ABSCISIC ACID

1952 Bennet-Clarke
Trying to discover new auxin A and B
TLC of the eluted chromatogram of alcoholic extract of plants gave, in addition to auxin-like compounds, compounds that were inhibitory in an Avena coleoptile growth test: “inhibitor beta”.

1950s Radley
Correlated the level of “inhibitor beta” with potato tuber sprouting (inverse relationship)

1963-5 Waring, Corning, Milborrow,
Isolated and crystallized an inhibitor “dormin” from birch leaves that caused bud to become dormant

1963-5 Addicott
Crystallized a compound from cotton fruit that accelerated cotyledon abscission at 0.01 ug applications; C_{15}H_{20}O_{4} used NMR to determine the structure. Purification was aided by high optical activity of this molecule

1966. Millborrow et al
Compared IR of dormin to abscisin, was indistinguishable.....11 days later they synthesized this compound to confirm their guess that dormin was abscisin. Synthesized molecules included racemic mix of both enantiomers: (+) S; (-) R
ABSCISIC ACID designated by agreement as the name

1968 Millborrow
Characterized beta inhibitor and found it to also be “abscisic acid”
ABA IMPLICATED IN

storage protein accumulation
desiccation protein accumulation in embryos
transport of photosynthate to growing embryos
promotes seed dormancy (w/out ABA get vivipary)
reversal of GA-induction of alpha-amylase accumulation in germinating grains
promotes bud dormancy
promotes leaf senescence
guard cell closure under water stress
increased root growth and decreased shoot growth under water stress

induces abscission when applied at high levels, but all evidence indicates ethylene
plays this role in planta

Sites of synthesis
Seed including seed coat, roots (esp. under stress), growing shoots.

Transport
mechanism not known, via xylem and phloem; also apoplastic
redistribution of ABA in leaf under water stress

Endogenous derivatives and related compounds
phaseic acid (slightly active) and dihydrophaseic acid (permanent inactivation)
monosaccharide esters (probably permanent inactivation)

Stereochemistry critical for activity

Biosynthesis
Isoprenoid pathway via carotenoid intermediates and an oxidative cleavage reaction

Subcellular sites of biosynthesis
IPP to 9’cis neoxanthin (40 carbon xanthophyll) plastids
9’cis neoxanthin cleaved to xanthoxin (15 carbon) plastids? cytosol?
xanthoxin to ABA cytosol
ABA SYNTHESIS

- C15 sesquiterpene
- ABA always found as the S enantiomer, high optical rotation
- the isoprenoid unit can be seen
- low levels of synthesis in normal vegetative tissue.
- 10-1000 fold higher levels in developing seeds, dormant buds, stressed tissue (particularly water or salt stress) so work with this tissue usually.
- Originally thought to be synthesized via FPP (C15), but strong genetic and biochemical evidence that it comes exclusively from carotenoids!

4xIPP(C5)->GGPP (C20)
condensation of 2xGGPP (C20)>violaxanthin (C40)>xanthoxin->ABA

A HISTORICAL NOTE:
Feeding studies to distinguish between the two pathways were difficult—reasons unclear at the time (now we know about the deoxyxylulose pathway to IPP in the plastids) radiolabeled MVA or CO2 precursors were incorporated into ABA only at a low rate in vivo. However, MVA was clearly demonstrated as a precursor of ABA, via terpenoid biosynthesis by in vivo and in vitro studies, using specifically radiolabeled MVA tritiated isotopes. Losses of pro R, pro S hydrogen stereospecific

*(2,14C (2R)-23H1) MVA
(2,14C (2S)-23H1) MVA
(2,14C (4R)-23H1) MVA-retain-indicates formation of cis before trans
(2,14C (4S)-23H1) MVA-loose-
(2,14C (5R)-23H1) MVA
(2,14C (5S)-23H1) MVA

****Show MVA, ABA, with expected H losses
when MVA is converted to IPP
-the 4proS H of MVA is always lost
-the 2proR and 2rpoS H of MVA are retained (but lost except at 6’ methyl group after ABA formed)
-the 5proR is always lost during ABA formation

indirect pathway.
get same stereospecific pattern of labeling of proR, proS hydrogens from MVA as for direct pathway

Biochemical Evidence FOR INDIRECT PATHWAY
-Violaxanthin endogenous
-Violaxanthin can be oxidized to xanthoxin (C15) (BUT at abnormally high photointensities)
-Xanthoxin shown to be endogenous to several plant species, although not demonstrated in others
-In vivo feeds of xanthoxin to tomato, bean show conversion to ABA
-Inhibitors of carotenoid biosynthesis also can inhibit ABA formation (fluridone, norflurizone).

This effect doesn’t always occur.
possible explanation: the pool of xanthophylls is so large that it doesn’t get depleted enough for ABA synthesis to be diminished.
(1) leaves growing in hv show ABA inhibition after exposure to fluridone,
leaves already mature (presumably w/large carotenoid pool) do not.
(2) Mature bean leaves pretreated w fluridone, exposed to 14C02 24h---leaves stressed for 14h.
Total and specific radioactivities of ABA, xanthophylls measured. xanthophyll level decreased, ABA total level same
ABA and xanthophyll specific RA reduced to similar level
Thus formation of ABA from a preformed precursor by fluridone not inhibited,
but formation of both ABA and xanthophylls from 14C02 both inhibited.
block carotenoid biosynthesis with inhibitors (fluridone; norflorazone; block conversion of phytoene to phytofluene) also inhibit ABA accumulation. These inhibitors also have multiple effects; photobleaching, chloroplast degradation;
dark-grown barley seedlings still have low ABA accumulation but apparently normal phenotype even following fluridone treatment. (Gamble, PE; Mullet, JE. 1986. Inhibition of carotenoid accumulation and ABA biosynthesis in fluoridone-treated dark-grown barley. Eur. J Biochem. 160:117-21).

Green bean leaves pretreated with fluridone, labeled w 14C02 24h, water-stressed for 14 h. ABA and several xanthophylls isolated, quantified. fluoridone didn't inhibit ABA accumulation, but specific activity was reduced to the same level as for the xanthophylls (Li, Y, DC Walton. 1987. Xanthophylls and abscisic acid biosynthesis on water-stressed bean leaves. Plant physiol. 85:910-15.)

-poor incorporation of MVA,C02 into ABA could be explained in vivo by the pools of carotenoids, specifically xanthophylls such as (violaxanthin, leutein, neoxanthin)

-maize vp (viviparous) mutants carotenoid deficient and also ABA deficient in leaves, roots

SO>>CONCLUSIVE PROOF THAT ABA SYNTHESIZED VIA CAROTENOIDS !!(????)
(No...because alterations in ABA could be due to indirect effects of reduced carotenoids and therefore (stress and) altered plastid function on ABA synthesis; however, one might expect increased ABA synthesis in this case)

STUDIES WITH 18O provide further evidence in support of the indirect biosynthesis of ABA
water-stressed bean and Xanthium leaves in presence of 18O2
02 is incorporated into ABA mostly at carboxyl group
this suggests
1) 1'-deoxy ABA isn't immediate ABA precursor???
2) ABA synthesised was derived from a preformed precursor with 1' and 4' oxygens of ABA already present (or else they'd already be labeled)
this is consistant with the labeled O2 being derived from the aldehyde of xanthoxin formed on cleavage of violaxanthin

SIMILAR EVIDENCE for non-stressed tissue (maize embryos)
Feed 18O2 to embryos:
kernels cultured on agar, thus no maternal ABA
(prob...what about stress of culture conditions??)
since no PHS occuring, carotenoid level may be unimportant to embryo physiology; event may be separable
GC-negative chemical ionization-MS= ??Is this a way to obtain predominantly everything in the parent ions?
GC-electron impact-selected ion monitoring = selected ions enable increased focus on particular characteristic ions, and thus higher sensitivity (very important for ABA)

<table>
<thead>
<tr>
<th>Relative abundance at m/z</th>
<th>278</th>
<th>280</th>
<th>282</th>
<th>284</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos</td>
<td>100</td>
<td>16</td>
<td>5</td>
<td>.5</td>
</tr>
<tr>
<td>agar</td>
<td>100</td>
<td>85</td>
<td>27</td>
<td>.9</td>
</tr>
</tbody>
</table>

embryos have most ABA non-isotopically labeled, next, just labeled at one carbon. GC-EL-SIM MS measurments indicated the 18O was at the carboxyl.

agar also contained ABA; MORE label, but similar ratio of label.
thus, maybe ABA newly synthesised is preferentially released into media, or maybe ABA newly synthesised gets either oxidized to phaseic acid, or secreted.
Against
Is ABA formed from xanthoxin in vivo??
is xanthoxin derived from carotenoids

avocados slices convert
14C MVA to ABA and carotenoids, xanthophylls
14C phytoene only to carotenoids, xanthophylls (compartmentalization??)

Fungus Cerospora rosicola excretes ABA synthesises it rapidly
14C acetate, 14C MVA converted by fungus to ABA
inhibitors of carotenoids didn't affect ABA synthesis, thus direct pathway implied
Intermediates in the putative Cerospora spp pathway fed to plants, with mixed results (Vicia faba, but not other plants tested, synthesised ABA from these compounds)
One late intermediate is endogenous to fungus
Fungal pathway seems to be direct pathway.

evidence from non-stressed tissue for carotenoid origin of ABA (1989).
carotenoid mutants are ABA deficient, show physiological symptoms of ABA deficiency.
(maize viviparous 2,5, and 9 = vp2, vp5, vp9; pink scutellum = ps = vp7; white seedling = w3; yellow = y3, y9)


Ultimately, a combination of genetics and biochemistry led to the conclusion the indirect pathway was probably the only pathway, or at least by far the most significant.

Viviparous mutants in MAIZE include ABA biosynthetic mutants
w3- blocked in the desaturation of phytofluene
vp5- blocked in the desaturation of phytoene
vp7- lycopene, delta and alpha carotenoid accumulate

aba mutants in Arabidopsis are ABA biosynthetic mutants

See BUCHANAN
ABA RESPONSE

ABSTRACT

LEA are a diverse group of proteins (about 20-100 genes) that accumulate in response to desiccation/ABA in maturing embryos of angiosperms, gymnosperms. Regulation of the accumulation of the mRNAs coding for such proteins is not identical. Classifies based on pattern of expression and sequence.

Gene expression: Some are induced by ABA and or desiccation in adult plant as well. wheat-Em is regulated by exogenous ABA in embryos; however 10 related genes exist cotton-detailed study of LEA expression. LEAs can be classified by expression patterns in developing embryos (6 different patterns of expression during embryo maturation and desiccation, and in response to ABA). Some LEAs are regulated by desiccation and expression in embryos is increased by ABA; some LEAs are regulated only by ABA; some LEAs regulated by ABA only at specific stage(s) of embryonic development. cotton-somatic embryos- mRNA for EMB-1 accumulates at earliest embryo development (globular stage)

LEAs probably function as protectants against desiccation although mechanisms as yet not known.

5-6 classes based on sequence homology
ALL are hydrophylic, small/medium, rich in arginine, lysine,
Class 1:
10,000 MW, highly conserved in monocots, dicots. wheat vs carrot proteins are 80% identical, 90% similar. High in gly-gly residues (6-8 in protein sequence) expected to disrupt alpha helix. Very hygroscopic proteins with random coil structure.
Class 2:
stretch of 8-9 ser residues
rich in gly,OH bearing aas, so probably little alpha helix
Class 3:
14-60 kD
11-mer unit repeated 5-16 times. lys-ala-lys-met-ala-lys-asp-thr-thr-met-gly

just before desiccation LEAs can be up to 30% of total protein
may bind water or replace water, thus maintaining protein function

ABA maintains embryo DORMANCY by blocking expression of germination genes and inducing expression of embryonic gene programs

- Fluridone blocks desaturation of phytoene to phytofluene.
- Fluridone added to isolated kernels or isolated cob-pieces blocks carotogenesis and reduces ABA levels - mimics vivipary in kernels
- add back ABA- get normal, dormant seeds, which are viable.
Mutants of *Arabidopsis seed development* (Koornneef et al, Plant Physiol 1989. 90:463-469) a subset of these are **ABA deficient mutants** all at same loci

*aba* - precocious germination, lower level of accumulation of specific proteins, increased stem, leaf, fruit (silique) withering, reduced seed dormancy normal seed development (also like *sit* mutant of tomato)

**ABI3 of Arabidopsis mediates seed and seedling specific ABA responses such as dormancy**

- **abi3 mutants**: seeds have reduced storage compound accumulation and dormancy, normal anthocyanin accumulation, seedlings don't germinate well

- **ABI3** expressed in siliques, not in adult vegetative organs

- **ABI3** protein has sequence homology to transcription regulators, particularly VP1 gene

- **ABA does not regulate transcription of seed storage protein genes**

**Viviparous mutants in MAIZE include ABA response mutants**

*vp1* - ABA-insensitive mutant with normal ABA levels but (pleiotropic effects) is viviparous accumulates reduced levels of LEA proteins, doesn’t accumulate anthocyanins in aleurone; normal germination; the rest of plant growth is normal. VP1 is expressed specifically in endosperm and embryos codes for myb-like transcription factor

**ABA interacts with GA in germination**

**barley aleurone layer**-

Aleurone layer one-three cells thick plays critical role in mobilization of endosperm to support the post germination seedling growth. Usually originates from endosperm cells (ie, the result of fusion of one egg nucleus with two sperm nuclei, does **not** have genetic makeup identical to that of embryo).

GA induces the synthesis and secretion of a large number of enzymes
- alpha amylases
- proteases
- 1,3;1,4 glucanase, endoxylanase--hydrolyze cell wall and facilitate enzyme movement
- nucleases

**good system for study because:**

- homogeneous cell population that responds to two hormones
- for GA, the source and target tissue are distinct--thus specific hormonal effects can be studied
- many enzymes are induced in concert
- protoplasts from aleurone respond to GA, can analyze secreted enzymes and facilitates fractionation of organelles
- antagonism between GA, ABA relatively simple, enabling studies of interactions of hormones
- mutants with altered sensitivities exist
- no cell growth or division
- response in isolation is similar to response in planta.

Aleurone response includes:
- GA induce the expression of a set of genes, and suppress the synthesis of another set.
- ABA has reverse effect on these gene sets
- induction of ABA- and GA-induced proteins is Ca\(^{++}\)-dependent
  - *abi1* is threonine/serine protein phosphatase that binds Ca++
  - Ca\(^{++}\) dependent pathway components: cyclicADP-Ribose mobilizes sequestered Ca\(^{++}\). phosphatase inhibitor also required, H+ as second messenger,
  - LT178 (an ABA-induced gene) is also induced by microinjected cyclicADP-Ribose plus phosphatase inhibitor
  - also Ca\(^{++}\) independent pathways at least for ABA signaling
  - catabolic enzymes induced about two hours after hormone application.

16 ABA-induced proteins identified after 2-d gel electrophoresis
mRNAs accumulation induced by ABA is detected at around 30 min.
ABA-induced proteins include:
- 11- aa repeated sequence-like class 3 LEAs
- 21 KD inhibitor of a-amylase (this is the only protease/amylase inhibitor shown to be specific for an endogenous amylase)

**Stability of alpha-amylase mRNA is decreased by ABA and phaseic acid.**
ABA later decreases stability of alpha-amylase, particularly high pI one, if added 12-16 h after GA application.
Phaseic acid (PA) causes inhibition of alpha-amylase synthesis if added 12-16 h after GA application (like ABA). probably acts to destabilized alpha-amylase mRNA (like ABA)
PA doesn’t induce transcription of ABA-induced genes

Water or salt stress imbibed seeds induces ABA levels, and induces the same group of proteins
fluridone inhibits the synthesis of the proteins and decreases the ABA level
possibly, in nature, stressful conditions cause an increase in ABA which triggers a variety of metabolic events that slow down the process of germination

Mutants effecting germination include
slender barley-functions as if always has saturating GA concentrations
viviparous mutants

**ABA response mutants**
Select seedlings that germinate normally on medium with 10 uM ABA.
abi mutants (ABA insensitive)

each mutant has different parameters of response. endogenous ABA levels slightly higher.

abi1  as adults transpire excessively and wilt (codes for threonine/serine protein phosphatase that binds Ca++; may act as Ca++ channel on plasma membrane that is modified via ABA, but does not directly bind ABA)

abi2  as adults transpire excessively and wilt (codes for threonine/serine protein phosphatase that binds Ca++; may act as Ca++ channel on plasma membrane that is modified via ABA, but does not directly bind ABA)

abi3  as adults normal water relations

abi4  as adults normal water relations (codes for AP2-domain transcription factor)

abi5  as adults normal water relations

• abi1, abi2 and abi3: very little dormancy, normal seed weight

Does this mean ABA is not needed for seed development??

drought stress induces stomatal closure and maintains this response; mediated by ABA. Externally applied ABA causes stomatal closure. But, since at pH of cell wall a significant proportion of ABA would be uncharged, and thus permeable to cell, not clear if receptors are on PM or inside cell.

**Root development and environment alters the growth of the shoot-role of ABA**

when the soil dries around roots, leaf growth decreases and stomata close. growing plants with a pressure vessel placed around the roots uncouples the effect of soil drying from the effect of reduced water uptake. If roots put in several containers, and in some let soil dry, the water relations of the shoot are similar, but the leaf growth rate is still reduced. When the soil dries, ABA moves from the root to the shoot in the transpiration stream. This ABA is thought to play a role in stomatal closure, and reduced leaf growth rate. Soil drying causes an increase in xylem sap pH; this would increase ABA concentration in the xylem, and could be the mechanism by which ABA moves into transpiration stream. A second, as yet unidentified molecule that influences stomatal closure also is found in the transpiration stream. Ion flow in the transpiration stream changes with drying, and these ions seem to increase the sensitivity of the guard cells to ABA. The sensitivity of the guard cells to ABA changes diurnally, with lowest sensitivity being in the early morning. Therefore, the time of day that experiments with roots are conducted will influence results.

Sucrose insensitive mutants may be impaired in ABA synthesis or ABA response genes
High sugar concentration effects the expression of over 40 genes, and ultimately effects development.

- **upregulation** of genes of carbon partitioning, shoot to root size, flower induction, tuberization, and anthocyanin and chlorophyll (in seedlings).
- **downregulation** of catabolic genes, photosynthetic genes in mature plants (RUBISCO, plastocyanin)

Sucrose response requires SNF-like protein kinase

Hexokinase –dependant pathway:
Hexokinase also implicated (overexpression of hexokinase->increase in sucrose sensitivity, and visa versa)
Hexokinase independent pathway

**Germination and growth on sucrose or glucose:**

- WT plants do not expand cotyledons or true leaves
- **sis** (sucrose insensitive; 19 isolated) and **sun** (sucrose unresponsive) mutants form expanded cotyledons and leaves. **sun1** has decreased repression of RUBISCO
- **gin1** (glucose insensitive) form expanded cotyledons and leaves (gin1 is downstream of hexokinase)

As with hormonal responses, sugar responses interact with responses to light, development, hormones.

- **gin1** defective in ethylene response
- **ctr1** (ethylene insensitive) and **eto1** (ethylene biosynthesis) mutants are glucose-insensitive
- Ethylene mimics **gin1** mutant phenotype
- **prl1** selected for increased sensitivity to sugars, also has increased sensitivity to ethylene, ABA, CK, auxin

- **sis4** is allelic to **aba2**
- **sis5/sun6** is allelic to **abi4** (AP2 domain transcription factor)

Components of ABA metabolism and response pathways are also shared by sucrose signaling response pathways

Some ABA response mutants are not sucrose signaling mutants (e.g., **abi2**, which has an abnormal ABA response phenotype and is an osmo-insensitive mutant)

**Multiple types of ABA receptors may be on plasma membrane and internal**

To investigate localization of ABA receptors, epidermal peels isolated from *Commelina* (succulent monocot, easy to pull off epidermis).

Closed guard cells microinjected with ABA. Neutral red dye (which is actively taken up by living cells) used to monitor viability of cells. Stomates opened anyway. External applications of ABA...stomates stayed closed. ABA was not metabolized.

In contrast, ABA microinjected into guard cells of open stomates...get closure.

Are there two mechanisms at work, with two receptors??
Similar study (Gilroy and Jones) of effects of ABA, GA on secretion of alpha-amylase by barley aleurone.

Protoplasts made,
- microinjected $^{14}$C GA or $^{14}$C ABA, no effect on secretion, but also not metabolized.
- external applications of GA caused both de novo synthesis and secretion of alpha amylase; response inhibited by ABA.

EXTERNAL ABA, GA is necessary for aleurone responses. (internal ABA, GA receptors could also mediate these or other responses)

These studies are some of the first using microinjection into plant cells. Difficult technique! Can microinject metabolite, or caged metabolite (light activated)