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Nucleotide Metabolism in 'Washington' Navel Orange Fruit: II. Pathway Capacities During Development

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Abstract. **Changes in the capacity of 'Washington' navel orange [*Citrus sinensis* (L.) Osbeck] fruit to synthesize (*de novo* or by salvage) pyrimidine nucleotides, but not purine nucleotides, appears to be related to the stage of fruit development. *De novo* pyrimidine synthesis in whole-fruit tissue increased 6-fold during Stage I of development (cell division phase), from 10 nmol [^{14}C]bicarbonate incorporated into uridine nucleotides during 5 hr per g dry weight whole-fruit tissue from ovaries harvested at flower petal drop to 57 nmol for 2-month-old fruit. Capacity of peel tissue to synthesize pyrimidine nucleotides *de novo* decreased following completion of Stage I, from 43 nmol [14C]bicarbonate incorporated into uridine nucleotides during 5 hr per g dry weight of peel tissue from 2-month-old fruit to 11 nmol for 5-month-old (Stage II) fruit. This decrease was not offset by increased salvage of uridine. Capacity of whole-fruit tissue to synthesize purines *de novo* increased 3-fold during Stage I. Synthetic capacity of peel tissue from Stage I fruit was half that observed for whole-fruit tissue and did not decrease significantly during Stages II (cell enlargement phase) and III (maturation phase). These observations suggest purine synthetic capacity may not be related to stage of development. Changes in protein or glucose contents, or respiratory activity of peel tissue, could not account for the observed reduction in pyrimidine synthetic capacity. Thus, the reduction observed in synthetic activity was specific for pyrimidine nucleotides. The capacity of fast-growing, 1-month-old fruit (high potential to set) to synthesize or catabolize either pyrimidine or purine nucleotides did not differ from that of slow-growing fruit (low potential to set), suggesting that nucleotide synthesis is not limiting to growth.**

'Washington' navel orange fruit growth occurs in three stages, predominated by cell division, cell enlargement, and maturation, respectively (2, 5, 7). Fruit set occurs during Stage I of development, the period from flower petal drop to 2 months past petal drop. Cell enlargement predominates during Stage II. Fruit approach full size during this ≈3-month period. Maturation occurs during Stage III. In this nonclimacteric fruit, maturity is judged against legal/commercial standards.

Increased pyrimidine nucleotide biosynthesis has been reported to occur in pea cotyledons during germination and early development (10), during the early phases of the mitotic cycle of synchronously dividing cells of *Vinca rosea* L. [now, *Catharanthus roseus* (L.) G. Don] (8), and during embryogenesis of *Daucus carota* L. cells in suspension culture (1). In at least the two latter species, it appears that growth by cell division is accompanied by increased pyrimidine biosynthetic activity. Further, activities specifically associated with growth by cell division might be expected to decline during periods of growth by cell enlargement and of maturation. Consistent with this interpretation, Kanamori-Fukuda (8) has reported decreased pyrimidine nucleotide biosynthetic activity of *V. rosea* cells in suspension culture following completion of the cell division phase of growth. The apparent relationship between growth by cell division and rate of pyrimidine nucleotide biosynthesis suggested this metabolic activity might change during the three stages of citrus fruit development.

Differences in growth rate exist among Stage I fruit (9, 13), permitting examination of nucleotide synthetic and catabolic capacities in relation to rate of growth during the cell division phase of growth. Fruit growth rate during early development is one of many factors associated with citrus fruit set (9, 13): Faster-growing fruit have a greater potential to set and survive to harvest, while slower-growing fruit tend to abscise early in their development. An association between rate of growth during Stage I and capacities of purine and pyrimidine nucleotide synthesis and catabolism would provide a physiological mechanism associated with fruit set.

To the best of our knowledge, this is the first study to assess the activities of the pathways for the *de novo* biosynthesis, salvage, and catabolism of purine and pyrimidine nucleotides in relation to stage and rate of growth in fleshy fruit.

Materials and Methods

Fruit were collected from 20-year-old 'Washington' navel orange trees on 'Troyer' citrange rootstocks or from cuttings (1- to 2-years-old) rooted from these scions. Trees were located at the Citrus Research Center and Agricultural Experiment Station, Univ. of California, Riverside. Fruit of known age (months past

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petal drop) were obtained from flowers in individually tagged inflorescences on orchard trees. Individual flowers within each inflorescence were observed twice weekly for petal drop during the 1982 spring bloom period. Fruit differing in potential to set and survive to harvest were distinguished based on growth rate (increase in transverse diameter per week) during the 4th week past petal drop. At least eight fruit were collected between 8:00 and 10:00 AM for each replicate and processed immediately. Fruit were taken at random from all areas of the trees between ≈0.5 and 2 m in height. Differences in many internal characteristics are associated with position of the fruit on the tree (3, 12). Therefore, high variability was expected using a random collection procedure to obtain representative population samples.

Fruit were washed, disinfested in 5% chlorine bleach solution, rinsed in distilled water, and sectioned submerged in 50 mM Hepes buffer (pH 8.5). Radial tissue slices (≈5 × 7 × 1 to 2 mm) containing either all the tissues of the fruit in proportion to their cross-sectional representation (whole-fruit) or only the isolated peel tissue, were used (11). Whole-fruit tissue was used in studies involving only Stage I fruit. The difficulty of obtaining similar-sized whole-fruit tissue slices throughout development was avoided by using peel tissue for these studies. Tissue slices were pooled in 50 mM Hepes buffer (pH 8.5) then 500-mg (fresh weight) aliquots were blotted, weighed, and quickly remoistened.

Nucleotide synthesis. The capacity of fruit tissue slices to synthesize nucleotides *de novo* was assessed at 30°C under optimal conditions (11) by measuring the incorporation of [^{14}C]bicarbonate (Na salt) at a saturating concentration (20 mM) and uniform specific radioactivity (9000 dpm/nmol for pyrimidine nucleotides and 2300 dpm/nmol for purine nucleotides) into either total uridine nucleotides (ΣUMP) or into the sum of adenine nucleotides, nucleoside, and base (ΣAde) isolated by co-crystallization (11). The formation of nucleotides from existing nucleosides and bases through activity of the salvage pathways was determined by measuring the incorporation of 2 mM [2-^{14}C]uridine into ΣUMP (isolated by co-crystallization) and of 2 mM [6- or 8-^{14}C]adenine into total adenine nucleotides (isolated by thin-layer chromatography) and into nucleic acids (estimated by radioisotope content of acid-insoluble material) (11).

Determination of protein and relative water contents. Protein content was determined on supernatant fractions from 25% to 33% homogenates of tissue slices (prepared as for incubation) in 50 mM Hepes (pH 7.5) using the Bio-Rad protein-dye binding assay. The assay was linear for protein concentrations of 200 to 800 μg bovine serum albumin/ml. Relative water content was determined by drying weighed aliquots of tissue to constant weight at 60°C in a convection oven and was calculated as 1 − (dry weight/fresh weight).

Data presentation and statistical analyses. Data are presented on a dry-weight basis as a mean ± SE with number of replicates (N) given in parentheses. Each datum represents net incorporation over nonenzymic (background) incorporation measured using heat-inactivated tissue. Statistical significance was established using one-way analysis of variance (ANOVA); least significant difference (LSD) was used where significant differences among three or more means were indicated by ANOVA (6).

Results

A 28-fold increase in fruit dry matter occurred mainly in the peel during the first 2 months of development (Table 1). Juice vesicles completely filled the locules of the endocarp during this period, but the endocarp contributed only 11.1% ± 0.2% (N = 4) of the dry matter of 2-month-old fruit. Endocarp plus central axis tissues underwent an 111-fold increase in dry matter during the last 6 months of development and were the major source of dry matter in fruit that met legal criteria for maturity [California law requires a total soluble solids : total (titratable) acid ratio of 8:1, referred to as 8-month-old fruit in Table 1]. Relative water content increased during development (Table 1). Expression of metabolic data on a dry-weight basis avoided bias due to the increase in water content.

Capacity of whole-fruit tissue to synthesize nucleotides during Stage I of development. The capacity of whole-fruit tissue to synthesize purine and pyrimidine nucleotides *de novo* increased 3- and 6-fold, respectively, during Stage I of development from petal drop to 2 months past petal drop (Table 2). No changes in uridine salvage or catabolism were detected during the second month of development (Table 2). Whole-fruit tissue protein content decreased slightly during the second month of development (Fig. 1).

Fruit transverse diameter, fresh weight, and relative water content were significantly different for two populations of 1-month-old fruit selected on the basis of their significantly different growth rates to represent "persisting" and "abscising" fruit (Table 3). However, significant differences were not observed between persisting and abscising populations in their capacity to metabolize pyrimidines or purines, with the possible exception of uracil catabolism (Table 3).

Capacity of peel tissue to metabolize purines and pyrimidines during development. Peel tissue isolated from 2-month-old fruit exhibited one-half the purine *de novo* synthetic capacity observed for whole-fruit tissue (Tables 2 and 4). Pyrimidine *de novo* synthetic capacities of peel and whole-fruit tissues were similar (Tables 2 and 4). Since peel tissues contribute 83% of the dry matter of whole 2-month-old fruit and all other incubation conditions were uniform, comparisons between the capacity of whole-fruit and peel tissues are justified. Equal uptake of the radiolabeled bicarbonate ion by these two tissues types is assumed in making such comparisons.

Incorporation of [8-^{14}C]adenine into nucleic acids decreased during development; i.e., 300 ± 105 (N = 2) nmol [8-^{14}C]adenine were incorporated into nucleic acids per g dry weight of peel tissue from 2-month-old fruit during a 3-hr incubation as compared to 37 ± 13 (N = 4) nmol incorporated by peel tissue from fruit collected during the last 3 months of development ($P < 0.05$). However, the capacity of peel tissue from Stage II and III fruit to synthesize purine nucleotides, either *de novo* or by salvage of adenine, was not significantly different from that for peel tissue from Stage I fruit (Table 4).

The capacities of peel tissue from Stage II and III fruit to synthesize pyrimidine nucleotides both *de novo* and by salvage of uridine was signficiantly less than those of peel tissue from 2-month-old fruit (Table 4). Uridine catabolism by peel tissue from these older fruit also decreased significantly compared to peel tissue from 2-month-old fruit (Table 4). Protein content of peel tissue decreased during development from 2 months past petal drop to commercial maturity (Fig. 1).

Discussion

Dry matter distribution between peel and internal tissues during the first 2 months of fruit development was consistent with previous observations (5, 7) and confirmed the duration of Stage I of development in the fruit used.

A 6-fold increase in the pyrimidine *de novo* synthetic capacity

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Table 1. Changes in contribution of fruit tissue to dry matter content of 'Washington' navel orange fruit.

Transverse diam (mm)	Fruit age (months)	Total dry wt (g)[z]	Percentage of total fruit wt		Relative water content	
			Peel	Endocarp + central axis	Whole fruit (%)[y]	Peel (%)
3.7 ± 0.1	0	0.04 ± 0.00[x]	74.9 ± 2.2	25.1 ± 2.2	68.9 ± 1.4	---[v]
11.9 ± 0.4	1	0.29 ± 0.03	77.5 ± 0.7	22.6 ± 0.6	74.3 ± 0.4	---
22.9 ± 0.6	2	1.1 ± 0.04	82.9 ± 0.4	17.1 ± 0.4[w]	77.3 ± 0.2	77.3 ± 0.4
52.8 ± 1.0	5	---	---	---	---	81.8 ± 0.5
65.6 ± 1.2	8	34.8 ± 0.5	38.8 ± 0.6	61.2 ± 0.6	---	87.1 ± 0.6
LSD (0.05)			3.4	3.4	2.6	1.5

[z]Dry weight of peel and endocarp plus central axis summed.
[y]Calculated as (fresh weight − dry weight)/fresh weight; tissue prepared as for incubation.
[x]Mean ± SE of two to five fruit collections.
[w]Endocarp and central axis assessed separately and summed.
[v]Not determined.

Table 2. Purine synthetic and pyrimidine synthetic and catabolic activities in whole-fruit tissues during Stage I of 'Washington' navel orange development.

Fruit age (months)	Incorporation of precursor into product[z] (nmol/g dry wt during 5 hr)			
	Purine metabolism	Pyrimidine metabolism		
	de novo	*De novo*	Salvage	Catabolism
0	98 ± 14[y]	10 ± 6	---	---
1	168 ± 26	20 ± 4	357 ± 44	653 ± 33
2	293 ± 71	57 ± 3	282 ± 50	603 ± 110
LSD (0.05)	118	14	146	320

[z]Precursors were [^{14}C]bicarbonate (purine and pyrimidine *de novo*) and [2-^{14}C]uridine (salvage and catabolism). Products were adenine nucleotides plus adenosine converted to and isolated as adenine (purine *de novo*), total uridine nucleotides converted to and isolated as UMP (pyrimidine *de novo* and salvage), and CO_2 (catabolism).
[y]Mean ± SE of three to five replicates.

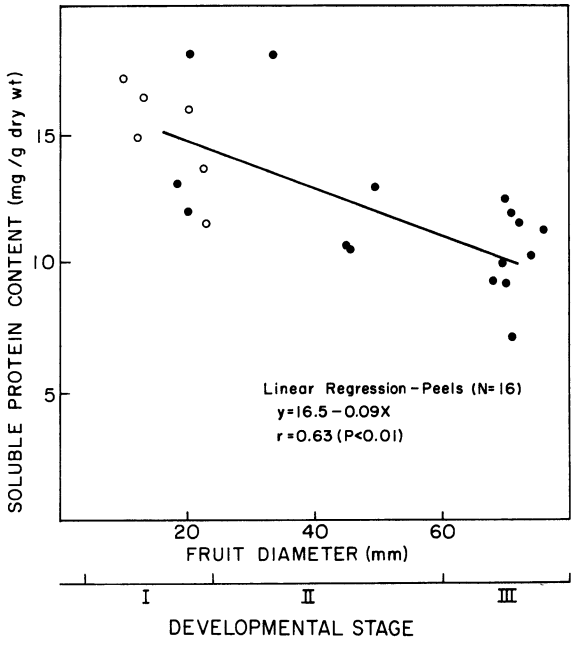

Fig. 1. Protein content of whole-fruit (○) or peel (●) tissue from 'Washington' navel oranges, expressed as milligrams of bovine serum albumin equivalents per gram dry wt, as a function of fruit transverse diameter and stage of development. Each point represents the average determination for a single collection of eight or more fruit.

occurred during the first 2 months of development. A 3-fold increase in purine *de novo* synthetic capacity occurred during the same period. Tissue protein content cannot account for these increases. Increased orotate pathway activity for *de novo* synthesis of pyrimidine nucleotides observed during early development apparently was not a response to decreased salvage or increased catabolism since uridine salvage and catabolism did not change significantly during the second month of development. Preliminary data (not shown) suggest that an increase in the activity of the final steps of the orotate pathway (conversion of orotic acid to UMP) occurs during the second month of development, which parallels the observed increase in pyrimidine *de novo* biosynthetic capacity from [^{14}C]bicarbonate: i.e., 50 nmol [6-^{14}C]orotic acid incorporated into ΣUMP per g dry weight of whole-fruit tissue from 13-mm-diameter fruit during a 3-hr incubation period (N = 1) vs. 298 ± 22 (N = 3) nmol per g dry weight of whole-fruit tissue from 20-mm-diameter fruit.

In general, the transition from growth predominantly by cell division to that by cell enlargement is centrifugal; that is, it proceeds from the interior to the exterior of citrus fruit (2, 7). The similarity of pyrimidine *de novo* synthetic capacity in whole-fruit and peel tissues from 2-month-old fruit suggests that peel tissue, where cell division predominates throughout the first 2 months of development, undergoes an increase in capacity. This increase is consistent with those reported for other plant tissues growing by cell division (1, 8). Further evidence of the association between growth by cell division and increased pyrimidine nucleotide biosynthetic activity is provided by the decrease observed in pyrimidine nucleotide synthetic capacity (*de novo* and by salvage) of peel tissue following the completion of Stage I. The decrease in capacity of peel tissue to synthesize pyrimidine nucleotides during development also was observed in subsequent years using fruit selected based on transverse diameter (unpublished data). The increase observed in the capacity of whole-fruit tissues to synthesize purine nucleotides *de novo* during Stage I, however, may be due to increases occurring in the endocarp and central axis. The capacity of peel tissue from 2-month-old fruit is lower than that of whole-fruit tissue from the same fruit and did not decrease significantly during the last two stages of development. A high capacity of internal tissues to synthesize purines *de novo* is consistent with the predominance of these nucleotides over pyrimidine nucleotides in juice of sweet oranges (4).

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Table 3. Characteristics defining Stage I 'Washington' navel orange fruit selected to represent "persisting" and "abscising" fruit and purine and pyrimidine metabolic activities of these two populations.

Parameter assessed	Population assessed: Persisting	Abscising
Characteristics defining populations		
Growth rate ($mm \cdot wk^{-1}$)	3.2 ± 0.3 (5)[z]a[y]	0.5 ± 0.1 (5) b
Transverse diameter (mm)	11.9 ± 0.4 (5) a	6.0 ± 0.4 (5) b
Fresh wt (g)	1.2 ± 0.1 (6) a	0.3 ± 0.0 (4) b
Relative water content (%)	74.3 ± 0.4 (5) a	65.6 ± 0.8 (4) b
Nucleotide metabolic activities (nmoles precursor incorporated into product per g dry weight during 5 hr)		
De novo synthesis		
[^{14}C]bicarbonate → [^{14}C]ΣUMP[x]	19 ± 8 (2) a	18 ± 4 (2) a
[^{14}C]bicarbonate → [^{14}C]ΣAde	168 ± 26 (3) a	112 ± 11 (2) a
Salvage		
[2-^{14}C]uridine → [^{14}C]ΣUMP	383 ± 62 (2)	305 (1)
[2-^{14}C]uracil → [^{14}C]ΣUMP	70 ± 14 (2)	48 (1)
[6-^{14}C]adenine → [^{14}C]nucleic acids	287 ± 64 (3) a	508 ± 210 (2) a
Catabolism		
[2-^{14}C]uridine → $^{14}CO_2$	685 ± 16 (2)	589 (1)
[2-^{14}C]uracil → $^{14}CO_2$	1314 ± 142 (2)	783 (1)
[6-^{14}C]adenine → $^{14}CO_2$	301 ± 133 (3)	174 (1)

[z] Mean ± SE of (N) replicates.
[y] Means in rows designated with a or b were separated by ANOVA ($P < 0.05$).
[x] Precursor → product. ΣAde, adenine nucleotides plus adenosine converted to an isolated as adenine; ΣUMP, total uridine nucleotides converted to and isolated as UMP.

Table 4. Purine and pyrimidine synthetic and catabolic activities in isolated peel tissue from 'Washington' navel orange fruit collected during each stage of development.

Fruit age (months)	Developmental stage	Incorporation of precursor into product (nmol/g dry wt during 5 hr): *De novo*	Salvage	Catabolism
		Purine metabolism[z]		
2	I	150 ± 18[y]	795 ± 165	10 ± 2
5	II	134 ± 28	836 ± 253	20 ± 3
8	III	106 ± 34		
LSD (0.05)		99	864	12
		Pyrimidine metabolism		
2	I	43 ± 7	264 ± 33	583 ± 51
5	II	11 ± 3	53 ± 18	59 ± 20
8	III	8 ± 1	41 ± 2	199 ± 59
LSD (0.05)		13	55	184

[z] Precursors used were [^{14}C]bicarbonate (*de novo*), [8-^{14}C]adenine (salvage and catabolism); products were adenine nucleotides plus adenosine converted to and isolated as adenine (*de novo*), total adenine nucleotides (salvage), CO_2 (catabolism).
[y] Mean ± SE of three to five replicates.
[x] Precursors used were [^{14}C]bicarbonate (*de novo*), [2-^{14}C]uridine (salvage and catabolism); products were total uridine nucleotides converted to and isolated as UMP (*de novo* and salvage), CO_2 (catabolism).

Reductions in activity of metabolic pathways can result from a nonspecific decrease in protein content or from a reduced availability of energy needed to fuel metabolic processes. The decrease observed in pyrimidine nucleotide synthetic capacity (*de novo* and salvage) following completion of Stage I could not be accounted for by the decreasing protein content of tissue. In addition, there were no parallel changes in free glucose content or respiratory activity of the tissue to account for the observed decrease (data not shown). The failure to observe a similar reduction in capacity to synthesize purine nucleotides provides additional evidence that the decrease observed is specific for pyrimidine nucleotide synthesis.

The majority of fruit abscission occurs during the first stage of growth, the potential of fruit to persist through this early, fruit-setting stage of development being associated with its rate of growth. These observations, taken together with the association observed between the capacity to synthesize pyrimidine nucleotides and growth by cell division, suggest that fruit differing in their potential to set also may differ in pyrimidine nucleotide biosynthetic capacity. However, the capacities to synthesize (*de novo* or salvage) either pyrimidine or purine nucleotides were statistically similar despite the demonstration of significant differences in other characteristics between these two populations. In addition, nucleic acid synthetic activities of the two populations were statistically similar. A difference in the potential to set between the populations of fruit used was verified in a parallel study on adjacent trees conducted by C.J.L. Of the 83 fast-growing fruit present 1 months past petal drop, 55% set (i.e., persisted through the second month past petal drop), while none of the 76 slow-growing fruit set. Thus, it is apparent that metabolic parameters other than pyrimidine and purine nucleotide synthesis are more important in determining the growth rate and fruit-set potential of navel orange fruit.

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Endogenous Gibberellins and Cytokinins in Spear Tips of *Asparagus officinalis* in Relation to Sex Expression

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Abstract. **Endogenous gibberellins (GA) and cytokinins (CK) were extracted from asparagus (*Asparagus officinalis* L.) spear tips, purified, and determined by lettuce hypocotyl and amaranthus bioassays, respectively. There was no quantitative difference in GA-like activity between heterogametic male and female spears. The major GA fraction in asparagus spears has 1 OH group. Asparagus spears contain 3 major fractions of CK-like activity. Fraction 1 eluted from Sephadex LH-20 and C_{18} HPLC columns with or before zeatin-riboside. Fractions 2 and 3 eluted in a similar pattern to IPA-riboside and IPA, respectively. There were higher levels of CK fraction 2 and trends toward higher levels of fraction 1 and total CK in female than in heterogametic male spears. There were also higher CK:GA ratios in female than in heterogametic male spears. The data support the hypothesis that sex in asparagus is controlled in part by CK levels or by CK:GA ratios.**

A. officinalis is a dioecious plant in a genus that contains both monoecious and dioecious species (1). Flowers from female plants of *A. officinalis* have rudimentary stamens and show little variation in flower morphology (23). Male plants have flowers with rudimentary ovaries and exhibit variations both within and among plants for the relative development of ovaries and stamens (18, 23, 30). Andromonoecious plants occur in low frequencies and are being used by plant breeders to develop all-male cultivars of asparagus (18), because male plants produce higher yields (24, 29) and have greater longevity in the field (10, 32). The sexes also differ in foliar morphology, which may influence plant productivity (2).

The mechanism of sex determination at the metabolic level is not understood. However, application of plant growth regulators have been shown to modify sex expression in asparagus. Lazarte and Garrison (17) showed that a cytokinin, *N*-(phenylmethyl)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine (PBA), promoted the development of styles and ovaries in male plants when applied to emerging spears at the time flower buds were differentiating. Gibberellin A_3 promoted the development of the rudimentary stamen of the female flower.

The literature contains many examples of the relationship between endogenous plant growth substances and sex expression in plants (4, 6–8, 25). Endogenous GA was higher in male plants while endogenous CK was higher in female plants of *Cannabis sativa* and in *Spinacia oleracea* (16).

There is increasing evidence that the ratios of endogenous plant growth regulators are as important in the control of plant development as the absolute levels of hormones. For example, the CK of in vitro plant tissue cultures interacts with auxins in controlling root and shoot differentiation (26, 31); the mechanism of apical dominance apparently is tied to an antagonism between auxins and CK (28); a current hypothesis on the bud dormancy of temperate woody plants is based on a GA–abscisic acid balance (19); and there appears to be an auxin–ethylene relationship to stem and root growth (19).

The ability to control the sex expression of *A. officinalis* or other dioecious species would facilitate the incorporation of desirable traits into breeding lines and allow for the routine development of inbred lines.

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