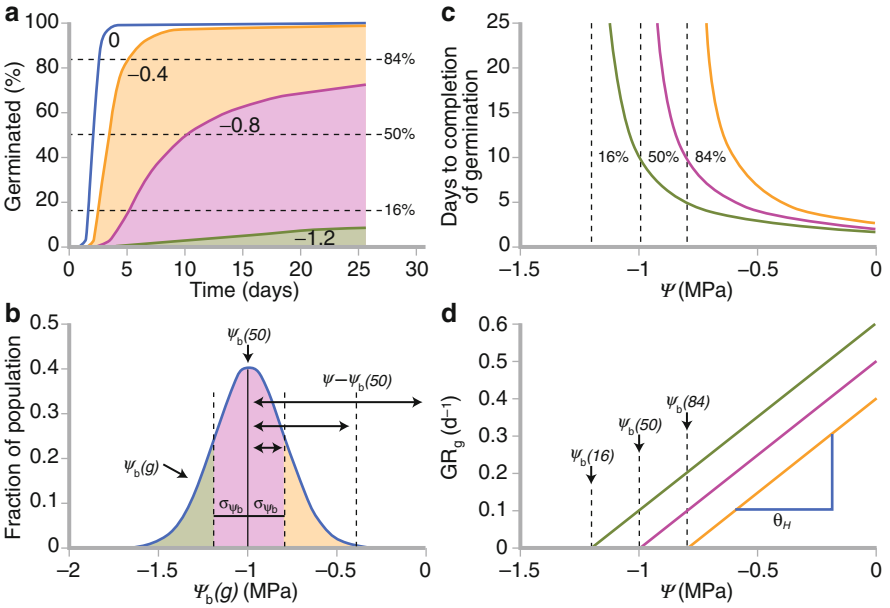

Fig. 7.4 Seed germination patterns in relation to water availability. (**a**) Germination time courses of seeds imbibed in 0, −0.4, −0.8 or −1.2 MPa, based upon the hydrotime model with values of $\theta_H = 2$ MPa d, $\psi_b(50) = -1.0$ MPa, and $\sigma_{\psi b} = 0.2$ MPa, all reasonable values for nondormant seeds. The colored regions refer to *panel b*, and germination percentages of 84, 50 and 16 are indicated by the horizontal dashed lines; these values represent ±1 $\sigma_{\psi b}$ around the mean. (**b**) The $\psi_b(g)$ distribution of threshold water potentials for the seed population. The normal distribution is indicated by the bell-shaped curve, which corresponds to the fractions of seeds in the population with the indicated $\psi_b(g)$ values. One standard deviation ($\sigma_{\psi b}$) on each side of the median ($\psi_b(50)$) is indicated. The difference between the seed ψ and the $\psi_b(50)$ is indicated for different values of ψ by the double arrows. At 0 and −0.4 MPa, essentially all seeds will complete germination. Between −0.4 and −0.8 MPa (*orange* region), a fraction of the population will not be able to germinate because the ψ is below their respective thresholds. This is shown by the reduction in germination percentage (and slower rate) of the *orange* shaded region in *panel a*. Similarly, reducing the ψ to −1.2 MPa prevents the germination of 84% of the seed population (*purple area* in *panel a*), and the remaining 16% germinate slowly as the ψ is close to their ψ_b (*green area* in *panel a*). (**c**) The times to germination of different fractions of the seed population imbibed at different ψ. At high water potentials, times to germination are similar, but increasingly diverge as ψ decreases. As the ψ approaches the ψ_b for a given seed fraction, times to germination increase very rapidly and asymptotically approach the ψ_b lines (*vertical dashed lines*). (**d**) Germination rates (GR_g), or $1/t_g$, for the curves illustrated in *panel c*. Plotting the inverse of t_g versus ψ results in lines with slopes of $1/\theta_H$ and intercepts on the *x*-axis of the ψ_b values for the specific germination fraction. For the fractions chosen, the intercepts differ by the value of $\sigma_{\psi b}$

The relationship between the $\psi_b(g)$ distribution and times to germination is determined by the difference between ψ and $\psi_b(g)$. If we consider the median seed of the population with a threshold of $\psi_b(50)$, then the difference between the threshold and the actual ψ is shown by the arrows in Fig. 7.4b. As ψ is reduced from 0 to −0.4 to −0.8 MPa, the difference $\psi - \psi_b(50)$ also decreases, which, according to

(7.1), means that the time to germination t_g must increase proportionately. When $\psi = \psi_b(50)$, t_g equals infinity for that fraction, which is the definition of the threshold, i.e., the ψ at which germination cannot be completed. For the example in Figure 7.4b, $\psi_b(50) = -1$ MPa with a standard deviation ($\sigma_{\psi b}$) of 0.2 MPa. Thus, at −0.8 MPa, a final germination percentage of 84 is expected, and at −1.2 MPa a germination percentage of 16. As shown in Fig. 7.4a, the respective germination time courses are approaching those values, but it will take a very long time for the asymptote to reach those percentages (~350 d), because the time to germination increases sharply as $\psi - \psi_b(g)$ gets very small (Fig. 7.4c).

Equation (7.1) can be rearranged in the following way to illustrate the relationship between ψ and germination rates (GR), or the inverse of time to radicle emergence for fraction g ($GR_g = 1/t_g$).

$$GR_g = 1/t_g = (\psi - \psi_b(g))/\theta_H \tag{7.2}$$

Thus, a plot of GR_g versus ψ gives straight lines with common slopes of $1/\theta_H$ and intercepts on the ψ axis equal to $\psi_b(g)$ (Fig. 7.4d). This is one way to determine the values of θ_H and of $\psi_b(g)$ for specific germination fractions. A more convenient method is to fit the original germination time courses at different ψ using regression models that allow the values of θ_H, $\psi_b(50)$ and $\sigma_{\psi b}$ to be determined. An alternative approach termed the "Virtual Osmotic Potential" (VOP) model shares the concept of a population distribution of $\psi_b(g)$, but assumes that progress toward germination is due to either accumulation of osmotic solutes to increase embryonic growth potential or a reduction in the restraint by enclosing tissues over time following imbibition (see also Sect. 4.6).

7.2.1.2 Hydrotime and Dormancy

The hydrotime model can reproduce the patterns of germination time courses that occur in response to reduced ψ. Interestingly, these same patterns are also encountered as seeds enter or leave dormancy, even when germinating on water. That is, there is generally an increase in both final germination percentage and speed of germination as a seed population loses dormancy. This is illustrated by the loss of dormancy due to after-ripening of botanical (true) potato seeds (Fig. 7.5). Initially, the seeds are partially dormant; germination is slow and the maximum, even in water, is only about 50% (Fig. 7.5a). After 7 or 30 days of after-ripening, germination increases to near 100% and is much more rapid and uniform (Fig. 7.5b, c). This is particularly evident when the seeds are germinated at reduced ψ, because −0.4 MPa almost completely prevents germination initially, but after 30 days of after-ripening, the same ψ only slightly delays germination. This change in sensitivity to ψ indicates that the $\psi_b(g)$ distributions of the seed population have shifted to lower values due to after-ripening. The $\psi_b(50)$ values go from −0.18 MPa initially to −1.89 in 30-day after-ripened seeds (Fig. 7.5d, f). Note that as far as the hydrotime model is concerned, there is no distinction between a constant threshold distribution and a

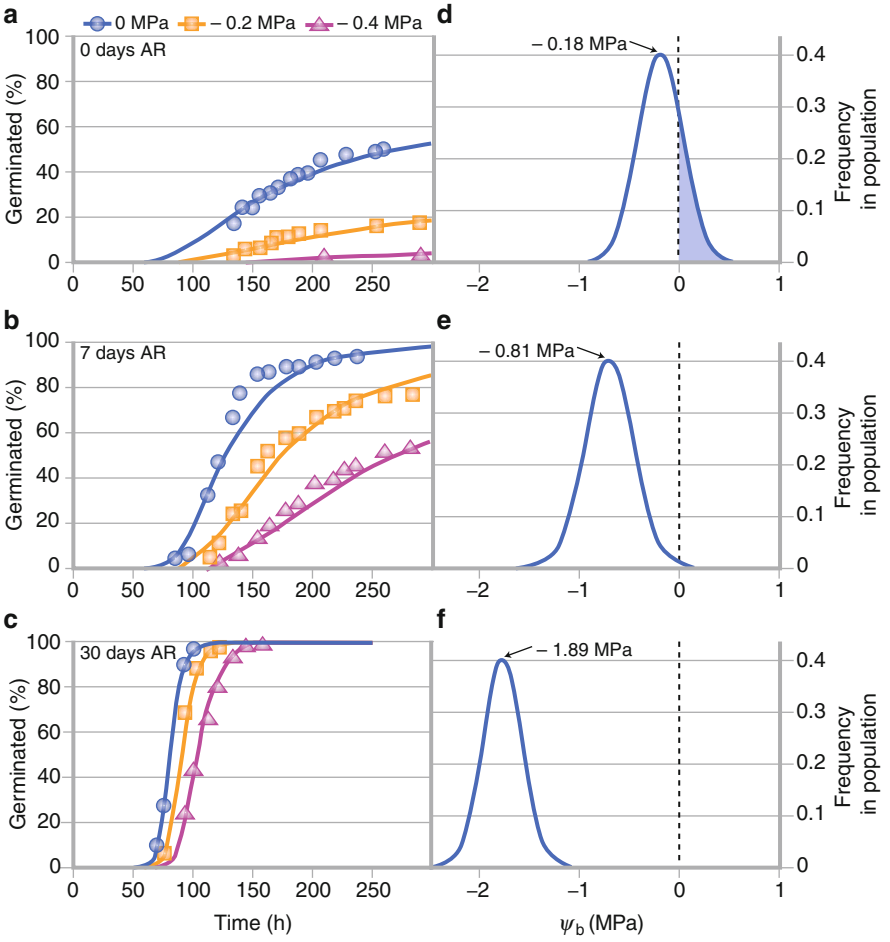

Fig. 7.5 Germination of botanical potato seeds at 14–15°C and different ψ (0, −0.2, −0.4 MPa) as affected by after-ripening (AR) for (**a**) 0, (**b**) 7 or (**c**) 30 days at 4% seed moisture content and 37°C. The symbols are the experimental data, and the curves are predicted from the hydrotime model based upon the $\psi_b(g)$ distributions shown in **d**, **e**, and **f**. Initially, the ψ_b distribution was high ($\psi_b(50) = -0.18$ MPa) and a fraction of the seed population was unable to germinate even in water (represented by shaded area under curve to the right of the dashed 0 MPa line in *panel d*). During after-ripening, the $\psi_b(g)$ distributions shifted to lower values ($\psi_b(50) = -0.81$ MPa, −1.89 MPa; *panels e* and *f*), corresponding to the more rapid germination and decreased sensitivity to inhibition by low ψ (*panels b* and *c*). Modified from Alvarado and Bradford (2005) and Allen et al. (2007)

change in ψ, or a constant ψ with an opposite direction change in the threshold distribution, because germination kinetics depend solely on the difference $\psi - \psi_b(g)$. Thus, a negative shift in the $\psi_b(g)$ distribution results in an identical effect on germination as an equivalent increase in ψ. Dormancy, the inability to germinate in

water, is equivalent functionally to a seed having a ψ_b threshold greater than 0 MPa, as indicated by the blue shaded fraction of the distribution in Fig. 7.5d.

Examples of both dry after-ripening and of moist chilling (stratification) affecting $\psi_b(g)$ threshold distributions have been reported. Thus, it may be a general phenomenon that the potential for germination (or alternatively, the depth of dormancy) is determined physiologically by shifts in the $\psi_b(g)$ distributions to higher or lower values, depending upon the environmental signals received by the seeds. As discussed previously (Sect. 6.6), hormones, particularly ABA and GA, are likely to be involved in regulating these transitions. It is consistent, therefore, that these hormones shift $\psi_b(g)$ distributions to more negative values when germination is promoted (i.e., in the presence of GA or absence of ABA) and to more positive values when germination is inhibited (in the presence of ABA or absence of GA). In fact, the sensitivity of seeds to hormones, oxygen, light and other factors can also be quantified by population-based threshold models based upon the hydrotime equation (7.1).

7.2.1.3 Ecological Applications of the Hydrotime Model

If the hydrotime model provides an accurate description of germination responses to dormancy-inducing and dormancy-breaking factors, what are the implications for seed ecology? In drier environments, seasonal or erratic rainfall is the primary determinant of seedling survival, and seeds from these environments often require dry after-ripening and are sensitive to inhibition of germination by high temperatures. These responses are illustrated by the germination and after-ripening of cheatgrass seeds in the dry Great Basin region of the western United States. Seeds are initially shed in early summer, and require several months of after-ripening to alleviate dormancy. Thus, at the time of shedding, their mean ψ_b thresholds are near 0 MPa, and are higher when set to germinate at warm temperatures (20/30°C) compared to cooler temperatures (10/20°C), indicating also the occurrence of thermoinhibition (Fig. 7.6). Values of $\psi_b(50) > 0$ MPa indicate dormant seeds. Over the summer months, the values of $\psi_b(50)$ for these populations after-ripening in soil under field conditions decline, indicating that the seeds are increasingly likely to germinate rapidly when water becomes available. The rate at which this decrease in $\psi_b(g)$ occurs is dependent upon the seed ψ. At high ψ (e.g., imbibed seeds), after-ripening does not occur and seeds either deteriorate or remain dormant. Between about −40 and −400 MPa, after-ripening proceeds, but at a decreasing rate as the ψ declines. At very low ψ (below −400 MPa), after-ripening does not occur. Thus, water availability (and temperature) influence the rate of after-ripening, and the consequence of after-ripening is a decrease in $\psi_b(g)$ such that germination rates and percentages will be higher the later that rainfall occurs in the fall. It has been noted that from an ecological perspective, slow germination is as effective as dormancy at unfavorable times in a fluctuating environment, because seeds are generally capable of tolerating dehydration if radicle emergence has not occurred. Maintaining a wide distribution of $\psi_b(g)$ values within the seed population assures that while some seeds will capi-

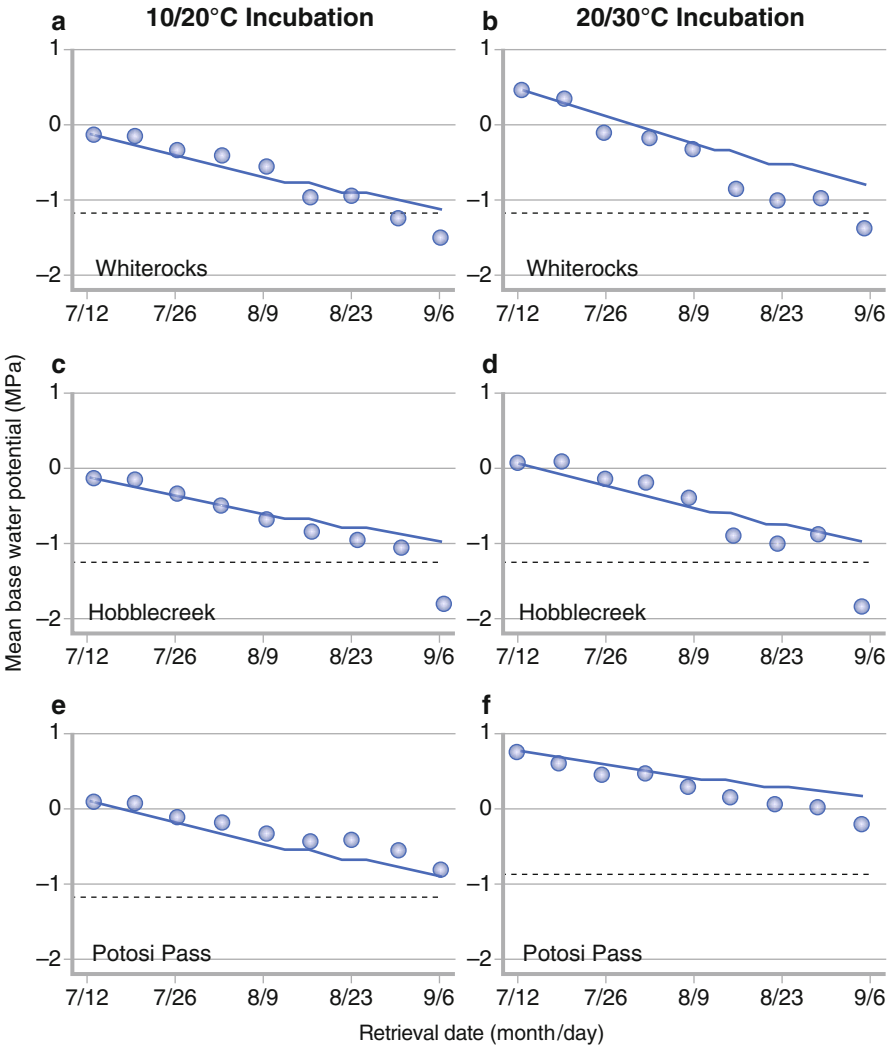

Fig. 7.6 Predicted and observed changes in $\psi_b(50)$ during field after-ripening of seeds of three populations of *Bromus tectorum* (Whiterocks, Hobblecreek and Potosi Pass) when measured at two incubation temperatures (10/20 or 20/30°C). Symbols represent values measured in the laboratory for seeds retrieved from the field, solid lines show values predicted from a simulation model of after-ripening rates for the environmental conditions (primarily affected by temperature), and the dotted horizontal lines represent the $\psi_b(50)$ values of seeds for each population when fully after-ripened and tested at the indicated temperature. Note that $\psi_b(50)$ values were generally higher when tested at higher temperatures, indicating that thermoinhibition of germination influences ψ_b distributions (see also Fig. 7.11). From Bauer et al. (1998). With permission of Oxford Univ. Press

talize rapidly on favorable conditions, others will be more conservative, committing to radicle emergence only after a much longer period of hydration or not at all. This "bet-hedging" strategy may be critical for survival of species in relatively unpredictable environments. In other cases, avoiding low temperatures and the danger of frost may be more important, requiring seeds to delay germination until after experiencing a cold period. This has also been associated with shifts in $\psi_b(g)$ distributions to more negative values after moist chilling.

There is an ecological rationality to having various environmental signals influence germination capacity via effects on $\psi_b(g)$. As seasonal and environmental requirements are met that indicate to the seed that an opportune time to germinate is approaching (e.g., after-ripening, stratification, light, nutrients, etc.), a negative shift in $\psi_b(g)$ will result in an increase in the fraction of seeds capable of germinating and in the overall speed of germination. However, since this increased capability is based upon the $\psi_b(g)$ threshold distributions, the seed population will still remain highly sensitive to the current local water availability. Even if the $\psi_b(g)$ distribution shifts to quite low values in a physiological sense (−1 to −2 MPa), a relatively small decline in soil ψ can still have a dramatic effect in delaying or preventing germination (c.f., Fig. 7.4). Thus, seasonal or environmental effects on the capacity for germination may act through physiological shifts in the $\psi_b(g)$ distribution, with the variation in $\psi_b(g)$ among individual seeds providing differential sensitivity to local conditions to ensure that there are both opportunistic and conservative individuals within the population. By responding to environmental factors via modification of their sensitivity to ψ, seed populations can achieve both long-term integration of their environmental history and regulation of their progress toward germination based upon current water availability.

7.2.2 Temperature

After water, temperature is the most important environmental determinant of seed germination. Temperature acts to regulate germination in the field in three ways: (a) by determining the capacity and rate of germination of nondormant seeds, (b) by removing primary and/or secondary dormancy, and (c) by inducing secondary dormancy. The latter two effects of temperature on dormancy release and induction largely determine the fraction of seeds that are potentially germinable in a given location at a specific time. The role of temperature in the induction and release of dormancy is discussed in Sects. 6.5, 6.6, and thus, attention here is focused primarily on the effects of temperature on germination rates and percentages of nondormant seeds.

7.2.2.1 Cardinal Temperatures for Seed Germination

It has been recognized since the mid-1800s that three "cardinal" temperatures (minimum, optimum and maximum) describe the range of temperatures (T) over which seeds of a particular species can germinate. The cardinal temperatures for

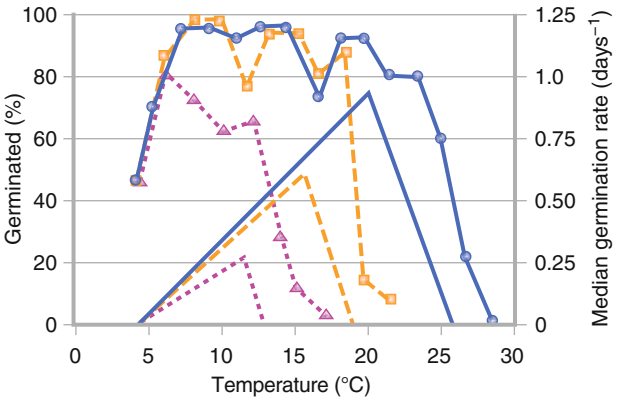

Fig. 7.7 Final germination percentages (*left y*-axis) of *Phleum arenarium* seeds in response to after-ripening at 15°C and 15% RH for 1 (*triangles*), 6 (squares) or 13 months (*circles*), illustrating the widening of the temperature window for germination as dormancy is alleviated. Superimposed are predicted median germination rates (*right y*-axis), which would increase linearly with temperature to a maximum, then decrease at inhibitory temperatures. Final germination percentage data from Probert (1992)

germination are generally related to the environmental range of adaptation of a given species and serve to match germination timing to favorable conditions for subsequent seedling growth and development. The minimum or base temperature (T_b) is the lowest T at which germination can occur, the optimum temperature (T_o) is the T at which germination is most rapid, and the maximum or ceiling temperature (T_c) is the highest T at which the seeds can germinate. The temperature range between T_b and T_c can vary with the dormancy status of the seeds, generally being more narrow in fresh or dormant seeds and widening as dormancy is lost. This is illustrated by *Phelum arenarium* seeds: fresh seeds germinated well only between about 5 and 13°C, but the upper temperature limit increased to 25°C following 13 months of dry after-ripening while the T_b remained constant (Fig. 7.7). Similar data are available illustrating reductions in the low temperature limit for germination following dormancy breaking (e.g., moist chilling), as in *Polygonum aviculare*. Since these relative temperature limits can vary with dormancy status, some consider that T_b and T_c should be defined as the minimum and maximum temperatures for germination of nondormant seeds of a species, with germination capacity approaching these minimum and maximum temperature limits as dormancy is alleviated.

7.2.2.2 Thermal Time Models

Germination rates (i.e., the inverse of times to germination for specific germination percentages) are also very sensitive to temperature, generally increasing with temperature to an optimum and then decreasing sharply at temperatures above the

optimum. Although total germination percentages tend to show a broad maximal range, germination rates more narrowly identify the optimum temperature for germination (Fig. 7.7). Germination rates of more dormant seed populations may also be slower compared to less dormant seeds at the same temperature. As the temperature window widens, germination rates will be similar at lower temperatures but continue to increase at higher temperatures as the T_o increases. Germination rates and percentages fall sharply as temperature increases above T_o.

Mathematical models have been developed to describe germination patterns in response to T. For suboptimal temperatures (from T_b to T_o), germination timing can be described on the basis of thermal time or heat units (i.e., degree-days). The basis of thermal time for suboptimal temperatures is that the T in excess of T_b multiplied by the time to a given germination percentage g (or t_g), is a constant for that percentage (the thermal time constant, $\theta_T(g)$):

$$\theta_T(g) = (T - T_b)t_g \tag{7.3}$$

or

$$GR_g = 1/t_g = (T - T_b)/\theta_T(g) \tag{7.4}$$

Since $\theta_T(g)$ is a constant for a given germination percentage, the closer T is to T_b, the correspondingly longer the time to germination, or the smaller the germination rate. As shown in (7.4), this model predicts that GR_g will increase linearly with temperature above T_b, as illustrated in Fig. 7.7. Although not always the case, T_b is often the same or similar for all seeds in the population, such that GR_g plots versus temperature all intersect the x-axis at the same point (Fig. 7.8). However, it is evident that the rate of germination is faster for lower percentages than for higher percentages, resulting in different slopes of the GR_g lines above T_b (Fig. 7.8). The inverse of the slopes equals the thermal time constants $\theta_T(g)$ for each fraction of the population (Fig. 7.8). As was noted previously for water potential thresholds (Sect. 7.2.1.1), the values of $\theta_T(g)$ are often normally distributed among seeds in the population, so they can be represented by a bell-shaped curve characterized by a mean ($\theta_T(50)$) and standard deviation ($\sigma_{\theta T}$) (Fig. 7.8 inset a). Thus, while all seeds in a population may have the same T_b value, they exhibit a distribution of times to germination ($\theta_T(g)$ values) that result in the familiar sigmoid germination time courses (Fig. 7.9a). When these time courses are plotted on a thermal (degree-hours) time scale (i.e., $[T - T_b]\ t_g$), they all fall on the same sigmoid curve (Fig. 7.9b). For most nondormant seeds, once the T_b, $\theta_T(50)$ and $\sigma_{\theta T}$ values have been determined, germination times for any fraction of the population at suboptimal temperatures can be estimated. T_b and $\theta_T(50)$ values characteristic of crop species are often used in conjunction with soil temperature data to estimate the days to seedling emergence after planting in the field. In many locations, weather data are available online already converted into degree-days units for various T_b values, making it easy to calculate accumulated thermal time since planting. Similarly, germination models

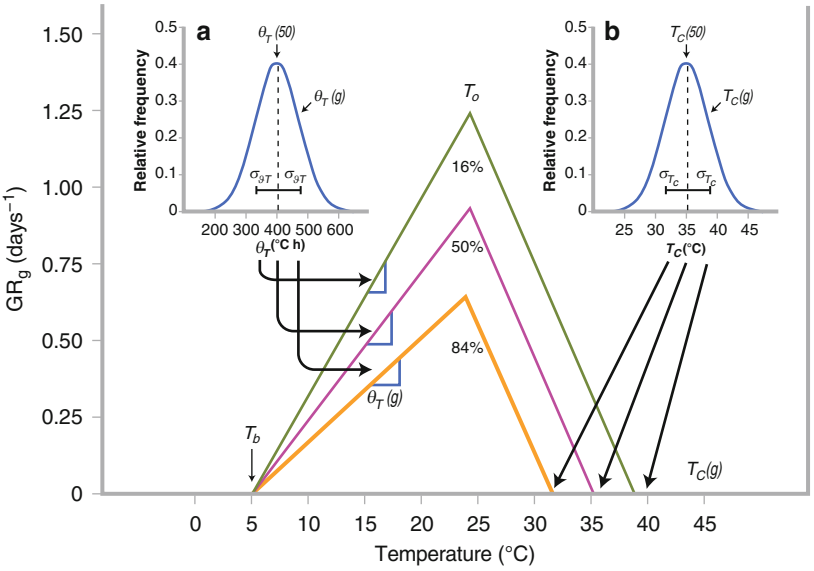

Fig. 7.8 Relationships between germination rates and temperature. At low temperatures, germination rates ($GR_g = 1/t_g$) for different percentages (g) of the seed population increase linearly with temperature above a common base temperature (T_b). The slopes of the lines are equal to the inverses of the thermal times to germination ($1/\theta_T(g)$), which vary among individual seeds in a normal distribution (*inset a*). The maximum GR_g occurs at the optimum temperature (T_o), and above this temperature GR_g decreases linearly. The ceiling temperatures for germination ($T_c(g)$) vary in a normal distribution among seeds within the population (*inset b*). Germination rates are shown for 16, 50 and 84%, which represent the median and ±1 standard deviation of the respective distributions. Modified from Bradford (2002)

based on thermal time are used for predicting weed seedling emergence in both cropping systems and non-crop landscapes.

Similar models have been developed to describe germination rates at supraoptimal temperatures (from T_o to T_c). In many cases, GR_g declines linearly with increasing T between T_o and T_c (Fig. 7.8). However, in contrast to a common T_b for all seeds in the population, it is generally observed that different fractions of the seed population have different T_c values. The following model accounts for this variation in T_c values:

$$\theta_2 = (T_c(g) - T)t_g \tag{7.5}$$

or

$$GR_g = 1/t_g = (T_c(g) - T)/\theta_2 \tag{7.6}$$

where θ_2 is a thermal time constant at supraoptimal T and $T_c(g)$ indicates that T_c values vary among fractions (g) in the seed population (Fig. 7.8 inset b). At supraoptimal temperatures, the differences in GR_g among seed fractions are a consequence of variation among seeds in their ceiling temperatures ($T_c(g)$), and the total thermal time is constant in the supraoptimal range of T for all seeds in the population.

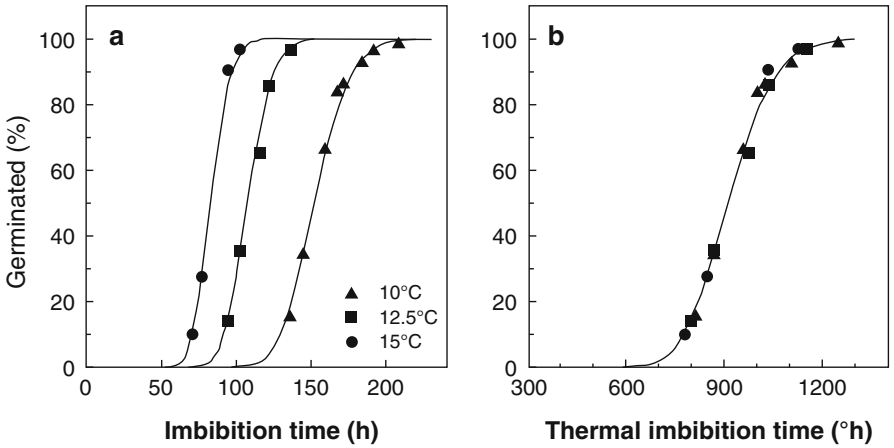

Fig. 7.9 Normalization of germination time courses across temperatures by thermal time. (**a**) Germination time courses of potato seeds at 10 (*triangles*), 12.5 (*squares*) and 15°C (*circles*). Symbols are data points, and curves are predicted from the thermal time model (7.3). (**b**) The same data as in *panel a* plotted on a thermal time scale, or $(T-T_b)\,t$ using a base temperature of 4°C. Thermal time or heat units accumulation (degree-hours) completely accounts for the differences in germination rates at these suboptimal temperatures. Original data of V. Alvarado and K.J. Bradford

7.2.2.3 Temperature and Water Interactions: Hydrothermal Time Models

As both T and ψ are critical environmental regulators of seed germination, it is convenient to combine their effects into a single "hydrothermal time" model. This can be done as follows for the suboptimal temperature range:

$$\theta_{HT} = (\psi - \psi_b(g))(T - T_b)t_g \tag{7.7}$$

Simply by multiplying the time to germination by $(T-T_b)$ the hydrotime model (equation 7.1) can be converted to a thermal time basis, with a new hydrothermal time constant (θ_{HT}). The conversion to thermal time largely accounts for the effect of suboptimal temperatures, while a normalization equation can also account for the effects of reduced ψ on germination. This equation converts germination time courses at any ψ into equivalent time courses in water (ψ=0 MPa) by normalizing for the delay in germination caused by reduced ψ:

$$t_g(0) = [1 - (\psi / \psi_b(g))]t_g(\psi) \tag{7.8}$$

where $t_g(0)$ is the time to germination of fraction g in water and $t_g(\psi)$ is the time to germination of fraction g at a lower ψ. The combination of this normalization function for ψ and thermal time can describe germination time courses at combinations of suboptimal temperatures and reduced ψ (Fig. 7.10). It is important to note that the normalization of time courses at different ψ to their equivalent in water is not a "hydrotime" scale in the same way that developmental events at different temperatures

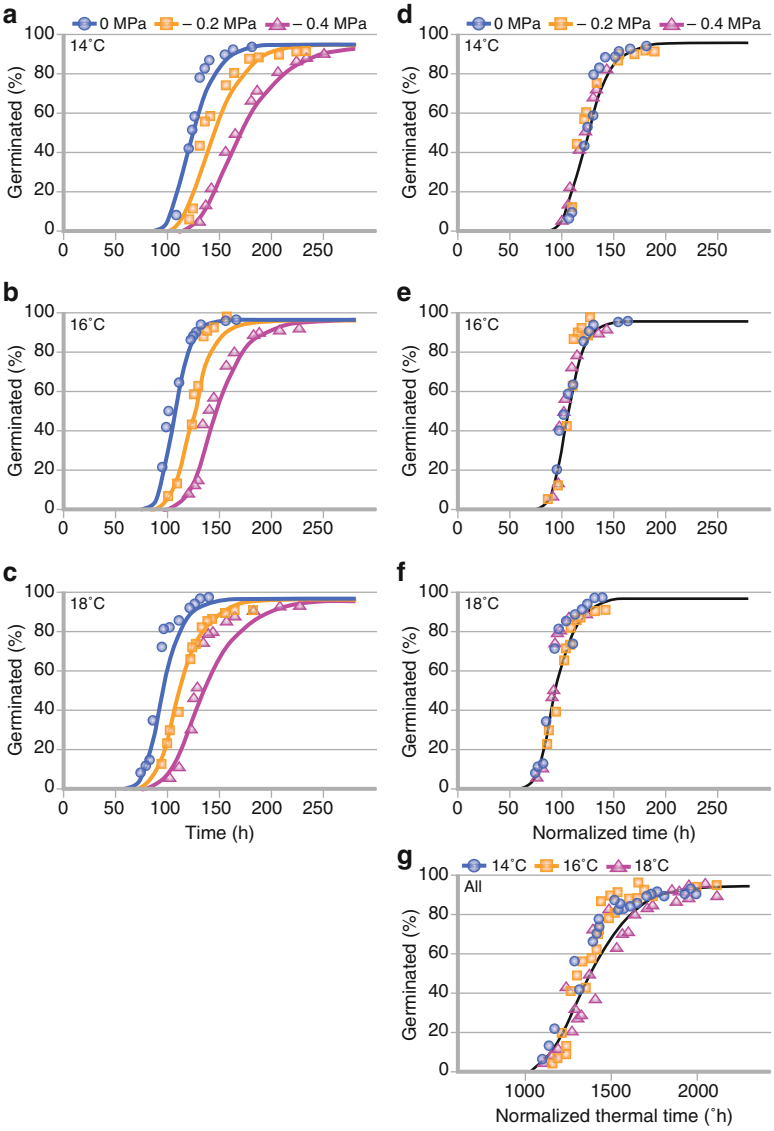

Fig. 7.10 Normalization of germination time courses across a range of suboptimal temperatures and reduced water potentials. **a**, **b** and **c** show germination time courses of potato seeds at 14, 16, and 18°C and at 0 (*circles*), −0.2 (*squares*) and −0.4 MPa (*triangles*) at each temperature. The curves are fit using the hydrotime model at each temperature (7.1). **d**, **e**, and **f** show the same data normalized to account for the delaying effect of reduced ψ according to (7.8). This function normalizes the time courses to match the germination rates in water (0 MPa). When these normalized curves are plotted on a thermal time scale (**g**), all of the germination time courses across both temperatures and water potentials fall on a common curve. Based on Alvarado and Bradford (2002)

can be plotted on a single thermal time scale (e.g., Fig. 7.9b). This is because the hydrotime to germination is the same for all seeds in the population; it is their ψ_b thresholds that vary among individual seeds.

As was discussed for changes in seed dormancy (Fig. 7.5), shifts in $\psi_b(g)$ distributions also contribute to temperature responses of seed germination. Recall that different fractions of seed populations exhibited different maximum temperatures for germination, or $T_c(g)$ values (Fig. 7.8). This can now be understood to be due to a shift of $\psi_b(g)$ to more positive values when T exceeds T_o. Different fractions of the seed population have different ψ_b values, so as the entire distribution shifts upward, those with the highest ψ_b values will reach 0 MPa, or be unable to germinate in water, at a lower temperature than seeds with more negative ψ_b values. For potato seeds, $\psi_b(g)$ values increase sharply at temperatures above T_o, intersecting the 0 MPa axis at the point where $GR(g)$ values also intersect the x-axis for the same fraction of the population (Fig. 7.11). Thus, the effect of temperature on $\psi_b(g)$ distributions appears to underlie the decrease in germination rates and inhibition of germination that occurs between T_o and T_c.

7.2.3 *Light*

Seeds may remain in the soil seed bank for many years, apparently unaltered, because germination of buried seeds is suppressed. If the soil is cultivated or natural disturbance takes place, as in a river bank, many of the seeds may germinate and a flush of seedlings results. This only occurs if seeds in the seed bank are nondormant, i.e., if they are sensitized by the previous seasonal conditions (Sect. 7.3). One investigation reported that weeds such as *Sinapis arvensis, Polygonum aviculare, Veronica persicaria,* and others increase from about 35 to 780 seedlings per square meter after the soil is turned over. Another study compared the occurrence of seedling flushes in two adjacent plots within a cultivated oats field. One plot was harrowed, as usual, during the daytime, whereas the other plot was cultivated in the same way but during the night. The difference in weed emergence was dramatic, with almost no emergence in the dark-cultivated plot and approximately 80% coverage by some 30 different arable weeds in the light-cultivated plot (Fig. 7.12). Light is primarily responsible for this effect. As little as 1 millisecond of exposure to full sunlight can cause many seeds to germinate and produce seedlings. This principle may be utilized to reduce the use of herbicides in weed management programs.

Since light controls dormancy and germination of seed banks, how effective is the soil as a light filter and how deep must seeds be buried to escape from its effects? Less than 2% of the light passes through 2 mm of sand, and the light that is transmitted consists only of wavelengths longer than about 700 nm (Fig. 7.13). A wide part of the spectrum can pass through clay loams, but if the soil particle size is small (when they will be closely packed), a layer 1.1 mm thick is virtually opaque. Inhibition of germination by other soils may require greater depths, however. About 8 mm of loam are needed to completely suppress germination of *Plantago major*, whereas light-promoted

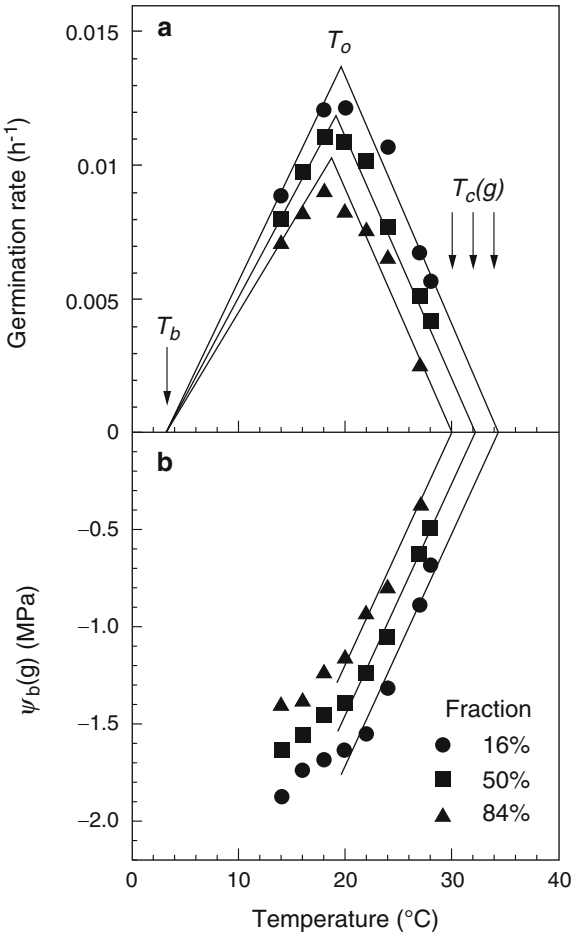

Fig. 7.11 The cardinal temperatures for germination of potato seeds. (**a**) Germination rates at different temperatures define the minimum or base temperature (T_b), optimal temperature (T_o), and maximum or ceiling temperatures (T_c). For this seed lot, T_b is 3.2°C, T_o is 19.3°C, and T_c varies with the seed fraction, being 34, 32, and 30°C for the 16th, 50th, and 84th percentiles, respectively. The symbols are the experimental data and the lines are germination rates determined by fitting the hydrotime model (equation 7.1) at sub- and supraoptimal T. (**b**) $\psi_b(g)$ increases linearly as T increases in the supraoptimal range. The $\psi_b(g)$ values calculated from the hydrotime model are plotted (*symbols*) along with the linear increases (*lines*) predicted by the model. The projected lines for different seed fractions (16, 50, and 84%) intercept the $\psi_b(g)=0$ MPa axis (where germination is prevented even in water) at the T_c values for these fractions. From Alvarado and Bradford (2002). Courtesy of Wiley

germination of *Digitalis purpurea* still occurs under 10 mm of sand, demonstrating differing sensitivities to light (Fig. 7.14). These examples also make clear that soil disturbance is of high ecological relevance, for without it the effective soil seed bank would be confined to the top soil layer of less than 1 cm.

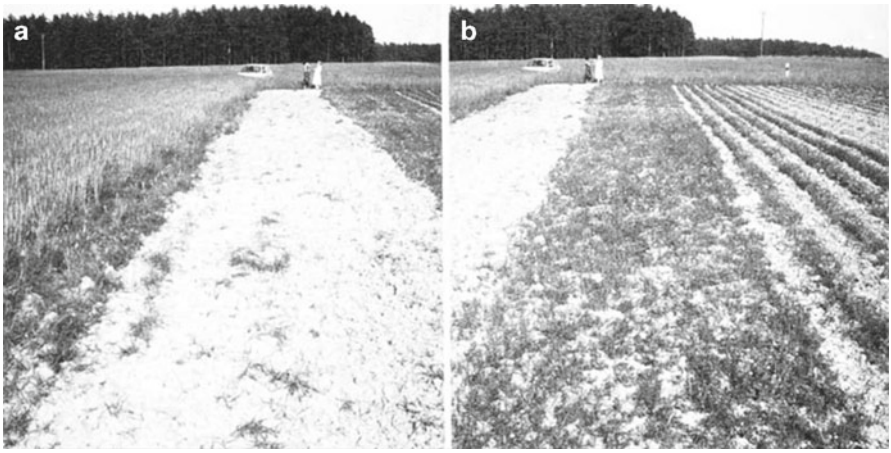

Fig. 7.12 Adjacent plots of about 55×3 m within an oat field in Germany. (**a**) All tillage was accomplished in the time span from 1 h after sunset until midnight. It was plowed in September, chopped in March and harrowed in April. The total coverage by weeds is about 2%. (**b**) All tillage was performed at noon, on the same days. The total area covered by weeds is about 80%. From Nezadal and Hartmann (1990)

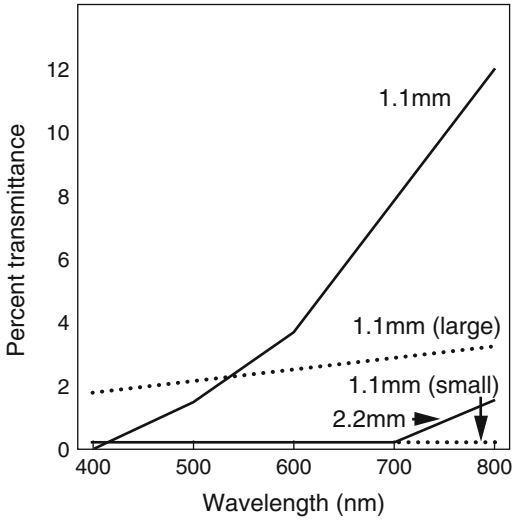

Fig. 7.13 Transmission of light through soils. Two soils were used, sand (——) and a clay loam (·····), at thicknesses indicated on the curves and, in the case of clay, at large (approximately 0.9 mm) and small (approximately 0.45 mm) particle size. Adapted from Woolley and Stoller (1978)

The quality of transmitted light is as important as the quantity. Since the longer wavelengths of light penetrate soil more easily, a buried seed experiences a relatively high proportion of far-red light (i.e., >700 nm), which affects the phytochrome photoequilibrium (ϕ) (Sect. 6.6.5.2). Light passing through 1 mm of dry, sandy soil results in a photoequilibrium of about 0.45, which should be sufficient to break

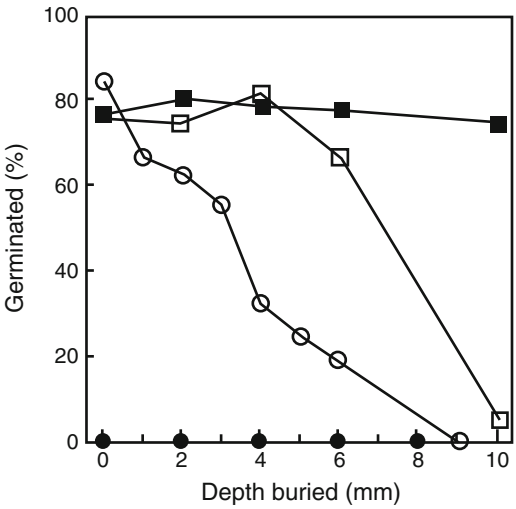

Fig. 7.14 Effect of burial on germination. *Plantago major* seeds were buried at different depths in wet loam held in the light (○) or dark (●). Seeds of *Digitalis purpurea* (■) and *Cecropia obtusifolia* (□) were buried at different depths in wet sand exposed to light. Germination of the last two species in darkness was almost zero. Adapted from Frankland and Poo (1980) and Bliss and Smith (1985)

dormancy in a high percentage of light-requiring seeds. That it does not necessarily do so may be because the very low fluence rates in the soil result in very low rates of Pfr formation. The germination of light-requiring seeds of some species is therefore restricted to the uppermost layers of soil where the light stimulus for dormancy breakage can operate. This is extremely important for small seeds with limited food reserves, because if germination is completed at too great a depth the seedling's reserves may be exhausted before it is able to reach daylight and begin to photosynthesize. Most species of light-requiring seeds are, in fact, small (<1 mg seed weight). This also implies that soil disturbance may lead to seedling death when the light-induced seed is reburied too deeply after the disturbance event. In addition, light requirements of seeds are modified strongly by other environmental factors, such as temperature and nitrate, which clearly will affect seed behavior in the field.

7.2.3.1 Phytochrome Responses

The role and mechanism of phytochrome in the breaking of dormancy and induction of germination were discussed in Chap. 6. In this section, phytochrome responses are viewed in an ecological context.

Light-induced germination often displays a biphasic response to the amount of photons or "fluence" of red (R) light (Fig. 7.15). The first phase is called the very-low fluence response (VLFR) and is present in seeds with extreme sensitivity to light. The VLFR is mediated by phytochrome A and is saturated by Pfr/Ptot ratios lower than 0.1%. This value of ϕ is also established by a pulse of far-red light (FR) because the absorption spectra of the active and inactive forms of phytochrome

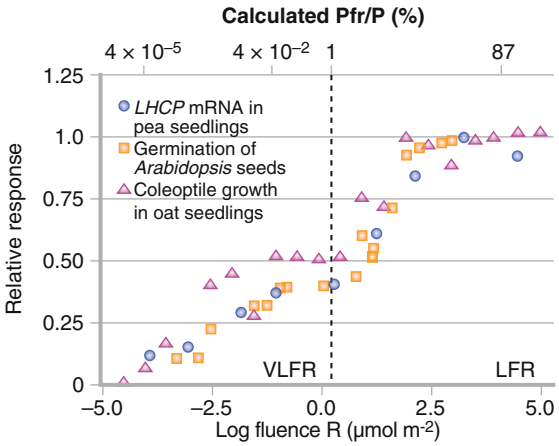

Fig. 7.15 VLFR and LFR influences on different physiological changes. Responses of seed germination in *Arabidopsis thaliana*, coleoptile growth in etiolated oat seedlings, and LHCP (light harvesting chlorophyll a/b-binding protein) mRNA expression in etiolated peas to the fluence of a R pulse and the proportion of Pfr established by these pulses. The commonality of the effects of VLFR (below 0.1% Pfr/P) and LFR (above 1% Pfr/P) are evident. From Casal et al. (1998). With permission of Oxford Univ. Press

overlap slightly (Sect. 6.6.5.2). Thus, these seeds do not show the classic R–FR reversibility. In the field, the VLFR may occur when the seeds have remained in the soil for a prolonged period of time. The second phase or low-fluence response (LFR) is saturated by moderate to high Pfr/Ptot ratios induced by R light. The LFR can be reversed when R is followed by a FR treatment. The main photoreceptor involved in LFR responses is phytochrome B. A third response to light is inhibitory to germination and is caused by the so-called high-irradiance response (HIR). The HIR can be induced by a prolonged irradiation with FR and may antagonize both the VLFR and LFR.

What is the ecological relevance of these different responses? The VLFR is thought to be involved in the promotion of germination by brief exposures to light during soil disturbances, as described above. The VLFR will also be the inducer of germination of seeds in the 1-cm top layer of the soil where very limited light penetrates. The LFR distinguishes itself from the VLFR by its R/FR reversibility. This has been established mainly under laboratory conditions and surprisingly little is known about the ecological relevance of the LFR.

Germination in many species is inhibited when seeds are on the soil surface. Often, the relative dryness of the soil accounts for this. Seeds of a large number of species, however, are affected by high fluence rates of light on the soil surface, which inhibit germination through the HIR. It has been argued that such sensitivity is a mechanism for discouraging germination under high solar radiation when the seedling would be subjected to harsh, drying conditions.

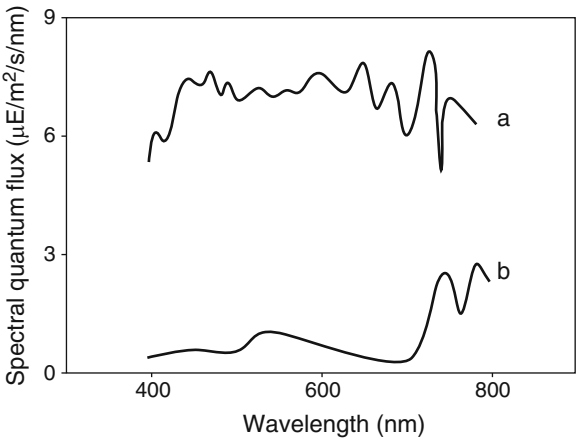

Fig. 7.16 Spectral energy distribution of sunlight and vegetational shade light. (a) Direct sunlight at 11:00 h., and (b) shade light (under sugar beet plants). The ratio of R to FR light (660/730 nm) in (a) is 1.18 and in (b) is 0.12. After Holmes and McCartney (1975)

Chloroplasts absorb light most strongly at approximately 675 nm and allow wavelengths >720 nm to be transmitted completely. Because of this, sunlight passing through green leaves has a very low R/FR ratio (Fig. 7.16). The phytochrome photoequilibrium (ϕ) established by such light can be as low as 0.15, depending on the thickness of the canopy. Most light-requiring seeds will not germinate under such conditions. Indeed, if they are exposed to this light for many hours over several days they will probably be induced into secondary dormancy. These seeds, therefore, become dormant as a result of irradiation with FR-rich canopy light and will germinate later only when a dormancy-releasing factor, such as chilling or light, has been experienced. Seeds of *Bidens pilosa*, for example, when forced into dormancy by canopy light, become light-requiring and subsequently germinate only when exposed to light of suitable quality to establish a relatively high photoequilibrium value of phytochrome; direct sunlight would, of course, suffice. This mechanism may account, for example, for the paucity of seedlings on forest floors and the flush that follows the appearance of a gap in the leaf canopy, brought about when individual trees die and fall or when tree clearance occurs. Thus, the seed bank in forest soils may be rich but germination is held in check, to a large extent by the canopy filtered FR light environment. In the field, vegetational shade is provided by grasses and herbs, of course, as well as by trees. For example, plants of *Arenaria* spp., *Veronica* spp., and *Cerastium* spp. establish themselves on vegetation-free ant mounds but not in the surrounding pasture. Seeds of these species are inhibited by light filtered through green leaves, but germinate in sites where the seedlings will face less competition with other plants for light for photosynthesis. Some species that are able to colonize shaded sites, e.g., *Centaurium erythrea*, are less sensitive to FR canopy light.

As the spectral energy distribution of canopy light is determined by the thickness of the canopy through which the light passes, the germination response of seeds under the canopy will vary according to the density of leaf cover.

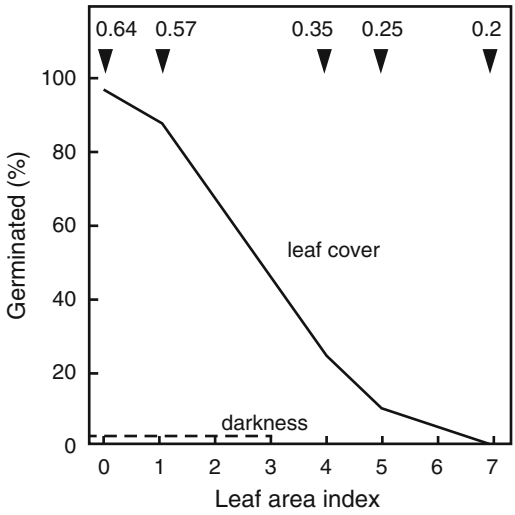

Fig. 7.17 Germination of light-requiring seeds under leaf cover. Seeds of *Plantago major* were sown beneath plants of *Sinapis alba* (*white mustard*) at different densities, as indicated by the leaf area index (i.e., unit leaf area per unit land area). Pfr/Ptot (ϕ) values (at soil level) at different densities are indicated at the top of the graph. (----) Germination in darkness. From data in Frankland and Poo (1980)

This has been simulated under experimental conditions using seeds of *Plantago major*. When placed under increasing leaf cover, provided by *Sinapis alba* plants, germination of these light-requiring seeds is increasingly inhibited as the cover becomes more dense (measured as the leaf area index) and the ϕ beneath the leaves decreases (Fig. 7.17). Variations in the density of a leaf canopy occur in the field as buds break and leaves expand and later senesce and fall. Thus, light-sensitive seeds among vegetation are exposed to changing light environments throughout the growing season, with concomitant effects on their dormancy breakage and germination. However, other environmental factors such as temperature are also influential, so that seed germination is not always predictable from the shade conditions. For example, there is a clear interaction between the different phytochromes and temperature (Sect. 6.6.5). This interaction may be decisive for the form of phytochrome (phyA, phyB, phyE) that will be engaged and, hence, for the ensuing response type. In addition, seasonal conditions during seed maturation may also influence phytochrome-mediated germination.

7.2.4 Nitrate

Nitrate ions have long been known to stimulate germination of seeds of many species, both monocots and dicots, as well as fern spores. Nitrate is part of the global nitrogen cycle and it is present in most soils, often within the range of concentrations that are effective in laboratory germination tests. Nitrate is therefore the foremost inorganic soil component that influences germination.

The ecological significance of nitrate must be considered in conjunction with environmental factors such as temperature, light, and the seed's sensitivity to nitrate

and other chemical soil constituents. Indeed, interactions between such factors and nitrate have been described for a large number of species. To add to the complexity, soil nitrate content, as well as the interacting factors and responsiveness of seeds to them, are all dynamic, displaying fluctuations over shorter or longer periods. In general, germination of most seeds is stimulated within a range of 0–0.05 mol L^{-1} nitrate. The nitrate concentration of the soil fluctuates within this range and therefore might play an ecological role in regulating germination of the soil seed bank.

In many weed species, light and nitrate interact to regulate germination responses. This interaction has been studied in more detail in seeds of *Avena fatua* and *Sisymbrium officinale*. In both species the efficacy of nitrate in stimulating germination depends on the amount of Pfr. In *S. officinale* the dependency on light appears to be absolute. In *Sisymbrium officinale* and *Arabidopsis thaliana*, the effects of Pfr and nitrate on seed germination are reciprocal, i.e., the light requirement of seeds in a soil with low nitrate is higher than that of seeds in nitrate-rich soils. Disturbance of the soil may result in a flush of germination when light is the limiting factor, but soil disturbance may also result in considerable release of nitrate ions. This phenomenon is probably a significant factor in the promotion of weed seed germination, such as *A. fatua*, when summer fallowing is a common practice.

The significance of soil nitrate for seed ecology seems obvious when we assume that a plant that will eventually grow from a seed requires nitrogen for optimal development. However, evidence for a relationship between nitrate concentrations that promote seed germination and those required by the growing plant is largely lacking. A more feasible explanation for the role of nitrate is the ability of seeds to sense changes in the amounts present in their immediate environment. Similar to the shading principle for light, established plants may lower the nitrate content of the soil around their root systems. Nitrate is consumed and nitrification may be inhibited. Thus, the seeds in the immediate environment are depleted of nitrate and germination probability will be reduced. Therefore, the seed's ability to sense both nitrate and FR light contribute to the detection of gaps in the vegetation and, hence, avoid potentially suicidal germination and seedling emergence among competitors, including the mother plant.

7.2.5 Oxygen and Other Gases

The gaseous phase of the soil occupies those pores that are not already filled with water. In addition gases may be dissolved in the soil moisture. Movement of gases through soil is primarily by molecular diffusion but when the soil is waterlogged, gas diffusion is entirely in solution, resulting in a drastic increase in diffusion resistance of several orders of magnitude. In heavy soils and especially in waterlogged soils the oxygen content of the gaseous phase may drop considerably below that of air. The gas phase is also affected by the presence of vegetation. Roots of plants will actively take up oxygen and produce carbon dioxide. In soils with a high organic

content and an active microflora, the oxygen–carbon dioxide balance may shift in a similar way. Although the proportion of oxygen in soil air rarely drops below 19% and carbon dioxide rarely exceeds 1%, extremes may occur at microsites such as those adjacent to plant roots or decaying organic matter, and in soils of flooded areas. In addition to oxygen, carbon dioxide, and nitrogen, soils may contain several other gases and volatile compounds, mostly related to anaerobic conditions and microbial activity. Soils may thus contain methane, hydrogen sulphide, hydrogen, nitrous oxide, and small amounts of carbon monoxide, ethylene, and ammonia.

(1) *Oxygen*. Germination and early seedling growth generally require oxygen at atmospheric levels. Oxygen diffusion can be strongly limited during the lag phase of germination (Sect. 4.4.2) because its diffusion rate is limited by its solubility in water. Moreover, oxygen is often utilized in seed coats and endosperms in non-respiratory reactions. Therefore oxygen concentrations in the embryonic axis may be rather low for some time and therefore seeds must be (temporarily) tolerant of anaerobic conditions. This is particularly true for seeds that germinate under water. Seeds of *Echinochloa crus-galli* germinate well under both anaerobic or aerobic conditions. This trait certainly contributes to its success as an aquatic weed in rice growing areas. The seeds of *Echinochloa* species are all tolerant to ethanol produced during anaerobic germination, which seems unrelated to dormancy breaking. In contrast, incubation under anaerobic conditions delays the induction of secondary dormancy in several species. Seeds of *Viola* species and *Veronica hederifolia* commence germination in 100% nitrogen or 2% oxygen but not in 8% oxygen. Oxygen is required, however, for the release of dormancy during chilling of *Ambrosia artemisiifolia* and during after-ripening of *Avena fatua* and other cereal grains. These examples show that the response to oxygen is highly variable, as is oxygen availability, which can vary due to soil characteristics, such as soil type, water content, and burial depth.
(2) *Carbon dioxide*. As for other gases, the concentration of carbon dioxide in the soil depends on depth, temperature, moisture, porosity, and biotic activity. Carbon dioxide increases with depth, ranging from 1 $dm^3\ m^{-3}$ at the top 10 cm of soil to 80 $dm^3\ m^{-3}$ at 50 cm. These concentrations may increase five- to tenfold during peaks of biological activity in the soil at warmer temperatures. As with oxygen, soil carbon dioxide concentrations are significantly influenced by moisture content, due to the restricted diffusion of gases. Respiratory activity of microorganisms, actively growing plant roots, as well as carbon dioxide evolving from decaying plant material increase with soil moisture content.

Soil carbon dioxide in the range of 20–50 $dm^3\ m^{-3}$ (2–5% by volume) may stimulate germination. This concentration is higher than what is usually found in the soil top layer. However, rainfall may cause an immediate rise in carbon dioxide, its concentration at a depth of 3 cm changing from 8 to 30 $dm^3\ m^{-3}$ (0.8–3%) within hours after rainfall. This rise in CO_2 concentration, rather than moisture content, light, ethylene, or nitrate causes the intermittent flushes of germination of *Echinochloa crus-galli* seeds. However, CO_2 concentrations in the soil are generally below those that stimulate germination. Therefore, it is not likely that the gas plays a significant role in the control of dormancy.

(3) *Ethylene*. Ethylene is a common soil constituent. The gas is produced by both aerobic and anaerobic microorganisms, as well as by plant roots. Ethylene concentrations of several parts per million have been recorded in soils, and vary markedly among soil microenvironments. Ethylene is known as one of the plant hormones (Sect. 2.3.1) that can have a strong influence on plant growth. Both promotive and inhibitory effects of this gas on seed germination have been reported. In addition, it may enhance the positive effect of light and nitrate on germination. In a study of the effect of ethylene on germination of 10 grass and 33 broadleaved weed species, in nine species this was promoted by ethylene in concentrations between 0 and 100 ppm, inhibited in two species; the other species were not affected. Interestingly, the nine species that responded positively to ethylene are now known to be responsive to nitrate as well. Interactions between ethylene and carbon dioxide have been reported also. Curiously, elevated amounts of carbon dioxide may enhance the effect of ethylene on germination, but high amounts generally have an antagonistic effect on its action.

An assessment of the ecological significance of soil ethylene in the germination of seeds in the field is difficult since the gas can both promote and inhibit germination. Perhaps the inhibitory properties of ethylene may be regarded as an adaptation to avoiding competition with neighboring plants. Ethylene concentrations are highest in the rhizosphere because microorganisms therein are capable of synthesizing ethylene. In addition, production of ethylene by microorganisms can be enhanced by compounds in root exudates. These signals from nearby roots via ethylene are apparently used by some parasitic weeds (e.g., *Striga*) to trigger germination. Consequently, injection of ethylene into field soils has been proposed as a method to induce germination of such weed seeds to allow them to be killed and reduce the soil seed bank prior to planting a susceptible crop. However, these seeds also detect specific chemical signals from roots (see next section) and may not respond fully to ethylene.

7.2.6 Other Chemicals

Soils contain a vast array of organic compounds, both volatile and nonvolatile, which are the products of decaying plant and animal remnants and the associated microorganisms. Living plants also produce a wide range of organics, usually in their root exudates. These compounds have the potential to inhibit or stimulate germination. Combustion of plant material by fire also results in the appearance of a number of compounds that may affect germination and seedling emergence.

(1) *Organic inhibitors.* Because the germination of light-independent seeds, which can occur in the dark, is often inhibited in the soil it has often been postulated that chemical inhibitors play a major role therein. These so-called allelopathic substances or allelochemicals form an important group of natural inhibitors. Allelopathy refers to harmful or beneficial effects of higher plants of one species (the donor) on the germination, growth, or development of plants of its own or another species (the recipient).

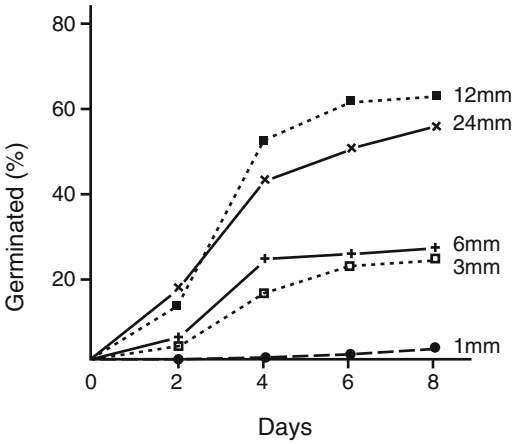

Fig. 7.18 Germination of *Parthenium hysterophorus* achenes placed at various distances apart. From Picman and Picman (1964). Courtesy of Elsevier

Allelopathic inhibition of seed germination may play a role in the regulation of plant succession. For example, pioneer weeds eliminate themselves over time through the production of toxins that inhibit germination of their own seeds (autotoxicity) and of accompanying weeds. Germination of *Aristida oligantha*, a grass that often follows weeds in succession, is not inhibited, however. Alfalfa plants also have an inhibitory effect on the germination of their own seeds. Inhibitors of germination have been detected in extracts from decaying material from, e.g., sorghum and sunflower. Experiments with soil samples have shown that the toxins also leach into the soil from living sunflower organs. However, the accumulated debris displayed greater toxicity than the leachate. Several allelochemicals have been identified. For instance, *cis*-dehydroxy-matricariaester (*cis*-DME) is the active compound in the underground organs of *Solidago altissima*, a weed that succeeds the pioneer *Ambrosia artemisiifolia* in old-field succession, and inhibits germination of its seeds. A soil block 10 cm in depth from the rhizosphere of *Solidago* has been determined to contain 5 ppm *cis*-DME, which is sufficient to inhibit *Ambrosia* seed germination. The inhibitor can persist in soil for several months without decomposition by microorganisms.

Allelochemicals leach from leaves, stems, and roots, and also from seeds. Those of *Parthenium hysterophorus* contain two major water-soluble sesquiterpene lactones: parthenin and coronopilin. Although the seeds (achenes) contain lower amounts than vegetative tissues, their concentrations are high enough to inhibit germination, which decreases with increasing seed sowing density (Fig. 7.18). Also, increasing the washing (leaching) period of seeds to remove the inhibitors increases subsequent germination, demonstrating that they have an autotoxic effect. The toxins are mainly located in the seed coat and may act as a rain gauge, allowing germination only when sufficient inhibitor has been washed out (chemical dormancy, Sect. 6.3.1.3). The germination inhibitors are also effective in the formation of inhibition zones, which deter competitors.

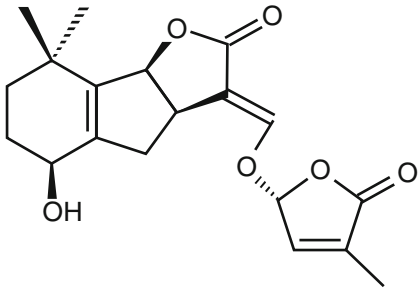

Fig. 7.19 The structure of the strigolactone (+)-Strigol, a germination stimulant of parasitic weed seeds

(2) *Organic promoters: parasitic weeds.* Parasitic weeds cause massive yield losses in agriculture. Broomrapes (*Orobanche* spp., Orobanchaceae) and witchweeds (*Striga* spp., Scrophulariaceae) are serious pests in many countries. *Orobanche* spp. are holoparasites that lack chlorophyll, and therefore obtain water, nutrients, and carbohydrates through the roots of their host. *Striga* spp. are hemiparasites, with reduced photosynthetic activity, and behave as holoparasites.

The first critical step in the life cycle of these parasites—germination of their seeds—is regulated by specific chemical signals exuded by the roots of their host plants, but even preceding this is the early and unambiguous recognition of the correct host. For *Striga* spp. several germination stimulants have been identified from host and non-host plants. Most of them are strigolactones, terpenoid lactones that are derived from carotenoids, including strigol (Fig. 7.19). The chemical communication between parasite and host is central to this recognition.

The physiology of the parasitic weed seed is complex. Newly shed seeds from such parasitic weeds as *Striga* and *Orobanche* spp. possess primary dormancy and require a period of moist conditions of several days to break dormancy and to become responsive to the chemical stimulants from the hosts, such as strigol and orobanchol. Contact with the host stimulant then induces germination. However, with prolonged conditioning, seeds may enter secondary dormancy and become less sensitive to the germination stimulant (Fig. 7.20). These changes in sensitivity suggest that this is a safety mechanism that restricts the period in which the seeds can respond to the germination stimulants produced by host plants. Indeed, there are several reports showing that a later crop-sowing date strongly reduces infection by parasitic weeds. This behavior resembles the "dormancy cycling" as described for nonparasitic weeds (Sect. 7.3.1).

Relatively little is known about the chemical stimulation of germination in root parasites. It is difficult to understand why some have a rather wide host range, while others exhibit an extremely narrow range. It is even more difficult to understand with our presently limited knowledge why false hosts also produce germination stimulants but are not infected. More definite information is needed on the chemical nature of other germination stimulants and their dose–response reactions.

(3) *Organic promoters*: *smoke.* Recurring fires are an integral part of several ecosystems and when such areas are protected from fire, their local ecology becomes severely disturbed. How does the occurrence of a fire stimulate the seeds of certain

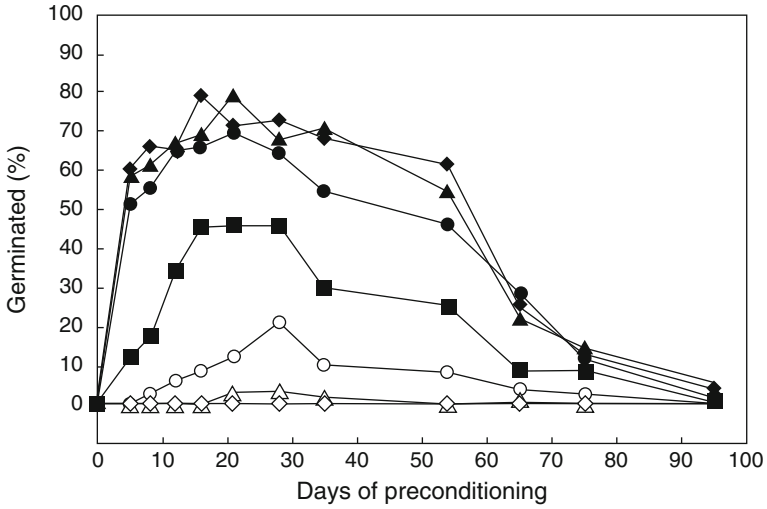

Fig. 7.20 Changes in germination of *Striga hermonthica* seeds in response to strigol with time after preconditioning. Seeds were moist-preconditioned at 30°C for the time indicated and then germinated at 30°C in the dark at a range of GR24 (a synthetic strigolactone) concentrations: 0 (◊), 0.00001 (□) 0.0001 (Δ), 0.001 (○), 0.01 (■), 0.1 (●), 1 (▲) and 10 mg L^{-1} (♦). From Matusova et al. (2004). With permission of Cambridge Univ. Press

species to germinate so quickly and lead to the emergence of new, green seedlings on the charred soil? There are many potential germination stimulants that change in the post-fire environment, including heat scarification, altered light levels (vegetation gaps) and increased nitrate in the soil. However, one of the most important inducers of germination in post-fire environments is smoke itself. Smoke induces germination both directly and indirectly by aqueous or gaseous transfer from soil to seeds. A principal group of active compounds in smoke has been identified as the karrikins (Sect. 6.6.7.3). The positive effect of smoke on seed germination is not limited to species that are native to fire-prone habitats. Smoke (and smoke extracts) appears to be an almost universal stimulator of seed germination and is now widely in use as an ecological and restoration tool throughout the world in a variety of conservation practices, in land management, and for the promotion of wild plants, including indigenous medicinal plants.

Smoke-stimulated germination occurs in 25 chaparral species, representing 11 families. None of these families is known to exhibit heat-shock-stimulated germination, i.e., by short exposures to high or extreme temperatures or temperature differences (Sect. 6.6.7). The quantitatively important gases generated by biomass smoke are generally not effective, except for NO_2. Seeds of smoke-stimulated species appear to have many similar characteristics that separate them from most heat-shock-stimulated seeds, including highly textured outer seed coats, a poorly developed outer cuticle, absence of a dense palisade tissue in the seed coat and a subdermal membrane that is selectively permeable, allowing passage of water but blocking entry of large solutes. It appears that smoke is involved in overcoming different

blocks to germination in different species. There is little doubt that the action of smoke-derived germination stimulants is subject to interactions with other abiotic environmental cues, such as temperature.

7.3 Secondary Dormancy and Seasonal Variation

Temperature acts to regulate germination in the field in several ways (Sect. 7.2.2) but also affects seed survival by determining the rate of deterioration (in moist seeds). Furthermore, temperature during seed development and maturation has a profound influence on performance of the mature seed after shedding (Sect. 6.6.2). Since temperature is relatively constant in its seasonal, monthly, and daily variations, it is arguably the most important environmental cue to synchronize seed germination with conditions suitable for seedling establishment. This is certainly valid for seasonal climate types, but in arid and semi-arid regions water may be the most important cue, whereas in the humid tropics variations in temperature and water availability appear to be virtually absent and hence will not synchronize seed germination with the environment, if this occurs at all.

7.3.1 Dormancy Cycling

In order to synchronize themselves with the seasons, seeds in the soil seed bank must be able to sense and perceive the continuous stream of information about the suitability of the environment for successful seedling emergence and subsequent plant growth. The ecophysiology of dormancy and germination deals with the perception of environmental factors by the seeds and how these are translated into signals within the seed and, ultimately, determine whether the seed will germinate.

Much progress in clarifying the internal processing of external information by seeds has been made with the large group of arable annual weeds of the temperate zones. In these regions seedling emergence is restricted to predetermined periods of the year, and in most cases (summer annuals) within a limited period in the spring, sometimes followed by additional flushes in summer. Examples of this group of summer annuals are *Ambrosia artemisiifolia*, *Polygonum persicaria*, *Chenopodium album*, *Spergula arvensis*, and *Sisymbrium officinale*. Species originating from climates with a hot dry summer and a cool humid winter, such as *Arabidopsis thaliana*, usually germinate in autumn whilst surviving the winter as rosette plants (winter annuals).

Dicotyledonous annual weed species often form large persistent seed banks. Survival of seeds in these seed banks may be as long as decades to perhaps even centuries. Emergence from soil seed banks is strongly stimulated by disturbance or wetting of the soil, e.g., by cultivation (Sect. 7.2.3), but the seasonal timing of emergence appears not to be affected. It therefore seems that the timing of emergence does not depend on prevailing conditions but on a seasonal fluctuation of the environment. In the temperate zones, temperature fluctuations over the year remain

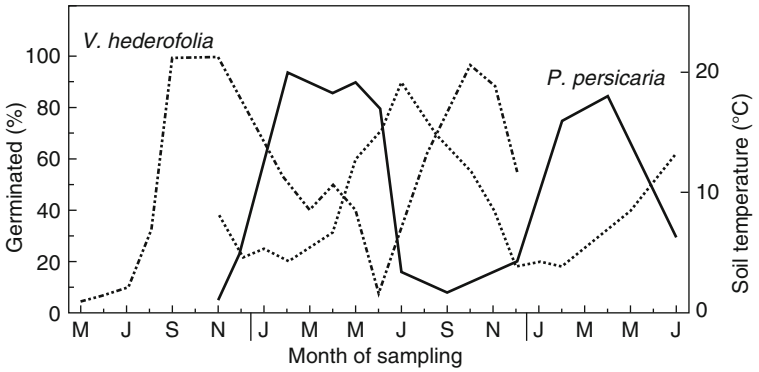

Fig. 7.21 Patterns of seed dormancy during burial. The patterns of germination and dormancy of *Veronica hederofolia* and *Polygonum persicaria* are shown. Seeds of both species were buried in sandy loam at 5 cm *(Veronica)* or 10 cm *(Polygonum)*. Germination at alternating temperatures in the light was tested at intervals. The dotted line shows the average morning soil temperature at the site of burial of *Polygonum*. *V. hederofolia* is a winter annual; *P. persicaria*, a summer annual. Note that the two species enter a deep secondary dormancy at different times of the year, in response to low and high temperatures, respectively. After Roberts and Lockett (1978) and Karssen (1980/81)

within narrow ranges, as opposed to, for example, rainfall, which can be more variable. Cold temperatures experienced by seeds in the seed bank in the winter of the temperate zones results in a fraction of nondormant seeds that may germinate in the spring when conditions for germination are permissive (Sect. 6.6.3). If the environment for germination is not favorable, seeds may gradually become dormant again, entering secondary dormancy. This is induced by the warm summer temperatures in summer annuals, such as *Polygonum persicaria*. In winter annuals, such as *Veronica hederofolia,* the summer temperatures break dormancy whereas the low winter temperatures induce secondary dormancy. Thus, summer annuals germinate in spring and winter annuals in autumn. However, many species may have characteristics of both. For example, *Lamium amplexicaule* and *Arabidopsis thaliana* are considered facultative winter annuals, which means that seeds from these species germinate not only in autumn but a small fraction also in the spring. Accordingly, seasonal fluctuations in temperature are considered the main regulatory factor of annual dormancy cycling (Fig. 7.21).

The range of conditions over which germination and emergence can occur widens during the alleviation of dormancy whereas it narrows during dormancy induction, indicating that dormancy is a relative phenomenon (Fig. 7.7), the expression of which is dependent on the prevailing environmental temperature. As indicated in Chap. 6, cold treatment, as well as after-ripening, also sensitizes the seeds to the environment to become responsive to light, nitrate and other factors. Therefore, germination in the field occurs when the germination temperature window overlaps with the actual field temperature (Fig. 7.22). In summer annuals the window is very narrow or even closed in dormant seeds at the end of the summer season and, hence, germination will not occur. When dormancy is terminated, as a result of the

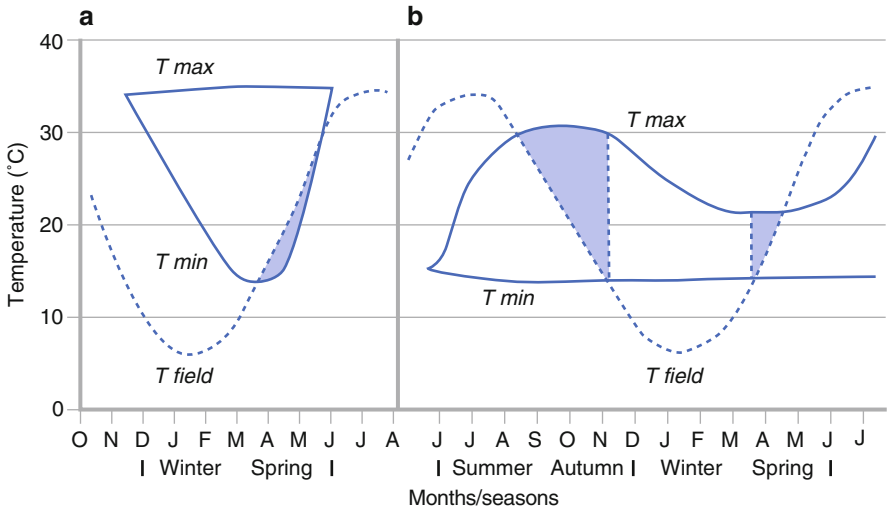

Fig. 7.22 Model to show the relationship between field temperature and the change in the range of temperatures over which germination can proceed. Solid lines represents the maximum (*T max*) and minimum (*T min*) temperatures at which germination is possible. The broken line indicates the mean daily maximum temperature in the field (*T field*). In the blue shaded area the actual and the required temperature overlap. (**a**) Strict summer annual and (**b**) facultative winter annual. From Probert (2000). Courtesy of CAB International

cool winter temperatures, the germination temperature window widens and will eventually overlap with the (increasing) field temperature in spring. However, germination will only occur when the requirements for germination, such as oxygen, water, soil components and possibly light are met. Conversely, induction of dormancy will be associated with a narrowing of the germination temperature window and a decreased responsiveness to environmental factors. Thus, dormancy cycling is a process of alternating perceptiveness of seeds to environmental factors that promote germination. The ecological relevance of dormancy cycling is to prevent seeds from germinating during short spells of favorable conditions in an otherwise unfavorable season. For example, a short spell of relatively high temperatures often occurs in autumn and could lead to germination if seeds are not dormant. Subsequent seedling emergence and establishment may become suicidal when temperatures drop again.

7.3.2 Dormancy Cycling: Mechanisms and Modeling

Based on the long-term effects of temperature on dormancy, i.e., dormancy breaking by chilling temperatures and induction of secondary dormancy by elevated temperatures, descriptive models have been developed. In this way the opening and closing of the "temperature windows" for germination can be modeled from the daily field

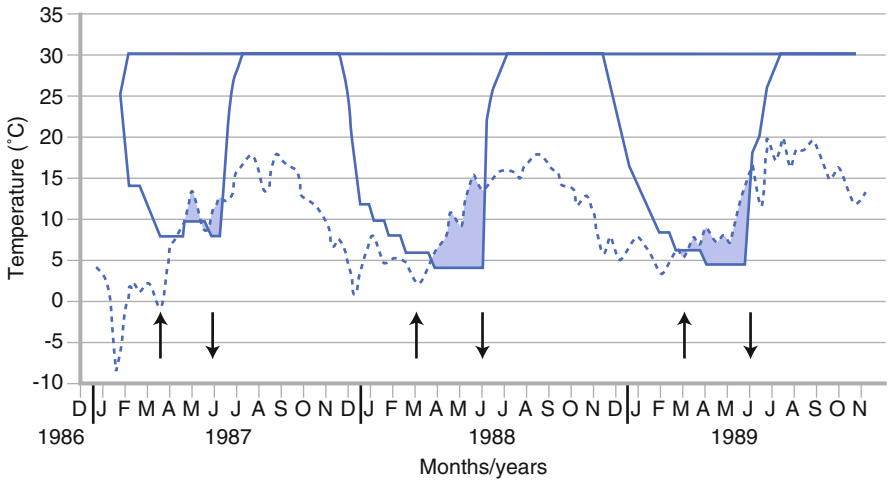

Fig. 7.23 Simulation of seasonal changes in the range of temperatures over which at least 50% of exhumed *Polygonum persicaria* seeds germinate. Solid lines represent maximum and minimum temperature required for 50% germination in water, calculated according to a descriptive model based on thermal time parameters. The dotted line indicates air temperature at 1.50 m. Blue shaded areas indicate overlap of field temperature and germination temperature range. Arrows indicate when germination outdoors in Petri dishes actually increased above (↑) or decreased below 50% (↓). From Probert (2000). Courtesy of CAB International

temperatures during a complete dormancy cycle. The models calculate the temperature sums from the number of days above or below the threshold temperatures for the induction or breaking of dormancy, respectively, during complete dormancy cycles in the field. In many cases this threshold temperature is approximately 15°C. Using a function that relates expected germination to heat and cold sums, temperature, and substrate, equation parameters are selected that provide the best similarities with germination data from laboratory experiments. From this the maximum and minimum temperatures to reach 50% germination are calculated and can then be compared with the real field situation to predict seedling emergence. These models are remarkably predictive of the timing of emergence for some species, but are much less accurate in predicting the number of emerged seedlings (Fig. 7.23).

Although it is now firmly established that temperature is the main driving force of dormancy and that dormancy cycling in the soil can be mimicked by descriptive models, a mechanistic explanation of this phenomenon is lacking. Only very few studies have been devoted to investigating the molecular aspects of dormancy relief and induction in the same seed batch and in the same study. So far reliable data have been obtained only from the *Arabidopsis thaliana* accession Cvi. This normally has a profound primary dormancy that can be broken by after-ripening and/or chilling and secondary dormancy can be induced by prolonged dark incubation at 20°C. An in vitro transcriptomics approach has revealed distinct sets of dormancy- and

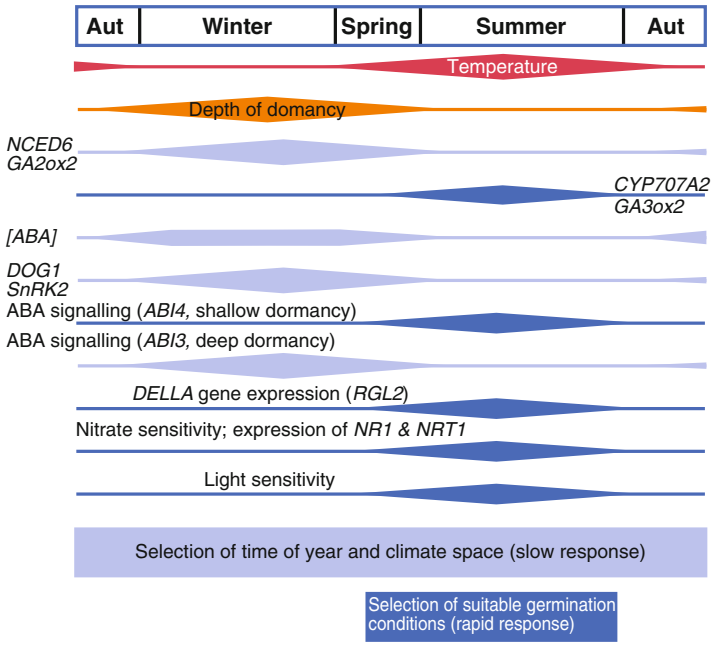

Fig. 7.24 Dormancy cycling in the field as monitored by depth of dormancy, light and nitrate sensitivity and expression of key genes of the winter annual *Arabidopsis thaliana* Cvi dormancy cycling in relation to annual fluctuations in soil temperature. The extent of each of the responses to seasonal variation is indicated by the height of the bars. The light and dark-blue bars at the base of the figure indicate the long- and short-term efficacies of dormancy breaking, respectively. From Footitt et al. (2011). Courtesy of the National Academy of Sciences, USA

germination-associated genes. Each of these consists of several hundreds of genes. It appears that the effects of different environmental factors that break dormancy, including after-ripening, cold, nitrate, and light, are very similar in that their effects on the different gene sets are additive.

The seasonal timing of physiological and molecular events associated with dormancy cycling of *A. thaliana* Cvi seeds in the field is primarily correlated with soil temperature and ABA concentration and sensitivity. The key enzymes of the GA–ABA balance (Sect. 6.6.1.4) identified in the laboratory behave as expected: expression of *NCED6* and *GA2ox2* genes (components of the ABA synthesis and GA inactivating pathways, respectively; Fig. 6.13) increases with increasing ABA concentrations and depth of dormancy during winter. In contrast, abundance of transcripts of the *CYP707A2* and *GA3ox2* genes (important in ABA inactivation and GA synthesis, respectively) decreases when dormancy is lost and ABA contents drop during spring and summer (Fig. 7.24). Similarly, transcripts of ABA-signaling genes from *ABI4* and *ABI3* are correlated with shallow summer dormancy and winter deep dormancy, respectively. *DOG1* and *SnRK* genes (Sects. 6.4.2, 6.6.1) are correlated

with deep winter dormancy only. *DELLA* gene expression, as well as nitrate- and light-sensitivity correlate with shallow summer dormancy. DOG1 is hypothesized to be involved in a thermal-sensing mechanism to influence the (temperature-driven) dormancy level by altering sensitivity to ABA. The abundance of two separate groups of transcripts correlates well with the previously mentioned "long-term" and "short-term" breaking of dormancy (Sect. 6.6).

7.4 Influences of Plant Life Cycle, Distribution and Origin on Germination

7.4.1 Plant Distribution

Different species may have different temperature requirements for germination, which are important in determining the distribution of plants, for they limit germination to regions that have suitable temperatures. It follows, also, that indigenous species of a particular region show characteristic temperature requirements, since they are adapted to the temperature conditions prevailing in their environment.

Members of one family, the Caryophyllacae, differ in their habitat and temperature requirements for germination, and are used here to illustrate this variable relationship. One way of documenting this is to determine, for each species, the time taken at each temperature to reach 50% of maximum germination to produce a germination "signature." The resulting signature curve thus indicates the low and high cutoff temperatures for germinated, or T_b and T_c. (Note that if the germination rates, 1/days to 50% germinated, are plotted rather than the times to germination, the patterns resemble the linear cardinal temperature relationships shown in Fig. 7.8.) Germination signatures of three Caryophyllaceous species are shown in Fig. 7.25, each typifying its geographical origin. The species from the continental grassland (steppe) *(Petrorhagia prolifera)* completes germination quickly at the favorable temperatures (12–40°C), with a high minimum (ca. 8°C) and maximum (ca. 42°C) (Fig. 7.25a, curve b). The Mediterranean species (*Silene echinata*) also completes germination fairly rapidly at median temperatures but, in contrast to the grassland type, has rather low minimum (<5°C) and maximum (ca. 25°C) requirements (curve a). Finally, the European woodland species (*Silene dioica*) is relatively slow to complete germination at median temperatures, with a high minimum (10–15°C) and moderate maximum (ca. 35°C) temperature requirement (curve c). The Mediterranean signature, belonging to the winter annuals, has been interpreted as favoring fall germination of the shed seed, in anticipation of the winter growing season. Seeds of the European woodland species, with a median temperature range, if shed in summer, would complete germination at once, whereas fall-shed seed would have to await the following spring. Here, germination in only spring/summer is encouraged. The grassland species have an opportunist signature and are able to germinate over a wide range of temperatures; they germinate when the seeds are shed in mid- to late summer. Of course, the same species may be found in a wide variety of climatic

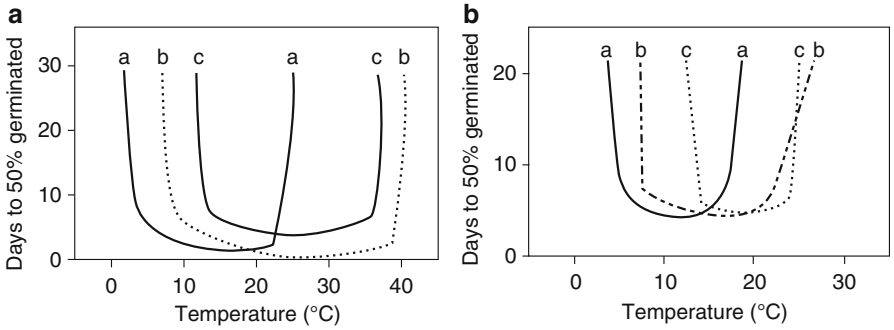

Fig. 7.25 (**a**) Germination "signature" curves. Nondormant seeds of three species were held at different temperatures, and the number of days taken to reach 50% germinated seeds was determined. a, *Silene echinata* (Mediterranean); b, *Petrorhagia prolifera* (continental grassland); c, *Silene dioica* (European woodland). Adapted from Thompson (1973b). (**b**) Germination "signature" curves of *Silene vulgaris* seeds from different regions. Seeds from three geographical localities were tested at a range of temperatures. The number of days for the germinated seeds to reach 50% was determined at each temperature. a, seeds from Portugal; b, seeds from Czechoslovakia; c, seeds from England. After Thompson (1973a)

regions: here, the germination behavior may differ according to provenance. The seed germination signatures of a single species, *Silene vulgaris,* from different regions are shown in Fig. 7.25b. Seeds from three origins show the typical germination signatures of the regional types (Mediterranean, continental grassland, and European woodland—cf. Fig. 7.25a), so clearly adaptation to the local conditions occurs within a species.

7.4.2 Seasonal and Flowering Interactions Affecting Dormancy

There is now developing an understanding that adaptation patterns of plants are highly dependent on perhaps just a few critical stages of the plant life cycle, namely, flowering and germination phenology (the term "phenology" denotes the timing of life cycle events). In populations of many species the timing of (field) germination largely determines their reproductive output (seed production) and how many generations can be completed in a given season or year. In addition, timing of germination is highly dependent on flowering time and, hence, depends on maternal environmental effects on germination and dormancy (Sect. 6.5). Evidently, these effects on dormancy and germination influence the timing of germination and, consequently, plant life cycle and population demography. Figure 7.26 shows several possible life cycles of annual plants. The winter annual life cycle occurs when seeds germinate in autumn (see also Sect. 7.3.1). Seedlings or rosettes overwinter and flowering, seed set and dispersal occur during spring and early summer. The dispersed seeds germinate again in autumn and the winter

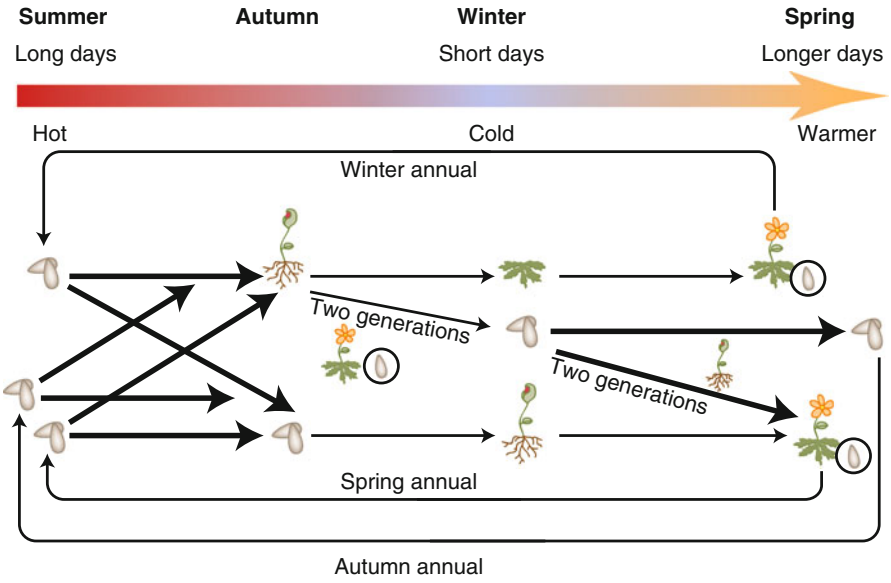

Fig. 7.26 Life cycles of annual plants. Bold lines indicate branch points determined by the germination behavior of seeds ( ) that matured during different seasons. The pathway from flower to seed represents the maternal effects on germination, and this path also completes the life cycle. The life cycle that is expressed depends on which path the seeds follow, which can be a function of when they matured and were dispersed. *Circled Seeds* indicate plants that have produced and shed seed. Adapted from Donohue (2009). Courtesy of the Royal Society of London

annual cycle begins again. Alternatively, the spring annual cycle starts when seeds germinate in spring and grow into mature plants that flower, set seed and disperse their seeds that same spring or summer. These seeds overwinter and do not germinate until the following spring. An autumn annual cycle occurs if seeds that mature in autumn remain dormant and do not germinate until the next autumn. If they germinate in the spring, then a spring annual generation could occur that set seeds in spring/summer and germinate in autumn, resulting in two generations per year instead of one. Many annual (and biennial) plants display such profound phenological variation. Thus, germination timing, along with capacity to flower in different seasons, determines the type of life cycle that is actually expressed.

Apart from the (maternal) environment, genetic factors also play a role in germination phenology, for example dormancy genotypes. Dormancy cycling is a determinant of germination timing, and together with the prevailing germination environment results in narrow seasonal time windows for seedling emergence. It is not known how the maternal environment of the maturing seeds affects dormancy and, consequently, dormancy cycling. However, it has become clear that DOG1 (Sect. 6.4.2) is a critical component in the expression of natural variation of dormancy in *Arabidopsis thaliana* and may be a mediator between the maternal environment and seed phenotype (Fig. 7.24).

Another example of the importance of germination timing can be found in desert populations. A study observing annual populations of 10 species in an undisturbed desert area for 22 years showed that delayed germination (i.e., dormancy) buffers the variation in reproductive success (germination and seedling survival). In other words, seeds remaining dormant in the soil may substitute for lower seed production or germination in a certain year, for instance because of a lack of precipitation. As noted above, in desert climates water and not temperature may be the limiting factor for seedling emergence.

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Section 7.3

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Chapter 8
Longevity, Storage, and Deterioration

Abstract Many seeds are capable of surviving dehydration at maturity, in which state they can survive for long periods (up to hundreds of years in some cases) and resume growth when rehydrated. However, deteriorative chemical processes continue in dry seeds, resulting in their gradual loss of vigor and eventual death. The rate of loss of seed viability is dependent primarily on their moisture content and the temperature at which they are stored. High temperatures and moisture contents accelerate seed deterioration, so low temperatures and moisture contents are used for long-term seed storage for germplasm conservation. While many processes may contribute to seed deterioration, it is likely that reactive oxygen species and related chemical oxidation are primarily responsible. Dormancy of some seeds that is alleviated by dry storage (after-ripening) may also be due to such oxidation events that could inactivate inhibitors or modify components of molecular regulatory pathways. Some seeds, including many tropical species, do not develop desiccation tolerance and are termed "recalcitrant." This property renders them difficult to store, complicating the preservation of their diversity in seed banks.

Keywords Longevity • Storage • Deterioration • Conservation • Diversity • After-ripening • Oxidation • Recalcitrant

The focus of this book is on seed development, germination and dormancy, and on the factors such as the environmental conditions that influence these. In this chapter an emphasis is placed on what could be considered the last stage of "development," the largely deteriorative processes that occur in seeds over periods of storage under various conditions. After relatively short periods of storage, the changes occurring in dry seeds can result in a different developmental state upon rehydration (i.e., loss of dormancy due to after-ripening; Sect. 6.6.2) that may be critical for the successful completion of the plant life cycle. With longer storage periods, the progressive loss of seed quality reduces the rate of germination and eventually results in

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loss of viability. For crop plants, preventing or minimizing the loss of seed quality and viability during storage is critical for maintaining seeds for planting in subsequent seasons. Long-term storage of plant germplasm is essential for conservation and breeding purposes, and this is best achieved by maintaining seeds under suitable storage conditions. This chapter describes the effects of storage conditions on seed longevity, the patterns and consequences of loss of seed quality during storage, and a current understanding of the mechanisms responsible for seed deterioration.

8.1 Ancient Seeds

There is probably no other aspect of seed biology that has been subjected to such fanciful claims as those for the existence of viable ancient seeds. A list of some of the more spectacular examples of these is presented in Table 8.1, along with reasons for rejecting them. Perhaps the most persistent myth concerning seed longevity is that viable grains of wheat and barley were uncovered during archeological excavations of ancient Egyptian tombs. Reports that "mummy" grains could germinate and produce seedlings were given considerable publicity and credence during the nineteenth and early twentieth centuries. But more recent scientifically rigorous studies show unequivocally that true ancient grains (particularly the embryos) have undergone severe morphological and physiological degradation (including carbonization) with accompanying total loss of viability. Some stored grains retain their original shape and even much of their cell fine structure, although upon hydration considerable disintegration occurs. It is worth reemphasizing, then, that there is no credible scientific evidence for the retention of viability by ancient cereal grains from the pharaohs' tombs. It is likely that any grains that germinated were either modern grains carried in by tomb robbers or explorers, or the collections were contaminated in transit or in storage, or that the claims were simply hoaxes.

The two most extreme claims for longevity, from the Pleistocene era, are for seeds taken from glaciers or permafrost deposits in the far north. One claim is for arctic lupin seeds that were discovered frozen and buried in the Yukon Territory in Canada. These seeds were removed from ancient rodent burrows containing remnants of a nest, fecal matter, and the skull of a lemming species. Dating of nests and remains of arctic ground squirrels found buried under similar conditions in central Alaska showed them to be over 10,000 years old, and hence it was concluded that the seeds in the Yukon must be of this age too. The highly circumstantial nature of the "evidence" has been debunked by more recent radiocarbon dating of the seeds, which set the time of their origin as being between 1955 and 1957 (in contrast, the lemming skull was about 23,000 years old)! A similar claim for long-term viability has been made for seeds of *Silene stenopylla* and two unidentified species of the genera *Polygonum* and *Arctuous,* retrieved from fossil burrows of ancient ground squirrels present in permafrost in the Siberian region of Russia. Radiocarbon dating of material in the burrow showed it to be around 28,000–32,000 years old; however, the seeds themselves were not dated, and it cannot be ruled out that, as with the

Table 8.1 Disputed or incorrect claims for viability of ancient seeds

Species	Location	Purported age	Status of seed	Comments
Barley	Tomb of King Tutankhamun	ca. 3,500 years	Nonviable	Extensively carbonized
Wheat	Ancient Egyptian tombs	>3,000 years	Not known	Claims made for viability, but age and source never verified
Wheat	Thebes, Egypt Feyum, Egypt	4,000–5,000 years	Nonviable	Some fine structure preserved, but degraded on rehydration
Arctic lupin	Miller Creek, Yukon	10,000 years	Viable	Radiocarbon dating showed they were modern seeds
Sacred lotus	Kemigawa, near Tokyo	3,000 years	Viable	Seeds in a submerged boat that was radiocarbon dated at 3,000 years. No seed measurements, which could have settled in the lake sediment after shedding from modern plants
Chenopodium album, Spergula arvensis	Denmark and Sweden, archeological digs	>1,700 years	Viable	No dating of seeds. Could be modern ones dispersed into the digs
Silene stenophylla Polygonum sp. *Arctuous* sp.	Siberia, Russia	>30,000 years	Viable	Present in fossil burrows in permafrost. Some contents of burrows radiocarbon dated, but not the seeds. Could be modern introductions

arctic lupin seeds, they are modern ones that became dispersed into cracks in the permafrost.

In the absence of reliable evidence from radiocarbon or alternative dating methods, other claims for extended longevity, e.g., sacred lotus in lake beds in Japan (3,000 years) and *Chenopodium album* and *Spergula arvensis* (~1,700 years) in archeological digs in Scandinavia, must be regarded with considerable skepticism.

More credible claims for extended seed longevity are presented in Table 8.2. The longevity of a *Canna compacta* seed, of the Cannaceae family, has been put at about 600 years. The seed was collected from a tomb in the high Andes in northwestern Argentina, enclosed in a *Juglans australis* nutshell forming part of a rattle necklace. Radiocarbon analysis of the nutshell and surrounding charcoal remnants were dated by radiocarbon analysis, but insufficient seed material was available for analysis. However, the only way for the *C. compacta* seed to have arrived inside the *J. australis* nutshell is for it to have been inserted there through the still-developing nutshell, while it was soft. Then the nutshell hardened and dried with the seed inside, forming a rattle. Hence the seed must have been at least the same age as the shell, and possibly older.

Table 8.2 Longevity of seeds for which there is authentication

Species	Location	Age	Status of seed	Comments
Canna compacta	Santa Rosa de Tastil, Argentina	ca. 600 years	Viable	Enclosed in a nutshell forming part of a rattle. Shell dated, but seed likely of similar age
Albizzia julibrissin	China to British Museum, London	200 years	Viable in 1940	Germination started accidentally. Viable seedlings produced
Cassia multijuga	Museum of Natural History, Paris	158 years	Viable	Wholly authenticated history from collection to sowing
Leucospermum sp. *Liparia* sp. *Acacia* sp.	Cape Floristic region, S. Africa	>200 years	Viable	National Archives, Kew. Germinated and produced healthy plants
Sacred lotus	Liaoning Province, China	1,300 years	Viable	Seeds aged from 200 to 1,300 years verified by radiocarbon dating
Verbascum blattaria, *Verbascum* sp. *Malva rotundifolia*	W.J. Beal buried seed experiment, Michigan, USA	120 years	Viable	Controlled burial experiment still in progress
Phoenix dactylifera (date)	Herod's palace, Masada, Israel	2,000 years	Viable	Radiocarbon dated. One seed germinated and produced healthy seedling

Some seeds stored in museums and herbaria are known to have survived for more than 100 years. Specimens of *Albizzia julibrissin* (family Fabaceae, legume) seeds collected in China in 1793 and deposited in the British Museum, London, germinated after attempts to quench a fire started by an incendiary bomb that hit the museum in 1940, indicating a life span of at least 147 years. Tests on old collections of seeds from the Museum of Natural History in Paris in 1906 and 1934 showed that some seeds retained viability after periods that ranged from 55 (*Melilotus lutea* [Fabaceae]) to 158 (*Cassia multijuga* [Fabaceae]) years (Table 8.3). The long life span of these and other specimens was attained even though the storage conditions were arbitrary (generally, warm temperatures and low relative humidity [RH]); longevity might have been greater had better conditions been used. Other seeds, also stored under suboptimal conditions, that have survived for at least 200 years are those collected from the Cape Floristic region of South Africa in 1802–1803. These were rediscovered in 2005 in the National Archives at Kew, UK, after prior storage in two other locations in London, including the Tower of London. Seeds of 35 species were in the collection, of which three (a *Liparia, Acacia* [both Fabaceae], and *Leucospermum* [Proteaceae] species) germinated and produced healthy plants.

The oldest authenticated surviving seeds are those of *Nelumbo nucifera* (sacred, Indian or Asian lotus, Nelumbonaceae) at nearly 1,300 years, and *Phoenix dactylifera*

Table 8.3 Viability record of some old seeds from the Museum of Natural History, Paris

Species	Date collected	% Germinated in		Longevity (years)
		1906	1934	
Mimosa glomerata	1853	50	50	81
Melilotus lutea	1851	30	0	55
Cytisus austriacus	1843	10	0	63
Dioclea pauciflora	1841	10	0	93
Trifolium arvense	1838	20	0	68
Stachys nepetifolia	1829	10	0	77
Cassia bicapsularia	1819	30	40	115
Cassia multijuga	1776	–	100	158

After Becquerel (1934)

(date, Arecaceae) at an amazing minimum of 2,000 years. The *N. nucifera* seeds were collected from Liaoning Province in China, preserved in the sediments of a Holocene dry lake, and several germinated and produced viable seedlings, although often with growth abnormalities. Radiocarbon dating of the fruit or pericarp verified that the antiquity of the collected seeds varied between 200 and 1,300 years. The *P. dactylifera* seeds were recovered from excavations of the Herodian fortress at Masada, Israel. Of the five seeds obtained, two were used for radiocarbon dating, and three were planted, of which one germinated and formed a substantial small plant; fragments of the seed obtained following germination were also dated, with similar results to the other two.

Thus of the longest-lived seeds, both monocot and dicot families are represented, with the highest number of species coming from the Fabaceae, a family of legumes. Several factors likely contributed to the ability of the seeds to survive for so long, but impermeable seed or fruit coats preventing imbibition may have been a key one. The environments from which the seeds were recovered were variable: *C. compacta* survived in the relatively cool but moister conditions of high altitude, although enclosed within a nutshell, while those of *P. dactyifera* were in hot desert-like conditions. *N. nucifera* would have been submerged for a considerable number of years before the water receded from the lake in which they were buried. Protective mechanisms preventing cellular changes that cause deterioration, thus increasing seed longevity (Sect. 8.3) could also have played a role.

A number of projects have been carried out, or are in progress, to determine longevity of seeds buried in soil. A study of buried weed seed, due to last for 50 years, was initiated in 1972. After only 2.5 years of burial, only 4 of the 20 species tested retained more than 50% viability. The longest controlled burial experiment to date is the one initiated by W.J. Beal in 1879. He selected seeds of 21 species of plants commonly growing in the vicinity of Michigan Agricultural College in East Lansing, USA. Fifty-seed lots of each species were mixed with moist sand in unsealed bottles and buried in a sandy knoll, with the neck of the bottle facing downwards. At regular intervals since, bottles have been unearthed and the germinability of the seeds tested. After 120 years, of the 21 species originally buried

Table 8.4 Longevity of some of the species of seeds in W.J. Beal's buried-seed experiment started in 1879

Species	Year 5	Year 20	Year 40	Year 60	Year 80	Year 100	Year 120[a]
Agrostemma githago	0	0	0	0	0	0	0
Brassica nigra	0	18	38	0	0	0	0
Capsella bursa-pastoris	100	42	0	0	0	0	0
Lepidium virginicum	94	58	2	0	0	0	0
Malva rotundifolia	2	6	0	0	0	2	2
Plantago major	0	0	10	0	0	0	0
Portulaca oleracea	38	14	2	0	0	0	0
Oenothera biennis	82	28	38	24	10	0	0
Rumex crispus	90	16	18	4	2	0	0
Verbascum blattaria[b]				68	70	42	46
Verbascum sp.							4

Data expressed as percentage germinated of the 50 seeds buried for each species for each year tested
Based on information in Telewski and Zeevaart (2002)
[a]120-year-old *V. blattaria* and *M. rotundifolia* seeds germinated and produced normal plants, flowered and set viable seed
[b]There is some question concerning the identification of this species up until the 50th year; it was previously called *V. thapsus*

only seeds of *Verbascum blattaria*, an unknown *Verbascum* sp. (Scrophulariaceae), possibly a hybrid, and *Malva rotundifolia* (Malvaceae) retained their viability, although many species were capable of germinating for up to 40 years (Table 8.4). Incidentally, *V. blattaria* does not possess an impermeable seed coat.

Many different factors determine the longevity of seeds buried in soil, even in controlled experiments. Soil characteristics such as moisture content and pH are important, as are seed attributes such as depth of dormancy and hardness or impermeability of the seed coat, which vary within any population of seeds of the same species (Sect. 6.3.2).

8.2 Longevity of Seeds in Storage

As described above, some seeds can retain viability for extended periods even when hydrated. However, the majority of species produce orthodox seeds that are desiccation tolerant at maturity (Sect. 2.5) and exhibit the greatest longevity when stored at low moisture contents. As might be expected, seed longevity is also extended at low temperatures. Quantitative relationships between the moisture content and temperature of seeds and their potential longevity have been developed that enable better prediction of the storage lives of seeds for agricultural or conservation purposes. Because recalcitrant seeds are unable to survive desiccation, they must be stored hydrated and generally have quite short storage lives (Sect. 8.6).

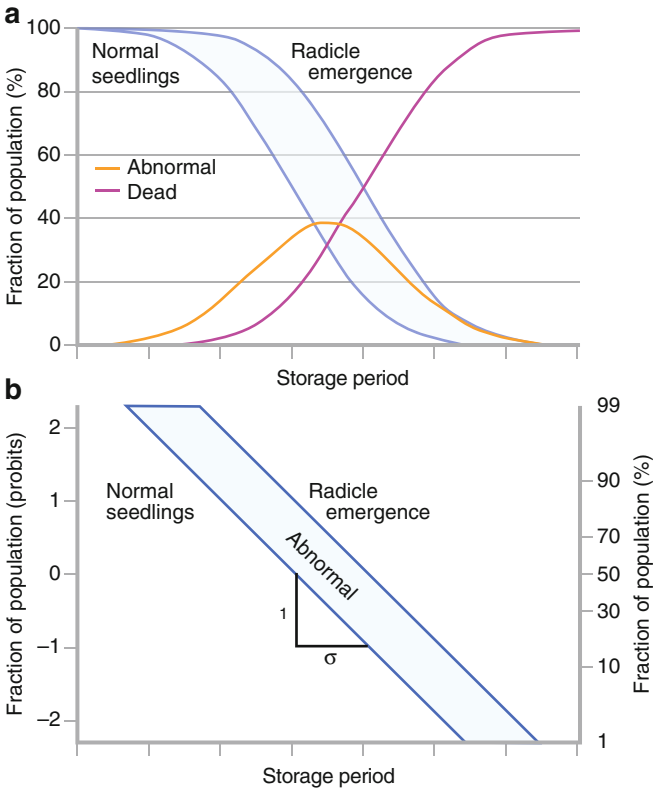


Fig. 8.1 Patterns of loss of seed quality and viability during storage. (**a**) The declines in the percentages of seeds capable of producing normal seedlings or only exhibiting radicle emergence following different periods of storage follow a sigmoid pattern, as does the increase in the percentage of dead seeds (i.e., those incapable even of radicle emergence). The differences between the normal seedling and radicle emergence percentages represent the percentages of abnormal seedlings, which have a normal distribution that peaks between the time of 50% normal seedlings and 50% radicle emergence. (**b**) When the sigmoid curves in (**a**) are plotted on a probit scale, which uses units of standard deviation (σ), they become straight lines with slopes of $1/\sigma$. The corresponding probability scale of percentages is shown on the right *y*-axis

8.2.1 Patterns of Seed Viability Loss During Storage

E.H. Roberts was one of the first to quantitatively analyze the patterns of loss of seed viability during storage. He noted that the loss of viability generally followed a sigmoid pattern and recognized that this could be related to a normal distribution of lifetimes among seeds in the population (Fig. 8.1). As seeds age, they will at some point lose their ability to germinate and form "normal seedlings," i.e., seedlings that have the capacity to form viable plants. Some seeds may be able to initiate germination or complete radicle emergence, but subsequently fail to develop into normal seedlings and are judged to be abnormal. Seeds incapable of any visible

germination are considered to be dead. As illustrated in Fig. 8.1a, the radicle emergence curve is offset from the normal seedling curve, as a given seed progresses from normal germination to abnormal germination to dead as storage time increases. The percentage of abnormal seedlings increases to a maximum after the normal seedling percentage falls below 50% (i.e., when the percentage difference between the normal seedling and radicle emergence curves is maximal) and then declines as the percentage of dead seeds increases.

The shapes of these curves are often symmetrically sigmoid once viability begins to decline, suggesting that the lifetimes of individual seeds are normally distributed. The probit transformation can convert a cumulative normal distribution to a linear form by converting the percentage axis to a probability scale (Fig. 8.1b). This scale uses standard deviation units, which essentially compresses the values around 50% and stretches the values near 0 and 100% (see right *y*-axis in Fig. 8.1b). Thus, percentages within 15.9 and 84.1% are within ±1 standard deviation (σ) of the median, percentages within 2.3 and 97.7% encompass $\pm 2\sigma$ of the population, and 3σ on each side of the median would include from 0.1 to 99.9% of the population. [Because the probit scale continues to both plus and minus infinity, it is not possible to represent 0 or 100% on it.] When plotted on a probit scale, the normal seedling and radicle emergence percentage curves during storage become parallel lines offset by a constant storage period but with a common slope of $1/\sigma$. The width of this difference between normal seedling and radicle emergence lines (representing the percentage of abnormal seedlings) varies among species. For soybeans, this range is relatively large, resulting in large percentages of abnormal seedlings as the seed population ages, whereas in lettuce, this period is short, and relatively fewer abnormal seedlings are generally observed as seeds age.

The probit transformation allows simple mathematical analysis of viability loss time courses. In mathematical terms, seed viability (v, on a probit scale) at any time can be related to storage period (p) according to the equation

$$v = K_i - p / \sigma \tag{8.1}$$

This equation states that the probit of percent viability after any storage period (v) is equal to the initial viability (K_i, or the intercept on the *y*-axis) decreased by the seed deaths that can be expected in that time period (p) according to the slope ($1/\sigma$) of the line. This seed viability equation provides two parameters to quantify the rate of loss of seed quality during storage: K_i, which is an indicator of the initial quality of the seed lot, and σ, which is related to the storage environment.

Different seed lots of the same species stored in an identical environment can lose viability at different rates. It is often observed that seeds show little change in viability for an initial period of storage, and then exhibit the characteristic sigmoidal loss of viability over a relatively short time (Fig. 8.2a). The length of this initial lag period is related to the initial seed quality. When these curves are plotted on a probit scale (excluding time points prior to the loss of viability), parallel lines result with the same slope ($1/\sigma$) but different intercepts (K_i values) (Fig. 8.2b). The intercepts on the probit scale represent unrealistic percentages, i.e., it would be impossible to

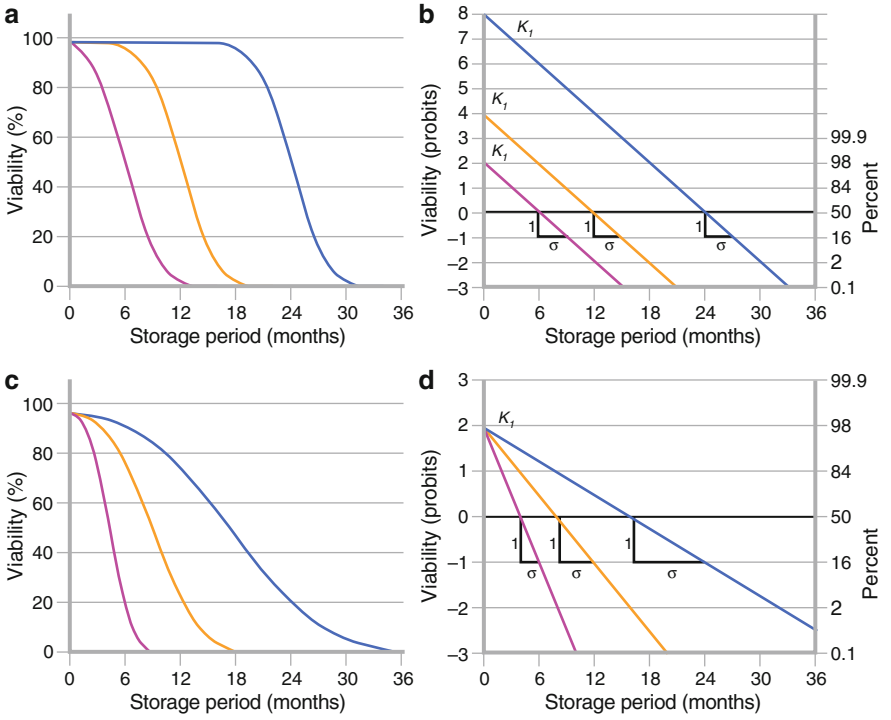


Fig. 8.2 Patterns of seed viability loss associated with variation in seed lots or storage environments. (**a**) Typical patterns of viability loss during seed storage, exhibiting a lag period of variable length with relatively constant viability, followed by a sigmoidal decline. Illustrated are three seed lots of differing potential longevity stored in the same environment. (**b**) When plotted on a probit scale, the curves in (**a**) become lines with a common slope of $1/\sigma$ but different K_i values, reflecting their differences in initial quality. (**c**) Patterns of viability loss where the same seed lot is stored in three different environments that result in different rates of viability loss. (**d**) When plotted on a probit scale, the K_i values are the same because the initial quality of the lot is the same. The slopes differ, however, indicating that the storage environment influences the standard deviation of seed deaths per unit time. The storage period is denoted as months, but the graphs apply to days or years also, depending upon storage conditions

determine experimentally the difference between a seed lot with 99.9% germination and one with 99.9999%. However, the K_i values on a probit scale can be used as indices of initial seed quality differences between the lots with respect to their potential longevity. The parallel slopes of the lines reflect the common environment in which the seeds are stored, for the rate of aging is consistent for a given species. Many factors can affect the initial seed quality before storage, particularly seed maturity at harvest, conditions during drying, subsequent handling, and any prior storage before viability began to be monitored.

If, on the other hand, a single seed lot is stored in multiple environments, such as at a range of temperatures or seed moisture contents, the K_i value will be unaffected, as it represents the initial quality of the seed lot, but differences in the rate of loss of

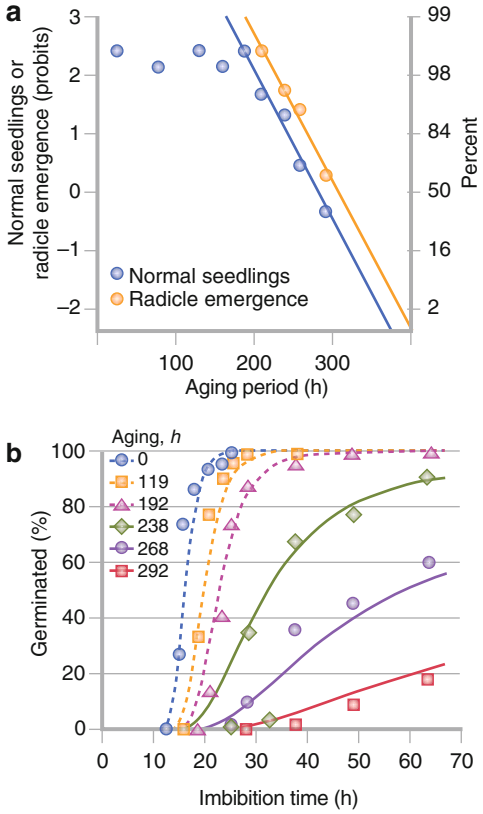


Fig. 8.3 Relationships between seed viability and germination rates during storage. (**a**) Normal seedling or radicle emergence percentages (on a probit scale) of lettuce seeds stored at 10% moisture content (82% RH) and 40°C (an accelerated-aging condition). Normal seedling percentage remains high until about 200 h of aging, and then declines, followed closely by radicle emergence percentages. (**b**) Germination (radicle emergence) time courses of seeds at different times of aging (hours) corresponding to (**a**). Symbols are experimental data, and dashed and solid curves are based on a population-based threshold model of seed aging. Different model parameters are required before (*dashed lines*) and after (*solid lines*) the loss of viability begins. Modified from Bradford et al. (1993)

seed viability over time will be evident in the slopes of the probit lines, or in σ (Fig. 8.2c, d). Equation (8.1) therefore provides a simple approach to quantify both the initial seed quality prior to storage (K_i values) and the environmental effects on the rate at which viability is lost (σ values). Some physiological treatments to seeds, such as seed priming, can shorten storage life by reducing both K_i and σ.

Seed germination rates are also affected by aging during storage, both before and after total viability begins to decline. Lettuce seeds, for example, exhibit a lag phase in which viability is maintained initially during accelerated aging at 10% moisture content and 40°C, followed by a rapid loss of their capacity to produce normal seedlings or radicle emergence (Fig. 8.3a). These data illustrate the parallel relationship

between these two indicators of germination capacity shown schematically in Fig. 8.1b. However, even during the lag phase when total viability is constant, germination (radicle emergence) is progressively delayed, and this delay becomes more pronounced once some seeds begin to lose viability (Fig. 8.3b). This effect of seed storage on germination rates has been described using a population-based threshold model (e.g., Sect. 7.2.1.1) of seed aging and represents an alternative method to quantify the effects of storage conditions and duration on seed quality.

8.2.2 Temperature, Moisture Content, and Seed Longevity

While various factors can influence seed longevity, the two most important ones are seed moisture content (or equilibrium RH, Fig. 2.29) and temperature. Consistent relationships among seed temperature, moisture content, and longevity have resulted in some general rules for seed storage. A commonly used practical rule for seed storage (known as James' Rule) is that the temperature (in Fahrenheit) plus the RH of the air (in percent) should total less than 100 for satisfactory seed storage. For example, if the RH is 50%, the storage temperature should be no greater than 50°F (10°C) for commercial (medium term) storage. Harrington's Rule states that storage life will approximately double for each 10°F (5.6°C) decrease in temperature and each 1% decrease in seed moisture content for temperatures between 0 and 40°C and moisture contents between 5 and 14%. Both of these rules emphasize the importance of low seed moisture content and temperature in extending the longevity of seeds, and in particular to avoid the combination of high temperature and moisture content simultaneously. However, they are limited by giving only relative indications of longevity in relation to temperature and seed moisture content.

To make equation (8.1) more quantitative and predictive, the relationship between σ values and the storage conditions must be known for different species. Extensive studies with many species have found that the following equation generally describes the dependence of σ on seed moisture content and temperature:

$$\log \sigma = K_E - C_W \log m - C_H T - C_Q T^2 \tag{8.2}$$

This is the Ellis and Roberts (1981) Seed Viability Equation, where K_E is a species constant that accounts for the inherent differences in storability among species, m is the seed moisture content (in percent fresh weight basis), and T is the storage temperature (°C). C_W, C_H, and C_Q are constants that must be empirically determined for each species from storage experiments at several temperatures and moisture contents. The protocol to determine these constants is to establish a range of seed moisture contents (by adding water or incubating in constant RH), hermetically seal the seeds, and then store them at constant temperatures. Periodically, samples are removed, germinated, and scored for radicle emergence or normal seedlings. Over time, the seeds lose viability, and the data from their survival curves can be used to derive the constants in the

viability equation. Work with eight species indicates that the temperature constants C_H and C_Q are similar for all of them. Furthermore, the value of the constant relating to moisture content (C_W) is common to many species when seed moisture is expressed in terms of equilibrium RH rather than seed water content.

Other equations, which do not depend upon a normal distribution of seed deaths in time and can accommodate the occurrence of the initial lag phase in the loss of seed viability, have also been used to describe seed viability loss time courses. However, the Seed Viability Equation has been widely used to quantify and predict potential seed longevity for many species and storage environments. The constants for the Seed Viability Equation along with utilities to calculate predicted longevities under different conditions have been compiled in the Seed Information Database (SID) maintained by the Royal Botanic Gardens, Kew (http://data.kew.org/sid/viability/). The effects of storage temperature and equilibrium RH on the longevity of soybean seeds are illustrated in Fig. 8.4. Seed longevity decreases exponentially as seed moisture content (or RH) increases (equation 8.2), resulting in short lifetimes at high moisture contents. This effect is exacerbated by high temperature, with the combination of high temperature and high RH resulting in very short seed storage lives.

The extreme reduction in seed longevity by the combination of high temperature and high humidity has been utilized in the development of "accelerated aging" or "controlled deterioration" tests. The viability period of a seed lot under extreme conditions (e.g., 75–100% RH and 40–50°C) can be correlated with its potential longevity under more favorable conditions, so the viability remaining after a relatively short period under accelerated aging conditions (a few days or weeks) can be used to rank seed lots for their relative potential longevity in storage. Since germination rate and other aspects of seed quality are also related to seed aging (Fig. 8.3), controlled deterioration tests are also used as indirect measures of seed vigor.

The seed viability equation and controlled deterioration tests can be used in various ways in seed quality assurance and inventory management programs. Some examples are:

- The optimal geographic locations for seed storage facilities can be determined based upon the prevailing temperature and RH.
- The seed viability equation can be used to predict the time it will take for percent viability to fall to any given level for a specific storage condition. For example, if the RH and temperature are known for a seed storage warehouse, the time that a seed lot can be expected to maintain marketable viability can be estimated.
- The results of laboratory-controlled deterioration tests can be converted into predicted storage life under warehouse conditions. This is useful in anticipating which seed lots may lose marketable viability during a particular sales period, even though all lots may currently have acceptable viability.
- The controlled deterioration test can be used as a vigor index once it is confirmed that the species behaves in a consistent manner across the range of temperatures and moisture contents experienced. This is useful not only for predicting storability of harvested lots, but also in comparing the effects of genotypes, production environments, seed maturity, and harvesting methods on initial seed quality.

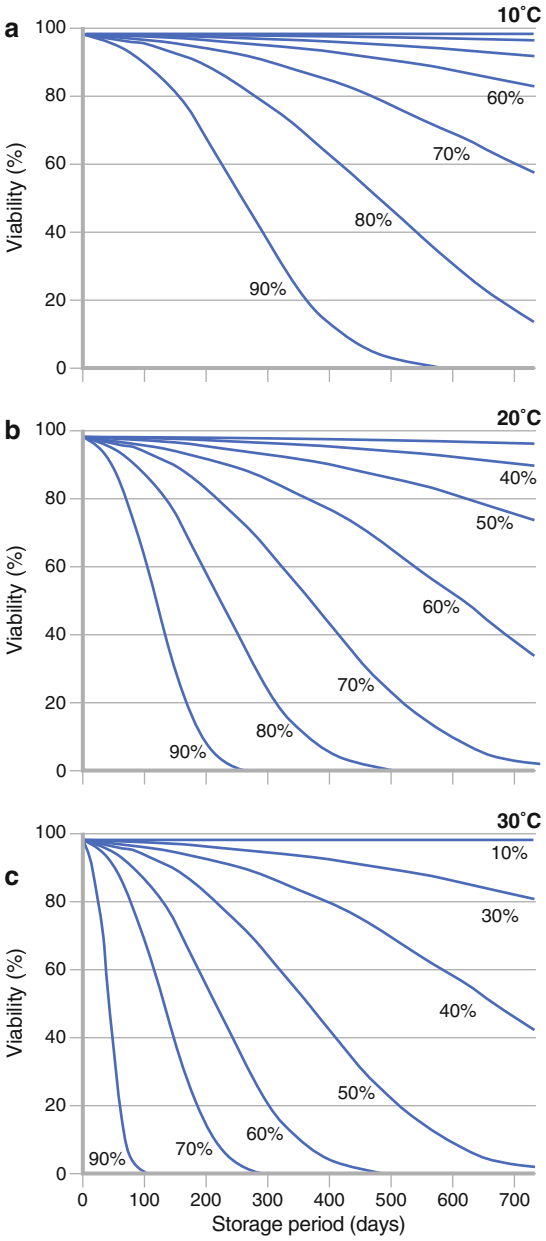


Fig. 8.4 Viability loss curves for soybean seeds stored at a range of equilibrium RH (resulting in different seed moisture contents) and at (**a**) 10°C, (**b**) 20°C or (**c**) 30°C as predicted from the seed viability equation. Note the very strong negative influence of higher RH on seed longevity, particularly at higher temperatures. Constants for the seed viability equation and utilities to calculate predicted viability are available at the Kew Seed Information Database (http://data.kew.org/sid/viability/)

Threshold quality levels could be established for specific aging conditions to simplify and speed the application of controlled deterioration tests.

- The effects of conditions during transport can be predicted. If a shipment must go through regions with high temperatures or RH, the consequences for subsequent seed quality can be estimated and appropriate steps taken to minimize its loss.

- Using the viability equation, it is possible to do cost/benefit analyses for alternative drying, packaging, or cooling scenarios for seed storage. The effects of such variables on seed storage life can be easily calculated to determine whether they are cost-effective.

8.2.3 Other Factors that Affect Seed Viability During Storage

While temperature and moisture content are the two most important conditions affecting seed longevity in storage, a number of other abiotic and biotic factors also contribute to seed longevity. As discussed in relation to seed development (Sect. 2.6), seed maturity at harvest or shedding can affect potential longevity, with more mature seeds tending to have greater longevity in storage. However, delaying harvest can also subject seeds to aging conditions in the field, particularly if seed moisture content is high, that will reduce subsequent longevity in storage. Seed drying conditions can also result in loss of seed quality if they are heated too highly at elevated moisture contents. For this reason, seeds to be used for propagation are generally dried initially at somewhat lower temperatures (e.g., <35°C) and this can be increased as seed moisture content decreases, but nonetheless not to exceed approximately 45°C.

Seeds of different species vary in their inherent storage potential, as indicated by the K_E value in the Seed Viability Equation (8.2). Some seeds, such as onion and soybean, inherently have short storage lives, while others, such as cereals or tomato, will store much longer under adequate storage conditions. Seed composition could influence storage life, and it was once thought that starch-storing seeds had greater inherent longevity than oil-storing ones. However, a broad survey across many species does not support this generalization. Genetic studies have identified quantitative trait loci (QTLs) that are associated with seed longevity, and some specific heat shock proteins appear to be able to enhance longevity. However, seed aging is a complex process that can occur via many possible avenues (Fig. 8.10); hence it is likely that multiple genes, biochemical pathways and products accumulated during seed development contribute to their longevity.

The gas composition of seed storage containers has received considerable attention with respect to longevity. Oxygen, in particular, might be expected to influence seed deterioration via oxidative reactions (Sect. 8.4.1). Seed mutants lacking enzymes responsible for the biosynthesis of antioxidants such as tocopherols exhibit greatly reduced longevity. While the evidence is rather mixed, in general, compared to storage in air, seed survival is somewhat extended in nitrogen or carbon dioxide atmospheres or in a vacuum, whereas elevated oxygen partial pressures can accelerate loss of viability. The latter effect has been developed into a controlled deterioration test in which seeds are stored in elevated partial pressures of oxygen, over 100 times atmospheric pressure, to cause rapid aging without increasing seed moisture content or temperature. The effect of oxygen depends in part on the seed moisture content, because it tends to promote aging at moisture contents below those at which

integrated metabolism is possible, but it may delay aging in seeds at higher moisture contents. This is likely due to the action of repair processes at higher moisture contents that require energy from aerobic respiration, while in drier seeds where integrated metabolism is not possible, oxidative damage can accumulate. The effects of temperature and moisture content on seed longevity are much larger than that of oxygen, but assuming that conditions are otherwise optimal, reducing the oxygen content in seed storage containers can provide additional protection against seed deterioration.

Biotic factors can also influence seed longevity in storage, particularly fungi and insects. Two types of fungi invade seeds: field fungi and storage fungi. The former invade seeds during their development or prior to harvesting while the plants are standing in the field. Field fungi need high seed moisture contents for growth (as high as 33% for cereals) and hence are infective only under conditions where seeds fail to follow their normal pattern of maturation drying. A period of high rainfall at harvest time, therefore, can result in extensive fungal infestation and grain deterioration. The main fungal species associated with wheat or barley in the field are *Alternaria, Fusarium,* and *Helminthosporium* spp., although several others have been recorded. Seeds that are sheltered from airborne pathogens by pods, fleshy fruits, or other surrounding structures (e.g., pea, tomato, melon, maize) are generally less susceptible to field fungi than seeds that are more exposed (e.g., wheat, oat, barley, sunflower).

Storage fungi, almost exclusively of the genera *Aspergillus* and *Penicillium,* infest seeds under storage conditions. Each species of storage fungus has a sharply defined minimum RH below which it will not grow, although other factors also determine virulence, such as the ability to penetrate the seed, condition of seed, nutrient availability, and temperature. The major deleterious effects of storage fungi are to: (1) decrease viability; (2) cause discoloration; (3) produce mycotoxins, byproducts that are toxic to mammals; (4) cause heat production; and (5) develop mustiness and caking. Fungi will not grow at seed moisture contents that are in equilibrium with an ambient RH below 68%; hence, they are not responsible for deterioration that occurs at moisture contents below about 13% in starchy seeds and below 7–8% in oily seeds (Fig. 8.5). Bacteria do not play a significant role in seed deterioration, as bacterial populations require free water to grow and are unlikely to increase in stored seeds unless water contents are very high. Microbial impacts on seed longevity are therefore best controlled by storing seeds at low moisture contents; conversely, they can be severe problems for seeds in open storage in climates with high ambient RH and temperature. In addition, some pathogenic bacteria and fungi (or their propagules) can survive for years on dry seeds, providing the opportunity for seed transmission of plant diseases.

Damage to stored seeds by insects and mites is a serious problem, particularly in warm and humid climates. Weevils, flour beetles, or borers are rarely active below 35% RH and 20°C, but are increasingly destructive as the RH and temperature increase. Mites do not thrive below 60% RH, although they have temperature tolerance that extends close to freezing. Fumigation and sealed storage are often used to kill insects present in stored seeds and to prevent reinfestation.

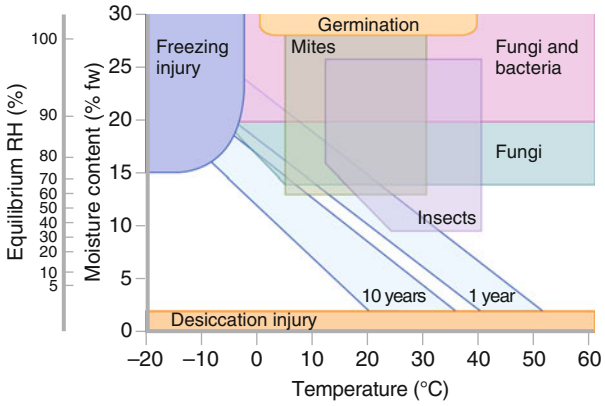


Fig. 8.5 Relationships between seed moisture content, abiotic, and biotic factors at different temperatures affecting seed storage. The different *colored* regions indicate storage problems that can occur within the ranges of moisture content (or equilibrium RH) indicated. The *light blue* areas show approximate ranges for 1 or 10 years of storage viability for different species. The relationship between RH and seed moisture content is based upon the wheat desorption isotherm; moisture contents will be lower for a given RH for oil-storing seeds. Growth of storage organisms is related to the equilibrium RH rather than to the seed moisture content per se. A region of desiccation injury at very low moisture content (<2%) is indicated; this may vary among species and the storage duration. Freezing injury occurs only in seeds at high moisture contents. Based on Roberts (1972)

The relationships between temperature, moisture content, storage longevity, and biotic factors affecting stored seeds are illustrated in Fig. 8.5. It is evident that storing seeds at low moisture content and low temperature is the best way to avoid biotic pests and to extend seed longevity. In hot and humid environments, this can be achieved only by additional drying of seeds below equilibrium with ambient conditions and subsequently storing the seeds in hermetic containers to prevent rehydration from the air. If this can be achieved cold storage may not be required for year-to-year storage of agricultural seeds, even in warm climates (Fig. 8.5). New methods using improved desiccants such as zeolites to absorb water from seeds stored in hermetic containers may enable better storage in humid climates.

8.3 Seed Storage and Conservation

The storage of seeds will be considered under two major headings: short-term storage, usually to maintain maximum viability of a seed population from one growing season to the next, and long-term storage in specialized facilities (gene banks) to preserve germplasm from domestic and wild species and thus ensure the future availability of broad genetic diversity. It is obvious from the previous sections that

seed stores should incorporate various features into their design to avoid conditions that result in loss of seed viability and thus minimize the chances for deterioration of their contents.

8.3.1 Short-Term Storage

While it is desirable to maintain seeds under optimum conditions between harvest and subsequent sowing, it is impractical on logistic and economic grounds to store huge quantities of commercial species (e.g., a cereal or legume) in controlled temperature and humidity facilities for such a short period. Storage of seeds under ambient conditions is possible if certain precautions are taken in the design of the storage structure:

- *Protection from water.* The roof, walls, and floors should be waterproof, with appropriate moisture barriers.
- *Protection from cross contamination.* For bulk storage, a separate bin should be provided for each cultivar. For bag storage, seeds of each cultivar should be stacked separately. All containers must be labeled with variety names and lot numbers.
- *Appropriate ventilation and aeration.* Fans or blowers are useful to provide air flow, thus reducing a buildup of heat and moisture, although any openings to the exterior should be covered with mesh to prevent animal or insect invasion. Seeds (open storage) or seed containers (e.g., bag storage) should be placed away from the walls to avoid reduced airflow.
- *Protection from rodents.* In developing countries stored seed destruction due to vermin, particularly rat infestations, is enormous; losses as high as 50–100% have been reported by family farms in some regions of Honduras, Laos, Bangladesh, and India, for example. Metal and concrete buildings, where affordable, normally provide good protection from rodents. Small isolated stores should have a floor raised about a meter above the ground, a rodent-proof door, and a removable entrance ramp. Metal, baked clay, or plastic storage bins with tight covers also protect against rodents. Examples of storage facilities utilized by small-scale farmers in developing countries as granaries and for saved-seed for planting are shown in Fig. 8.6.
- *Protection from insects.* The larvae of storage pests such as beetles (bruchids), grain borers, and weevils bore even into dry seeds and consume their reserves. A storage facility should be fumigated each time it is emptied, as should bins and boxes in which seeds are stored. An entrance constructed of a door leading into a small annex, from which there is a door entering into the main storage area, also cuts down direct access of insects to the seeds, as well as minimizing interior temperature and humidity fluctuations.
- *Protection from storage fungi.* Most fungi grow best under warm, humid conditions and thus damage can be minimized by drying seeds to safe moisture contents and holding them under dry or well-ventilated conditions. Treatment of seeds with fungicide may control some storage fungi (although many fungicides are most effective on soil fungi), and spraying the storage area when emptied may be advantageous.

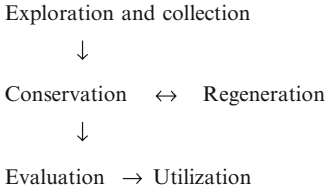
Fig. 8.6 Examples in developing countries of innovative, inexpensive structures for short-term storage of seeds, for consumption and for replanting. (**a**) Container for field corn grain in Malawi. The storage structure is raised above ground to avoid rodent infection and flooding, and shelter is provided to minimize exposure to the sun. Courtesy of H. F. Schwartz, Colorado State Univ., http://www.bugwood.org. (**b**) Woven bamboo storage silo, Mozambique, covered with clay, with caps to cover the openings for filling and emptying. It stands on a base that incorporates vermin traps and under a simple shelter to maintain cool, dry, and even conditions. Courtesy of HELVETAS Swiss Intercooperation. http://www.flickr.com/photos/44345799@N05

8.3.2 Long-Term Genetic Conservation: Ex Situ Seed Gene Banks

To meet the demands of increasing population and industrialization, society has developed an array of plants for consumption by people themselves or by domesticated animals. Yet only about 15 species actually feed the world; these include five cereals (rice, wheat, maize, barley, and sorghum), two sugar plants (sugar cane and sugar beet), three subterranean crops (potato, sweet potato, and cassava), three legumes (bean, soybean, and peanut), and two tree crops (coconut and banana). Of these, just three species, wheat, rice, and maize, produce nearly 70% of the world's food calories. Thus the fate of billions of lives hangs on the precarious balance of the genetic systems of these three crops, their diseases and pests, and their interactions with their environment. In addition, the majority of population growth in the next several decades is expected to occur in sub-Saharan Africa where other crops, including sorghum and cassava, are more important than wheat, rice, and maize. While the development of high-yielding crops through plant breeding and genetic engineering has increased yields and staved off famines, the replacement of traditional land races and local varieties has reduced the genetic diversity of the crops being grown. Genetic resources are being lost also by cultivation and industrialization of undisturbed lands where wild progenitors might be found, by abandoning old farming systems, and by the discarding of cultivars no longer in use. This loss of potentially valuable genes that could be used for future plant improvement, particularly in relation to pest, disease, and stress resistance, is a cause for concern. Steps have been taken to assemble germplasm resources for at least some of our cultivated plants and their wild relatives in order to preserve them in germplasm banks or "gene banks." Ideally, conservation should include a wide range of genetic diversity of all species, particularly those that have

actual or potential economic importance. Clearly this is impossible, for even ignoring the problem of deciding the potential value of species not yet investigated it would be necessary to sample a known species through its complete range of genetic diversity. This requires at least $10–20 \times 10^3$ samples per single crop species, and probably more for the major world crop cereals. Moreover, each sample should contain about 3,000 seeds to encompass the normal genetic variation within a population. Thus, there are practical limits to the number of samples that can be handled effectively in programs for the conservation and utilization of crop genetic resources. These limits are imposed by financial and personnel considerations, and since governments and world bodies have been generally reluctant to commit themselves to genetic conservation programs, gene banks are few and, on a global basis, distressingly inadequate. The international network of agricultural research centers collectively known as the Consultative Group on International Agricultural Research (CGIAR) maintains large germplasm collections (over 650,000 accessions) for many major agricultural crops, and a number of countries have national germplasm repositories. However, coverage is inadequate for many minor crops and renewed and continuous efforts to collect and conserve germplasm of both cultivated and wild plants is urgently needed.

Several stages involved in the establishment of a gene bank are outlined in the following flow chart.



Exploration and collection of accessions in general is inadequately supported relative to the magnitude of the need. Global gene bank accessions for major cereals, pulses, root crops, vegetables, forages, fiber, and sugar crops contain relatively good coverage of cultivated landraces (estimated at 50–95% for most), but coverage of related wild species is much less, generally less than 20% except for a few crops. The Millenium Seed Bank Project in the United Kingdom, an international conservation project coordinated by the Royal Botanic Gardens, Kew, set goals for collecting seeds of all of the UK's native flora and of 10% of the world's dryland flora (24,000 species); the latter was achieved in October 2009. The fact that this 10-year effort has collected only 10% of the global flora illustrates the magnitude of the challenge to collect and preserve the extant plant diversity.

After collection, seeds must be processed and cleaned, dried to low moisture contents, and packaged for storage in suitable containers, e.g., hermetically sealed cans or laminated foil packages. A general rule of thumb is that for heterogeneous materials 12,000 seeds are required for storage, and for homogeneous material 4,000 seeds. These should make up a "base" collection, i.e., one that is undisturbed and specifically laid down for long-term conservation. A "working" collection of the same seeds is also desirable, and these seeds can be used for medium-term

storage, regeneration, evaluation, and distribution to other users. Prior to storage, germination tests must be carried out to determine initial viability. Since germination testing is a destructive process, tests must use the minimum number of seeds to yield statistically significant results and, at the same time, not deplete the stored stocks; in fact, the greatest loss of stored seeds in seed banks results from their periodic use in tests to evaluate seed lot viability. As the intent of seed banks is to preserve the genetic diversity present in a sample, it must be regenerated before viability falls below about 85%, else genetic loss and drift can occur. The need to regenerate large numbers of accessions annually to maintain both viability and genetic diversity is one of the major expenses and challenges for gene banks. For example, if a large gene bank contains 500,000 accessions, and a seed sample can survive 50 years under optimal conditions before requiring regeneration (many do not achieve this), then a minimum of 10,000 accessions must be regenerated each year. Further genetic drift can occur during regeneration, as the species will likely not be reproduced in their native environments and selection pressures will be different. Thus, it is important to identify conditions to extend as long as possible the lifetimes of seeds in storage to reduce the frequency of sampling needed to monitor viability and to limit the number of regeneration cycles.

Gene banks are present in many countries and vary in their size and design. In the National Center for Genetic Resources Preservation in Fort Collins, Colorado, USA, for example, most seeds are pre-equilibrated at 25% RH and stored in hermetic containers at 5 or −18°C. The accessions are arranged in numbered steel trays and placed in numbered steel racks; each cold storage room has a capacity of about 180,000 cans of 500 ml volume. Storage of seeds in the Millenium Seed Bank, Wakehurst Place, UK is in large and medium-sized air-tight glass storage jars and smaller glass vials (Fig. 8.7a). Cryogenic storage in liquid nitrogen vapor (−160°C) is being utilized increasingly (Fig. 8.7b) and can greatly extend seed viability. Experiments to determine how long seeds can be preserved at this extremely low temperature (and in the absence of oxygen) are in progress, and unsurprisingly such experiments require very long timelines. Results confirm that seed quality and longevity will be greatly extended, but even at −160°C, seed deterioration occurs at a detectable rate. The Svalbard Global Seed Vault in Norway has aroused interest because it is located in a remote Arctic region about 1,300 km from the North Pole, buried in permafrost in a mountain, although the storage vaults are refrigerated to −18°C. It is not strictly a gene bank, however, because it does not collect, process or regenerate the accessions that it stores, but instead contains replicate samples from traditional gene banks and provides a back-up against accidental loss of germplasm (even as a result of nuclear war, hence the sobriquet “Doomsday Vault”!).

While the primary objective of gene banks is to conserve genetic diversity, the accessions that they contain are useful to breeders only when they are evaluated and characterized for their phenotypic traits. Many accessions will carry “passport” information, such as the location, season, and habitat, in which they were collected. Further observations may be made on growth characteristics, disease susceptibility, or other traits during regeneration for seed production. Users of the germplasm are also encouraged to report their phenotypic observations back to the gene bank. In

Fig. 8.7 (**a**) Storage of seeds at the Millenium Seed Bank, Wakehurst Place, UK in containers at −20°C. Collections focus on preserving seeds of the world's wild species. (**b**) Cryo-storage of seeds at the Agricultural Research Service's National Center for Genetic Resources Preservation (NCGRP) in Ft. Collins, CO, USA. Seeds are placed in the vapor phase over liquid nitrogen (−160°C). NCGRP focuses on storing seeds as diverse genetic resources to support breeding in agriculture. Like many gene banks both store seeds at 5°C for short-term accessions, at −20°C and over liquid N_2 for long-term accessions, testing them for viability at determined intervals, followed by regeneration of the stocks if necessary. (**a**) *Upper*, copyright of RBG Kew; lower, copyright of W. Stuppy. (**b**) Photo by S. Ausmus, courtesy of the USDA Agricultural Research Service

general, however, insufficient resources are available to comprehensively phenotype the stored accessions. A lack of adequate information on the phenotypic characteristics of accessions means that there must be extensive screening by breeders looking for particular traits of interest. For crops with large collections, smaller diversity panels are often assembled to more efficiently screen for potentially useful traits. The advent of inexpensive DNA sequencing and genomic analyses could facilitate the analysis of genetic diversity in germplasm collections and guide where further collection and conservation should be focused. Sequencing of the genomes of gene bank accessions could eventually lead to more direct screening methods, such as identifying only accessions containing sequence variation in a gene of choice known to be involved in a particular phenotypic trait.

For some species, particularly those whose seeds have low storability, the formation of seed gene banks is inappropriate, and the storage of other tissues or cells is required, e.g., meristems, cultured cells, pollen, seedlings, or living plants. The storage of recalcitrant seeds (Sect. 8.6) that can withstand only limited water loss and moderate temperatures provides considerable challenges.

Fig. 8.8 The location of Vavilov Centers of Diversity/Origin identifying areas of highest diversity of wild crop relatives. A few examples of conserved species are given in parentheses, with the number of endemic plants with agricultural relatives listed where known. (1) Mexico-Guatemala (maize, beans, squash, upland cotton). (2) Peru-Ecuador-Bolivia (62, potato, tomato, beans). (2a) Southern Chile. Chiloé Island (potato, strawberry). (2b) Southern Brazil-Paraguay (peanut, Brazil nut, pineapple). (3) Mediterranean (84, celery, chickpeas, durum wheat, clover). (4) Near East (83, wheat, barley, alfalfa, apple, cabbage, rye). (5) Ethiopia (38, coffee, sorghum, millet, wheat, barley). (6) Central Asia (43, almond, flax, lentil, wheat, beans). (7) India-Myanmar (117, cucumber, rice, mango, cotton). (7a) Malaysia-Thailand-Java (55, coconut, banana). (8) China (136, millet, buckwheat, beans, soybean)

8.3.3 Long-Term Genetic Conservation: In Situ Centers of Diversity

One of the first ex situ seed gene banks was set up in Leningrad (now St. Petersburg) by Nikolai Vavilov, a Russian geneticist and plant breeder; this famously survived the 28-months siege of the city in World War II, and several curators starved to death rather than eat the collected seeds. But Vavilov also recognized the importance of conserving plants in situ, at their centers of domestication (centers of origin) from wild species. Thus a number of Vavilov Centers of Diversity or Origin have been recognized in regions originally identified by him (Fig. 8.8). Although they have no defined borders, they are where the highest number of cultivated types and wild relatives exist, harboring a multitude of gene variants (alleles). Genes for disease, pest and stress resistance, and nutritional quality are just some of the resources that are inherent to plants in these regions.

8.4 Mechanisms and Consequences of Deterioration in Seeds

The patterns of loss of seed germination capacity and the factors that influence seed longevity provide some clues as to the mechanisms of deterioration that lead to loss of viability. Seeds generally exhibit an initial period of deterioration under dry storage during which germination percentage is relatively constant, but germination rate decreases (Fig. 8.3), indicating that a loss of vigor precedes the loss of viability. In addition, this pattern resembles "threshold" kinetics, such as occur with autocatalytic or cooperative chemical reactions, which proceed rapidly once a critical concentration of substrate accumulates, or mechanical failures in materials after a period of stress. One can envision that as cellular damage accumulates in dry seeds, repair processes following imbibition can successfully restore a functional physiological state, up to a certain point, and allow germination to be completed. The increasing delays in germination as seeds age during the lag period can be attributed to the longer time required for such repair to be effected as more damage accumulates. In most cases, although germination is delayed for such lower vigor seeds, the resulting seedlings exhibit normal growth rates following germination, indicating that the damage accumulated during this lag phase is fully reversible. However, once sufficient damage has occurred to critical functional components that repair processes are unable to restore them, the progression from only delayed completion of germination to abnormal germination and death occurs over a relatively short time period for a given seed. Nonetheless, there is still considerable variation among individual seeds in the extent to which they can avoid or repair damage, resulting in the normal distribution of deaths over time (Fig. 8.1).

This chapter can only briefly address current understanding of the mechanisms by which seeds can be damaged during dry storage and the consequences of such damage for germination upon subsequent imbibition (for more details see appropriate reviews in References, Sect. 8.4). Some key points about studying mechanisms of seed aging should be emphasized, and will be pointed out in particular instances. First, it is important to distinguish between the types of processes that can occur in dry seeds and those that can only occur upon subsequent hydration. At the low water contents at which seeds should be stored (e.g., <50% RH), integrated cellular processes, including respiration, ATP generation, transcription, translation, and enzyme activity, are either completely prevented or can operate at only a fraction of their capacity in hydrated cells. However, many studies of seed aging have been conducted under accelerated aging conditions at seed water contents that are in the region where enzyme activity and some metabolism are possible (75–100% RH). Caution must be exercised not to extrapolate to the situation in dry seeds those processes and mechanisms that can occur in relatively hydrated tissues. As a corollary to this, it is equally important to distinguish between damage that occurs in the dry state and that which occurs subsequently upon imbibition. For example, damage to membrane phospholipids could occur via chemical processes in the dry state, but the consequences of membrane leakage or loss of organellar compartmentation have meaning only in a hydrated cell in which liquid water is present. This complicates

investigations, for it is often difficult to detect changes occurring in dry seeds, which are subsequently inferred by their effects following imbibition. An important further complication derives from the fact that seeds vary in their resistance to aging and therefore in their vigor and viability after a given period of storage. For example, a seed lot that can produce 50% normal seedlings after imbibition may also contain 20% dead seeds and 30% partially viable seeds (resulting in abnormal seedlings) (Fig. 8.1), and even the normal seedlings will result from seeds at a range of vigor levels. A bulk sample of this seed lot may show excellent correlations with seed viability for a given seed component or activity, but it is difficult to distinguish cause and effect. For example, if only dead seeds accumulate (or lose) a given component (e.g., an oxidation product or antioxidant activity), there will be a very high correlation between its level in a bulk seed sample and loss of seed viability, but it might not be a cause of seed death but rather a consequence. It is still not possible to reliably distinguish viable from nonviable dry seeds and to segregate them into quality categories prior to sampling or imbibition. It is therefore important to keep the underlying population structure of seed aging in mind when interpreting the causes and consequences of seed damage during storage.

8.4.1 Deterioration Mechanisms in Stored Seeds

Across all of biology, while some microorganisms, plants, and animals have evolved mechanisms to survive desiccation to very low water contents, there is little evidence that any organisms can function metabolically at water activities below those in equilibrium with about 70% RH, or −50 MPa. Thus, this would seem to provide an approximate threshold to divide "dry" from "wet" mechanisms. As shown in Fig. 8.9, this is in hydration level II, in which only chemical reactions will be possible. Some enzymes have activity near this range, but the rates of reaction are extremely low or are limited to the lipid phase. Below an equilibrium RH of about 50% (depending upon the temperature), glass formation can occur, further increasing viscosity and limiting mobility of molecules and substrates that are required for integrated metabolism. Between 70 and 90% RH (hydration levels II and III), some enzyme activities can be detected (at very low rates) and substrate-level respiration may be detectable at the high end of this range. The minimum limit for respiration is about 90% RH (−15 MPa) or hydration level III, but higher rates of respiration and protein and nucleic acid biosynthesis only become possible as water availability increases to hydration level IV (>−5 MPa). Active physiological processes associated with turgor, growth, and germination only occur in hydration level V, or above 99% RH (~−1.5 MPa). Thus, in seeds stored under conditions where longevity will be promoted (i.e., <70% RH), only chemical reactions or radiation (e.g., cosmic rays) will be the primary sources of deteriorative changes. In the hydration range where seed deterioration is rapid, and is often used for accelerated aging studies (75–100% RH), other mechanisms can also come into play, particularly as the high water content is often

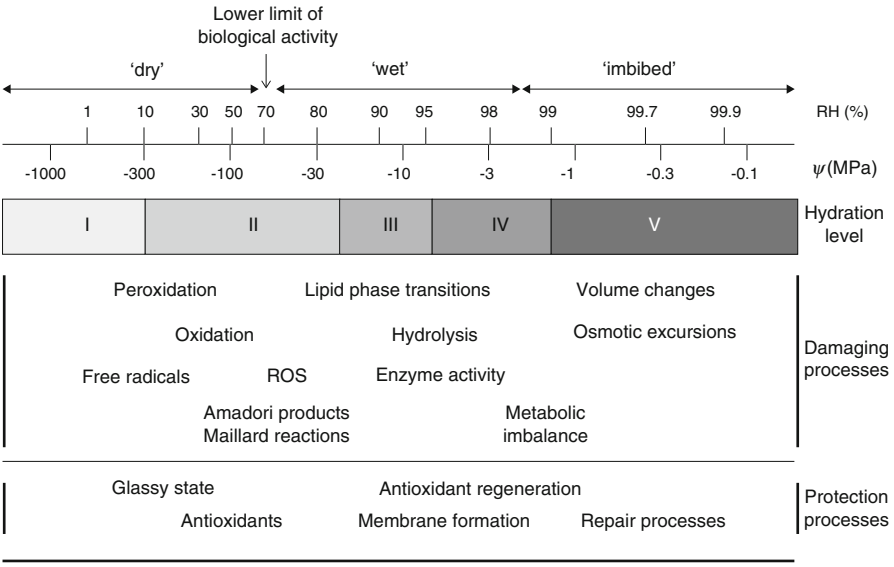


Fig. 8.9 Hydration levels and deterioration and protection mechanisms in seeds. The scales at the top indicate the equilibrium relative humidity (RH), water potential (ψ), and hydration levels as in Fig. 2.29. Below are shown a number of processes that are involved in the deterioration of seeds during storage at different hydration levels, as well as some protection and repair mechanisms. As metabolic/biochemical processes do not occur below about 70% RH, this is indicated as the approximate division between "dry" processes and "wet" processes. The highest hydration levels (IV and V) are achieved mainly by contact with liquid water, and are therefore indicated as "imbibed." ROS, reactive oxygen species. Adapted and modified from Walters et al. (2002)

combined with elevated temperatures. This is also the region in which seed water content increases rapidly with RH (Fig. 2.30). Thus, "dry" seeds will refer to those at water contents in equilibrium with 70% RH or less, while seeds at higher hydration will be termed "wet" and seeds in contact with liquid water or very high RH (>−2 MPa) will be termed "imbibed" (Fig. 8.9).

There is general consensus that oxidative and peroxidative processes play the primary roles in initiating the damage that occurs in dry seeds. Free radicals can be generated spontaneously, and can trigger oxidation of various seed constituents. At the lowest seed moisture contents, the extreme viscosity of the glassy state restricts the molecular motion and diffusion of substrates and reactants required for other types of chemical reactions, but the availability of oxygen and its tendency to form reactive oxygen species (ROS, including hydrogen peroxide and hydroxyl radical) allow free radical reactions to occur, albeit at slower rates than would occur in the absence of the glassy state. On the other hand, the presence of solvent water tends to quench free radical mechanisms and enable antioxidant mechanisms to be effective. Thus, damage due to free radicals and ROS is most prevalent in dry or the lower range of wet seeds (hydration levels I to III)

(Fig. 8.9). Peroxidation of lipids, initiated by the abstraction of a hydrogen by a hydroxyl radical, can result in a chain reaction that causes breakdown of the lipids (particularly unsaturated lipids) and release of by-products such as reactive aldehydes that can cause further damage to proteins and nucleic acids. Changes in membrane lipids due to peroxidation may be involved in the increase in membrane permeability and cellular leakage that is associated with seed aging. Reactive carbonyl by-products of peroxidation can also result in Amadori products, which are sugar-protein complexes and intermediates in the Maillard reaction that result in the nonenzymatic oxidation of biological molecules. Protein modifications such as nonenzymic glycosylation by reducing sugars can alter their structural integrity and inactivate enzymes that are required during early imbibition. ROS can also damage DNA, and chromosomal abnormalities increase in frequency as seeds age. These types of free radical or nonenzymic chemical reactions can occur at the low water contents present in dry seeds and are thought to be largely responsible for the deterioration of seeds stored under such conditions. Antioxidants, such as tocopherols, phenols, and ascorbate, can destroy free radicals and stop their propagation, reducing damage to the seed. However, at the low water contents of dry seeds, enzymic mechanisms to regenerate antioxidants are inoperative, so it is expected that they would eventually be exhausted, enabling ROS activity to become damaging.

At somewhat higher seed moisture contents (hydration levels II to IV), additional mechanisms of damage become possible. In the "wet" seed range, which is often used for accelerated aging treatments, sufficient water is available for some enzymes to become active and some aspects of metabolism to be initiated (Fig. 8.9). Hydrolytic reactions become possible as free water becomes available. However, as was discussed with respect to desiccation tolerance (Sect. 2.6.3), this is a dangerous hydration range for seeds, as inefficient respiratory activity can generate ROS and antioxidant protection and regeneration mechanisms may be inefficient. Such "metabolic imbalance" has been proposed as a possible cause of damage during dehydration, and similarly could cause damage to seeds held in this moisture content range. Lipid phase transitions also occur in this range of hydration, as sufficient water is present to reverse the water replacement by sugars and amphiphiles that stabilize bilayer membrane structures in dry seeds. However, as this is occurring, fusion of membranes and other problems can occur that could result in damage, particularly to critical functions such as respiration, which requires intact mitochondrial membranes and the proteins associated with them for electron transport and ATP synthesis. Holding seeds in hydration level III results in rapid deterioration, particularly at elevated temperatures, because all of the damaging reactions are possible (e.g., chemical, ROS, enzymic, some respiratory or metabolic) (Fig. 8.9), but insufficient water is present to enable the protective mechanisms available in a fully hydrated molecular environment.

As seeds attain higher levels of hydration (levels IV to V), their volumes increase sharply. This can cause stresses as spatial relationships among organelles and cellular constituents are reformed, particularly if membrane systems are impaired and compartmentalization is compromised. At the higher range of

wet seeds and fully-imbibed seeds, the consequences for seed quality become dependent more on functional repair processes than on water content per se. DNA repair is activated soon after imbibition commences (Fig. 4.9), and if repair is insufficient, the transcription and/or function of damaged genes will be compromised. Proteins damaged during storage, such as by the nonenzymic conversion of L-aspartyl residues to L-isoaspartyl residues during dry storage, can be repaired by a specific enzyme, protein L-isoaspartyl methyltransferase (PIMT), thereby restoring protein function. The enzyme is produced during seed development and its activity during imbibition is associated with improved survival of storage. If they are beyond repair, damaged proteins must be recognized by chaperone proteins and removed or recycled by the ubiquitin-proteasome system. A seed-specific transcription factor that regulates heat shock protein expression (HSFA9) also is involved in seed longevity, although it is unclear whether the expressed heat shock proteins act during storage to prevent damage or during subsequent imbibition to reduce the effects of aging.

8.4.2 Consequences of Storage on Germination

The mechanisms of damage, protection and repair in stored and imbibed seeds result in the consequences that are observed during germination. The kinetics of imbibition per se are not affected (Phase I in Fig. 4.1), but dead seeds often achieve a higher plateau water content than do viable seeds, apparently due to the absence of intact membranes, inability to generate turgor pressure and continued uptake of water into seeds in response to their osmotic solute content. This difference in imbibed seed density has been used to separate viable from nonviable seeds and upgrade the quality of seed lots. In the case of seeds with selectively permeable structures enclosing tissues or cell walls (e.g., endosperm or perisperm), nonviable embryos can leak solutes inside these envelopes and cause osmotic water uptake and swelling (Sect. 4.3.2). One of the earliest symptoms of seed deterioration is a delay in radicle emergence, followed by a progressive loss of capacity for normal germination (Fig. 8.3b). The root meristem is often particularly sensitive to deterioration, resulting in failure of continued radicle growth even if radicle protrusion has occurred due to cell expansion (Sect. 4.6.2). As discussed above, it is unlikely that there is a single master cause of seed deterioration. Instead, damage that accumulates while protection or repair processes are insufficient or inactive likely impacts many constituents of the dry seed, although some are more susceptible to oxidation or modification than others. When damage to key functional systems (e.g., membranes, organelles, nucleic acids, enzymes) exceeds the capacity of the seed to repair them, those systems begin to fail and initiate a deteriorative spiral toward death, resulting in the "threshold" kinetics of viability loss. Figure 8.10 illustrates the diverse and interacting mechanisms by which seeds may progress from viable to nonviable, and emphasizes the important role of water in the types of processes that can occur.

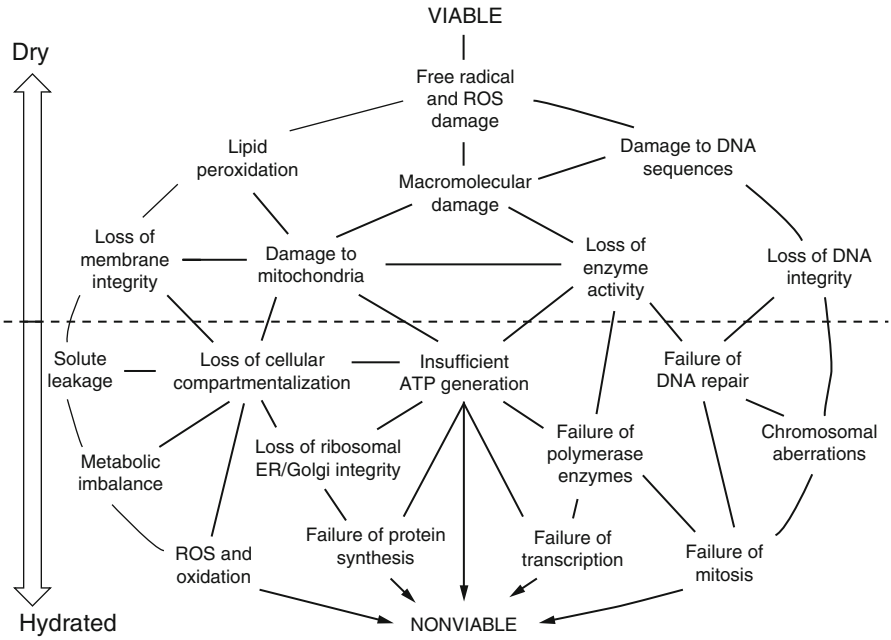


Fig. 8.10 A scheme to illustrate the variety of causes and pathways that can result in the loss of viability in stored seeds. Events above the horizontal dashed line can occur in dry seeds, while events below that line only occur and become evident at higher hydration levels following imbibition. ER, endoplasmic reticulum. Modified from Osborne (1980) and McDonald (1999)

8.5 Mechanisms of After-Ripening in Dry Seeds

The loss of dormancy that occurs during dry seed storage is termed after-ripening (Sect. 6.6.2). Changes occur in dry seeds during storage that influence whether or not they exhibit dormancy upon subsequent imbibition. The loss of dormancy during after-ripening is generally promoted by the same factors that affect seed aging, i.e., after-ripening is accelerated at higher temperatures and water contents. In fact, after-ripening could be considered to be the first stage of aging in seeds exhibiting primary dormancy. As discussed above, the types of chemical and biochemical reactions that can occur during seed storage, and therefore during after-ripening, depend upon the hydration level (Figs. 8.9, 8.10). The preponderance of evidence from across biology indicates that integrated metabolism, including respiration, does not occur below water contents in equilibrium with about 70% RH, and that complex processes such as transcription and translation would be very unlikely to occur at water potentials below about −5 MPa (96% RH). As after-ripening can proceed even at quite low seed moisture contents, processes that can occur at low hydration levels apparently are involved in the alleviation of dormancy, including

oxidation of various cell components and Maillard reactions that can alter protein structure or function (Fig. 8.9).

There are claims that gene expression, including both transcription of mRNA and translation of proteins, can occur in air-dry seeds (i.e., 50–85% RH). However, it is more likely that the observed changes to transcripts in the dry seeds during storage are the result of modifications by processes such as oxidation/fragmentation, rather than by their de novo synthesis. The evidence is not convincing that transcription can occur in dry seeds (i.e., those in equilibrium with <70% RH). De novo synthesis of proteins has also been claimed to occur in dry seeds, but again changes to the proteins could be the result of other processes such as oxidation, glycosylation or other modifications that can occur at low water activities.

Care must also be taken to ensure that studies on stored "dry" seeds are conducted at carefully controlled moisture contents, otherwise unrelated changes will be observed. In tobacco seeds stored under fluctuating moisture contents, for example, the changes in mRNA and protein content only occur when moisture contents are higher than required for after-ripening, and thus may not be essentially linked to this process.

Using magnetic resonance microimaging, it has been reported that the internal distribution of water within a dry tobacco seed is uneven, and that some areas (i.e., the testa and associated underlying tissues) have higher water contents that could support transcription and translation during after-ripening. This concept of "water pockets" of high hydration in dry seeds has subsequently been invoked to support claims of metabolic activity in dry seeds. However, relative tissue water contents based upon NMR microimaging must be interpreted with caution at low water contents, because lipids are also detected with this technique. In addition, it is difficult to conceive of a mechanism that would allow some tissues within a dry seed (i.e., 70% equilibrium RH or less) to accumulate and sequester sufficient water against a water potential gradient to the adjacent tissues without a permeability barrier separating the tissues. No such barrier is evident during imbibition of dry seeds, so the presence of water pockets in a dry seed sufficient to allow hydration to the level required for integrated metabolism would seem to be precluded on thermodynamic grounds.

The mechanism of after-ripening is unique and intriguing because it can occur in seeds under conditions that almost certainly preclude integrated metabolism, although it can also occur at higher water contents. A dry but after-ripened seed is likely in a developmentally distinct state relative to a fresh dry or imbibed seed, based upon gene transcription patterns in subsequently imbibed seeds. Thus, something occurs during dry after-ripening that upon imbibition results in a gene expression pattern associated with germination rather than with dormancy. What changes occur is certainly one of the key remaining mysteries in seed biology. After-ripening is a widespread phenomenon in nature with significant ecological implications. And seeds in the environment may encounter either brief wetting or high RH that could elevate their moisture contents into the range where metabolism is possible. Studies of the mechanism of after-ripening, however, must be conducted under carefully controlled seed moisture contents, to defined levels, so that it is possible to know the types of processes that can occur and therefore be responsible for after-ripening. In particular, after-ripening experiments (and those on aging mechanisms generally)

should avoid the use of high moisture contents associated with accelerated aging conditions, because after-ripening (and deterioration) can occur at low moisture contents, indicating that mechanisms that require higher hydration levels are not essential for it. Appropriate terms should also be used to describe events occurring in dry seeds, because terms such as "gene expression" are misleading with respect to changes that occur in transcriptomes or proteomes in dry seeds. Based on what is known about their physical state, such as a lack of a cytoskeleton (Fig. 4.9) and absence of free water (Fig. 2.29), it is difficult to conceive how transcription, translation and subsequent protein degradation could occur in a dry seed.

8.6 Recalcitrant Seeds

There is a class of seeds which at maturity are not capable of withstanding water losses to the extent discussed in previous Sections. These so-called recalcitrant seeds constitute about 3–47% of the flora, depending on habitat, and are mainly confined to woody species, many of which are of considerable socioeconomic importance in both tropical and temperate environments. Recalcitrant seeds pose the ultimate ex situ seed conservation challenge because they must maintain relatively high moisture contents to remain viable. But even when these seeds are stored under moist conditions, their life span is frequently brief and only occasionally exceeds a few months. Some species that produce recalcitrant seeds are listed in Table 8.5. Included are several large-seeded hardwoods (e.g., *Corylus, Castanea, Quercus* and *Aesculus* spp.), *Salix, Araucaria,* and *Juglans* spp., and important plantation crops like coffee, kola nut, cacao, tea and rubber. Seeds of most aquatic species (e.g., wild rice and some mangroves) also rapidly lose viability in dry conditions (but see below).

The inability to store seeds of recalcitrant species is a serious problem, for while vegetative propagation is possible for some species and is the usual practice, the retention of a viable seed stock is desirable to preserve maximum genetic diversity. Unfortunately, methods of storage other than drying may also be detrimental to recalcitrant seeds; e.g., low-temperature storage is inappropriate for some species, particularly for seeds of tropical plants, although cryopreservation of seed parts (i.e., embryonic axes) seems to have become a successful storage option. Grains of wild rice present a different problem, for although they may be successfully stored under moist conditions at low temperatures, they eventually lose dormancy and sprout.

At present, the optimal storage conditions for recalcitrant seeds can be determined only by trial and error, which is a tedious, time-consuming, and expensive approach. As our knowledge advances, it is apparent that some seeds (e.g., those of lemon) that were once thought to be recalcitrant can now be classified as orthodox—either the original method of seed drying or germination testing was at fault. Wild rice is recalcitrant only if dried at temperatures below 25°C followed by quick rehydration, but seeds survive and can be stored when first dried at temperatures above 25°C and are subsequently rehydrated slowly. A category intermediate between orthodox and

Table 8.5 Some species that produce recalcitrant seeds and examples of appropriate storage conditions

Species	Longevity and (% germinated)	Storage conditions	Damaging conditions
Avicennia marina	Few days	Moist	Drying
Corylus avellana (hazel)	6 months+	1°C in polyethylene bag	Drying
Castanea crenata (Japanese chestnut)	6 months	0–3°C in ventilated can or polyethylene bag	<0°C; excessive water or drying
Quercus borealis (red oak)	20 months + (50)	5°C in sealed tin	<20–40% moisture content
Juglans nigra (black walnut)	4 years	3°C in outdoor pit	Drying
Hevea brasiliensis (rubber)	4 months (3)	7–10°C in damp sawdust in perforated polyethylene bag	<20% moisture content
Cocos nucifera (coconut)	16 months	Ambient temperatures and high RH%	Drying
Coffea arabica (coffee)	10 months (59)	25°C in moist charcoal at 92–98% RH	<8–35% moisture content and <10°C
Cola nitida (kola nut)	5 months (80)	Ambient, heaped in open and kept moist	Drying
Theobroma cacao (cacao)	8–10 weeks	21–27°C in pod, coated with fungicide	<13°C, drying
Zizania aquatica (wild rice)	14 months (86)	1°C in water	Drying at <25°C

From information published by Berjak et al. (1989), King and Roberts (1979) and Kovach and Bradford (1992)

recalcitrant is now recognized (e.g., coffee) in which seeds survive desiccation but become damaged during dry storage at low temperatures.

All orthodox seeds can withstand dehydration to around 5% (0.053 g H_2O/g dry material [g/g]). Any seed that does not behave in this way cannot be considered orthodox. This means that the seeds of a large number of tropical species may accordingly be nonorthodox, which can be described as either recalcitrant or intermediate according to their storage behavior. Recalcitrant seeds are shed in a hydrated state, but the water content can generally be anywhere in the range from 0.43 to 4.0 g/g. In terms of desiccation sensitivity, such values relate to how recalcitrant a particular seed is. There appears to be a continuum of recalcitrant seed behavior, from species that are highly desiccation- and chilling-sensitive, to those that can tolerate drying to a lower water content that corresponds with recalcitrant seed behavior and can also tolerate relatively low temperatures. This concept of a continuum of desiccation sensitivity then extends to the intermediate seed species that do not react adversely to low temperatures (e.g., coffee seed), and to orthodox seeds that can generally tolerate less or more extreme dehydration. However, much work still lies ahead to establish clearly which species are truly recalcitrant and then to define quantitative relationships between storage conditions and viability. There

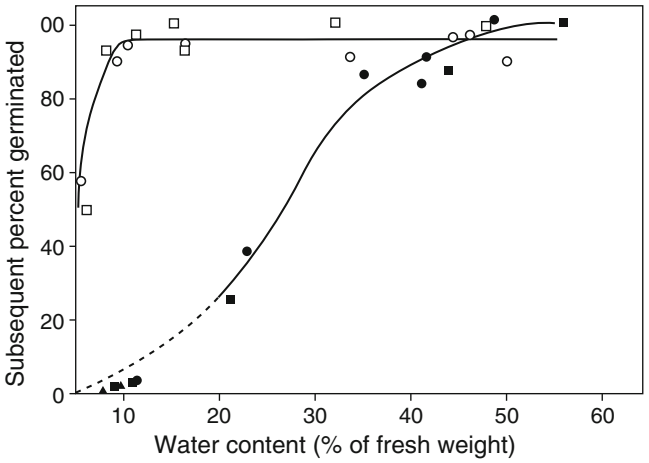


Fig. 8.11 Effect of loss of water on subsequent germinability of an orthodox and a recalcitrant *Acer* sp. Seeds (□, ■) or fruits (○, ●) of recalcitrant *Acer pseudoplatanus* (*closed symbols*) and the orthodox *A. platanoides* (*open symbols*) were dried to different water contents and their germination was then tested. The decline in germination of *A. platanoides* after drying to below 4% water content is unexpected and unexplained. After Dickie et al. (1991). With permission of Cambridge Univ. Press

is still an incomplete understanding of the physiological and biochemical bases for desiccation sensitivity in recalcitrant seeds but information is rapidly becoming available which is now considered briefly.

In many cases the sensitivity of recalcitrant seeds to drying is so great that just a relatively small change in water content is enough to cause damage. As an example, the response to loss of water of the recalcitrant *Acer pseudoplatanus* seed is very different from that of its orthodox, close relative *Acer platanoides* (Fig. 8.11). In the former, a reduction in water content from 60% to 50% lowers germinability by approximately 10%; this continues to fall as seeds dry, and no seed can survive drying to 10% water content. The ability of *A. platanoides* seeds to withstand drying (i.e., whether or not they behave as the orthodox type) depends on the stage of maturation at which they are dried, an observation that applies to other species too. Thus seeds may appear to be recalcitrant if they are dehydrated before they have fully acquired desiccation tolerance. A truly recalcitrant seed, of course, never becomes completely tolerant, at least under natural conditions of drying. This latter qualification is made because under certain conditions some recalcitrant seeds do survive dehydration. For example, isolated axes of some species (e.g., sycamore) show more tolerance than do intact embryos or seeds. The rate of drying is also important and in many cases slow dehydration is more damaging than rapid, "flash" drying. This is illustrated by *Landolphia kirkii* (a vine-like shrub of South-East Africa) whose seeds suffer lethal damage when the axis water content is reduced slowly to about 0.8 g/g, although the isolated axes themselves are unharmed by flash

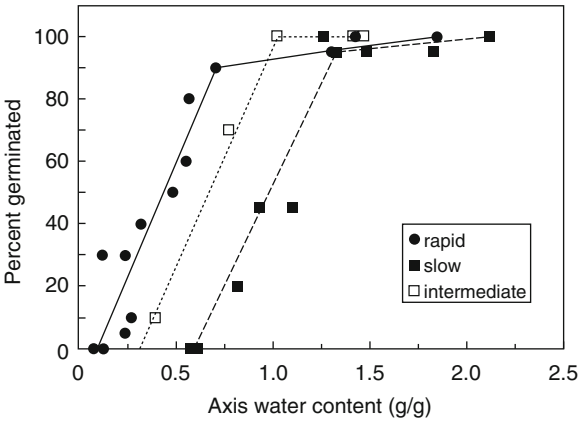


Fig. 8.12 Percent of rehydrated seeds that germinated of *Ekebergia capensis* previously dried at different rates to a range of axis water contents. From Pammenter and Berjak (2000). With permission of the Brazilian Society of Plant Physiology

drying to 0.3 g/g. In accordance with this, slow drying of seeds of *Ekebergia capensis* results in a loss of viability at considerably higher water contents than does rapid drying (Fig. 8.12). Thus, the rate of drying has a pronounced effect on the water content that is tolerated. The reason is because embryonic cells (particularly those of the axis) of the seeds that were dried slowly, causing considerable loss of viability, displayed advanced degradation of subcellular membrane structures. In contrast, seeds that were dried rapidly to an even lower water content, but still were largely viable, showed well-preserved membrane structures. It appears that different drying rates involve different deteriorative processes, which can only be explained by assuming that some damaging events occur at relatively high water contents. Slowly-dried seeds remain for a longer time at intermediate water contents than rapidly dried ones and hence will accumulate more damage. Thus the faster the drying, the less damage that accumulates and the lower the water content that can be tolerated. However, recalcitrant seeds still cannot tolerate the low water contents typical of orthodox seeds, irrespective of the drying rate.

To understand the physiological basis of recalcitrance it is necessary to recall the basis of desiccation tolerance and intolerance. As detailed in Sect. 2.6.3, water in seeds exists in several different states. Very few studies have been carried out to investigate which of the various water fractions are specifically important in recalcitrance, but in one case, *Landolphia kirkii*, biophysical evidence suggests that its seeds survive the loss of "freezable" water only if it is removed very quickly, by flash drying. The loss of "nonfreezable" water cannot be tolerated, however, because of the consequent loss of integrity of cellular components.

Late embryogenesis abundant (LEA) proteins, sucrose, certain oligosaccharides, and abscisic acid (ABA) are associated with the acquisition of desiccation tolerance (Sect. 2.5.2). LEAs are present in a wide range of recalcitrant seeds, mostly temperate, e.g., English oak and chestnut, and also in those of some tropical species. It is possible that they contribute to the slightly greater desiccation tolerance and chilling tolerance of some recalcitrant species. However, they are not present in

all recalcitrant seeds and are absent from the axes of several tropical wetland species. During their development, recalcitrant seeds of several species (sycamore, cacao, and wild rice) contain ABA at concentrations comparable with those in orthodox species. Sugars, including oligosaccharides such as stachyose, occur in many recalcitrant seeds (e.g., those of the mangrove *Avicennia marina*) and sucrose accounts for 11% of the dry weight of the recalcitrant tea embryonic axis! It is possible that intracellular glasses (Sect. 2.5.2.1) form in the presence of these sugars upon dehydration. However, they are clearly not effective protectants because water loss is fatal and recalcitrant seeds die at water contents that are higher than those at which these protective mechanisms are thought to operate. Therefore it must be concluded that whatever are the roles of LEAs, ABA, and sugars in orthodox seeds, they are not applicable to recalcitrant seeds.

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Glossary of Species Names and their Nomenclature

In the text, the common names of species are in English, but in both English and non-English speaking countries these are often different; hence the equivalent Latin name is given.

A

Alfalfa, Lucerne (*Medicago sativa*), 19, 20, 43, 44, 52–54, 327, 362
Almond (*Prunus dulcís*), 15, 24, 362
Anemone (*Anemone* spp.), 3
Apple (*Malus domestica*), 30, 250, 253, 257, 279, 300, 362
Arabian fenugreek (*Trigonella arabica*), 144
Arabidopsis, Thale cress (*Arabidopsis thaliana*),6, 28, 30–32, 34, 42–49, 54–57, 64–66, 72, 124–125, 156, 161–166, 168, 172, 174, 183–184, 202, 212–213, 217, 241, 250, 253–254, 258, 261–272, 274–280, 282, 286–289, 292–294, 321, 324, 330–331, 333, 334, 337
Arctic lupin (*Lupinus arcticus*), 342, 343
Ash (*Fraxinus* spp*., Fraxinus excelsior*), 3, 5, 253, 281
Autumn crocus (*Colchium sativum*), 24
Avens (*Geum* spp.), 3

B

Banana (*Musa* spp.), 358, 362
Barley (*Hordeum vulgare, H. distichon*), 8, 9, 11, 17, 18, 21–23, 31, 33, 41, 55, 61, 66, 68, 86, 88, 89, 92, 93, 98, 99, 105, 118, 120, 123, 161, 173, 184, 190, 192–202, 221–225, 232, 234, 253, 257, 263, 269, 276, 278, 291, 342, 343, 355, 358, 362
Barnyard grass (*Echinochloa phyllopogon, E. crus-galli*), 158, 160, 184, 325
Beech, Beechnut (*Fagus sylvatica*), 280
Beet. *See* Sugar beet
Birch (*Betula pubescens*), 250, 257, 282
Black cherry (*Prunus serotina*), 21
Black gram (*Vigna mungo*), 156, 204, 226
Black pine (*Pinus nigra*), 175
Brazil nut (*Bertholletia excelsa*), 3, 5, 17, 19, 22, 120, 122, 123, 362
Broad bean. *See* Faba bean
Buckwheat (*Fagopyrum esculentum*), 3, 13, 184, 219, 225, 229, 362
Bulrush (*Typha latifolia*), 158
Burdock (*Arctium* spp.), 300

C

Cabbage (*Brassica oleracea* var. *capitata*), 3, 174, 362
Cacao (*Theobroma cacao*), 14, 23, 370, 371, 374
Calabar bean, Ordeal bean (*Physostigma venenosum*), 23, 24
Carob, 6, 13, 203, 235
Chinese cabbage (*Brassica rapa ssp. pekinensis*), 34, 35

D

Dandelion (*Taraxacum officinale*), 3, 50, 300
Date palm (*Phoenix dactylifera*), 6, 11, 184, 185, 207, 344, 345

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