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Steroid profiles of transgenic tobacco expressing an *Actinomyces* 3-hydroxysteroid oxidase gene

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Abstract

Previously, we have shown that the expression of a 3-hydroxysteroid-oxidase gene in transgenic tobacco initiated a series of biochemical events leading to the conversion of sterol to stanol. As a result, the plants maintained a diminished sterol pool and a modified relative sterol ratio but demonstrated no observable morphological abnormalities. The maintenance of normal higher plant physiology in the absence of particular sterols or in the presence of modified sterol ratios is controversial. In this report, we present additional biochemical and physiological characteristics of transgenic tobacco expressing an *Actinomyces* 3-hydroxysteroid-oxidase gene. The total steroid accumulated in the transgenic plants is 6-fold higher than in control plants and consists of sterol, 3-ketosteroid and stanol. The relative abundance of sterols within whole plant and individual organs is grossly altered as ethylated side chain sterols account for 99% of the total sterol pool in the transgenic tobacco. Stigmasterol is readily apparent in all tissues and cholesterol is found at measurable levels in specific organs, while campesterol and sitosterol are detected at trace levels in the transgenic plants. Stanols and 3-ketosteroids accumulate in all tissues and represent 77% of the measurable steroid pool in the transgenic plants. The sum of sterol, the respective 3-ketosteroid plus stanol provide a relative abundance of steroid, which is similar to the abundance of sterol accumulated in control tissue. In vitro photosynthetic electron transport measurements demonstrate altered activity of chloroplasts under a variety of reaction conditions, indicating a link between the modified steroid pool and a modulation of chloroplast membrane function.

Keywords: *Nicotiana tabacum*; Tobacco; Transgenic; Cholesterol; Campesterol; Sitosterol; Stigmasterol; Stanol; 3-Ketosteroid; 3-Hydroxysteroid oxidase; Chloroplast; Photosynthesis

1. Introduction

Sterols are thought to stabilize plant membranes, with the specific structures of accumulated sterols playing large roles in conferring stability to membranes and modulating the activity of resident membrane enzymes (Schuler et al., 1991; Cooke et al., 1994; Popp et al., 1995 and Grandmougin-Ferjani et al., 1997). The most commonly occurring sterols in higher plants are those, which possess a double bond at C5 in the B-ring of the sterol nucleus (see Fig. 1). These sterols include sitosterol (**7**), stigmasterol (**10**), and campesterol (**4**), and they predominate in most-higher plant species examined, including our tobacco model (Fig. 1). These three sterols differ from one another with respect to saturation of the side chain and the level of alkylation of

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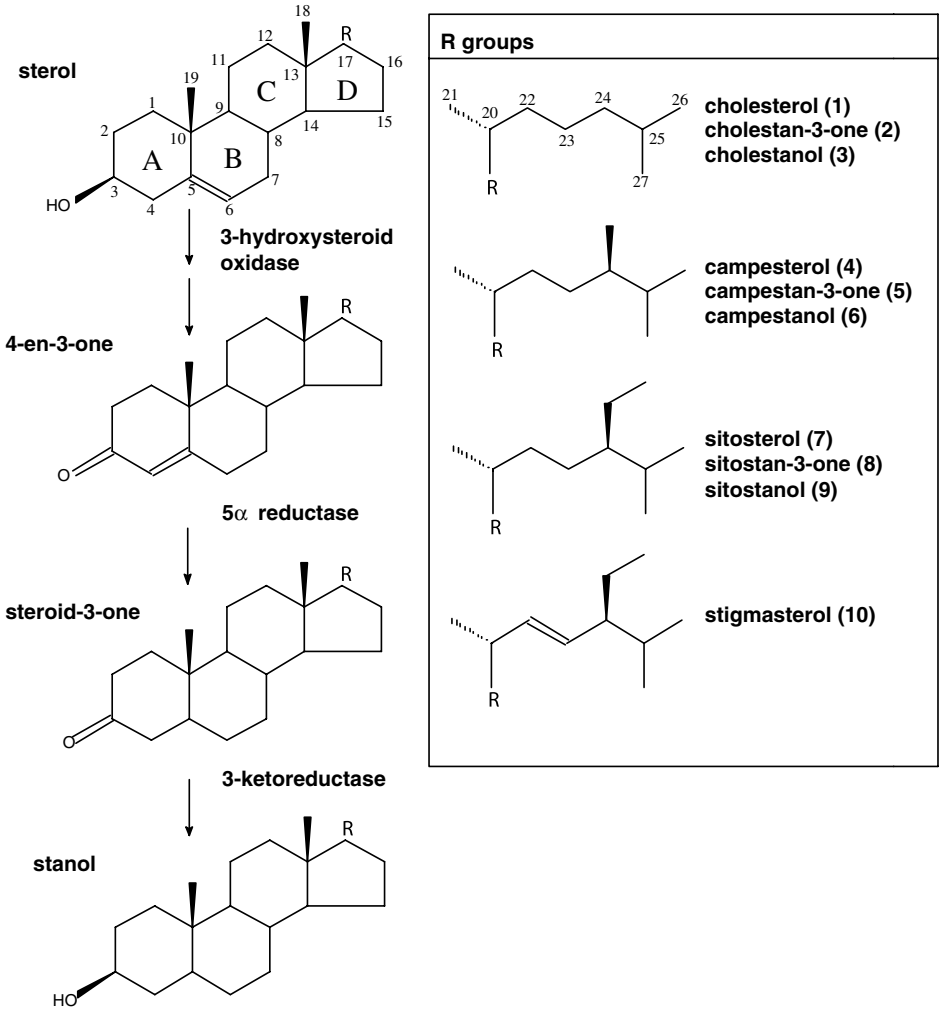

Fig. 1. Proposed pathway for the production of stanol in transgenic tobacco plants expressing a chloroplast-targeted 3-hydroxysteroid oxidase gene. The 3-hydroxysteroid oxidase catalyzes the initial oxidation and reduction of the sterol while the subsequent reduction of the 3-ketosteroid to stanol occurs by an uncharacterized endogenous plant enzyme. The aliphatic groups in the side box show side chain structures of the four 3-hydroxysterols detected in the transgenic tobacco. 3-Ketosteroids and stanols with side chain structures corresponding to cholesterol (**1**) (cholestan-3-one (**2**) and cholestanol (**3**)), campesterol (**4**) (campestan-3-one (**5**), campestanol (**6**)) and sitosterol (**7**) (sitostan-3-one (**8**) and sitostanol (**9**)) were observed in transgenic tobacco expressing the chloroplast-targeted 3-hydroxysteroid oxidase. No steroid was observed having both the unsaturated stigmasterol (**10**) side chain and a saturated nucleus.

the side chain at C24; campesterol (**4**) maintains a methyl addition, while stigmasterol (**10**) and sitosterol (**7**) maintain ethyl additions, and stigmasterol (**10**) is unsaturated at C22 (Fig. 1). The requirement for particular sterols in specific tissues for normal plant growth and development has become a popular view (Maillot-Vernier et al., 1991; Chappell et al., 1995; Schaller et al., 1998 and Fujioka and Sakurai, 1997). The observations that: (1) sterols accumulate in a developmental fashion, (2) modified ratios of sterols decrease plant growth rate, and (3) the requirement of sterols for the production of essential plant hormones have provided convincing evidence for the requirement of specific sterols in certain tissues for normal plant ontogeny. It is well established that unique sterol structures modulate the activity of enzymes within the membrane environment, as demonstrated by Cooke et al. (1994) and Grandmougin-Ferjani et al. (1997), who showed that cholesterol (**1**) and stigmasterol (**10**) stimulate the activity of the plasma membrane H^+-ATPase 2- and 1.5-fold, respectively. Additionally, Schaller et al. (1998) demonstrated that modification of the relative ratio of campesterol (**4**) to sitosterol (**7**) correlated with a 10–50% reduction in stem length in tobacco. However Corbin et al. (2001) reported healthy and vigorously growing transgenic *Nicotiana tabacum* which maintained less than 50% of wild type sterol levels, while the levels of other steroids, including stanols, were markedly elevated. Considering the strict relationship demonstrated between sterol structure and higher plant ontogeny reported in previous studies (Schaller et al., 1998) it is curious that a modification of the

standing sterol pool of this magnitude has no obvious impact on plant ontogeny.

Stanols are commonly occurring molecules that have been reported in many groups of organisms, including higher plants (Ida et al., 1981; Teshima and Patterson, 1981; Corbin et al., 2001; Moreau et al., 2002). Like their sterol counterparts, stanols maintain planar stereochemistry, suggesting similar function(s) in higher plants. Stanols, however, maintain a molecular nucleus that is devoid of a double bond (Fig. 1). The importance of the double bond to steroid function is speculative, however all higher plants accumulate sterols bearing a double bond within the B-ring, implying the evolution of a highly conserved structure–function relationship (Moreau et al., 2002). A physiological function for stanol in higher plant ontogeny has been recently described, as advances in Brassinosteroid (BR) research revealed a primary function for campestanol (**6**) as an intermediate in BR biosynthesis in higher plants (Fujioka and Sakurai, 1997; Noguchi et al., 1999). Furthermore, Moreau et al. (2002) and others demonstrated the accumulation of sitostanol (**9**) and campestanol (**6**) in cereal grains, suggesting physiological roles for these molecules. However, a direct relationship between membrane stanol levels and higher plant ontogeny has not been well established. Recently, Corbin et al. (2001) reported the conversion of 55% of the steroid pool to stanol in an analysis of mature, transgenic tobacco. The transgenic plants lacked observable morphological alterations stemming from the altered sterol pool suggesting: (1) the presence or absence of the double bond within the steroid nucleus does not influence its function in higher plant ontogeny, (2) the levels of sterol are maintained in excess of levels required to support basal physiological needs in this system, and/or (3) the stanol is sequestered in a fashion that mitigates any potentially negative impact on physiology or development. Regardless of the assertion, these results have confounded the relationship between sterol structure, distribution, titer and sterol effects on higher plant physiology.

As a further step in our continuing examination of the relationship between steroid structure, titer and function in tobacco, we assessed transgenic plants expressing a 3-hydroxysteroid oxidase gene for steroid accumulation during vegetative development. Our research group has examined the side chain and nuclear chemistry of bulk steroid in organs of mature control and transgenic tobacco to document the levels and the relative abundances of steroids in transgenic tissues accumulating altered steroid profiles yet maintaining normal ontogeny. Furthermore, due to the enhanced levels of steroid observed in the chloroplasts of these transgenic plants, we have seized upon the opportunity to assess the physiological ramifications of a modified steroid pool on select membrane associated processes in the chloroplast.

2. Results and discussion

2.1. Steroid content of control and transgenic tobacco expressing a 3-hydroxysteroid oxidase gene

Previously, it was shown that the *Actinomyces* sp. 3-hydroxysteroid oxidase enzyme expressed in transgenic tobacco facilitated the metabolism of a variety of sterols to their respective stanols without altering normal ontogeny (Corbin et al., 2001). In this report, we demonstrate that the action of 3-hydroxysteroid oxidase facilitates the modification of the sterol budget of the aerial tissues of mature, vegetative tobacco, with a dramatic reduction in observable levels of sitosterol (**7**) and campesterol (**4**). The transgenic tobacco described in this study accumulates sterol, oxidized sterols and stanol totaling approximately 27 mg of steroid per gram dry weight (Table 1 and Fig. 1). This level of accumulation represents an approximate 6-fold increase in total steroid levels in the transgenic tobacco compared to control tobacco (Table 1). The level of total steroid reported in these transgenic tobacco plants is consistent with data reported by Maillot-Vernier et al. (1991), Gondet et al. (1994) and Chappell et al. (1995), who demonstrated up to 10-fold increases in total steroid accumulation in tobacco in response to various genetic manipulations targeting sterol biosynthesis. Leaf and stem tissue from our transgenic and control tobacco plants were harvested between 45 and 55 days of growth for steroid analysis. The levels of campesterol (**4**), isofucosterol and sitosterol (**7**), which typically combine to account for approximately 40% of the representative sterol pool in control tobacco, occur at only trace levels in the transgenic tobacco, making up less than 0.001% of the representative steroid pool. Stigmastanol, which was reported by Venkatramesh et al. (2003) as a product of the *Streptomyces* 3-hydroxysteroid oxidase in transgenic *Glycine max* and *Brassica napus*, is not observed in our system, likely due to a difference in the specificity of the two enzymes. The *Actinomyces* enzyme may be less effective in utilizing stigmasterol (**10**) as a substrate compared to sitosterol (**7**), campesterol (**6**) and cholesterol (**1**). Five additional oxidized steroids, which account for 6 percent of total steroid, are present in the transgenic tissues but have yet to be structurally identified (data not shown). Control *N. tabacum* xanthi, transformed with an empty transformation vector, used in this study maintain a bulk accumulation of sterols, including: isofucosterol (~1%), cholesterol (**1**) (~9%) campesterol (**4**) (~20%), sitosterol (**7**) (~15%) and stigmasterol (**10**) (~50%), constituting approximately 5 mg total sterol per gram dry weight, which is consistent with values reported by Chappell et al. (1995) and Schaller et al. (1998) (Table 1 and Fig. 1). Stanol and oxidized steroid, present in abundance in the transgenic tobacco plants, were determined to occur at trace or

Table 1
Phytosteroid composition[a] of control *N. tabacum* and transgenic *N. tabacum* expressing a 3-hydroxysteroid oxidase gene

	Sterol	3-Ketosteroid	Stanol	% of total steroid pool
Control[b]				
Isofucosterol	63	n.d.[h]	n.d.	1
Cholesterol (**1**)	424	n.d.	n.d	9
Campesterol (**4**)	1038	n.d.	n.d.	21
Sitosterol (**7**)	734	n.d.	n.d.	15
Stigmasterol (**10**)	2651	n.d.	n.d.	54
Total	4910	n.d.	n.d.	100
Line 500103[c]				
Cholesterol (**1**)	300(5)[d]			1
Stigmasterol (**10**)	6031(95)[d]			22
Cholestan-3-one (**2**)		3380 (25)[e]		12
Campestan-3-one (**5**)		4339(32)[e]		16
Sitostan-3-one (**8**)		5947(43)[e]		22
Cholestanol (**3**)			1719(24)[f]	6
Campestanol (**6**)			2355(33)[f]	9
Sitostanol (**9**)			3159(43)[f]	12
Total	6331(23)[g]	13666 (50)[g]	7233 (27)[g]	100

[a] Values shown are averages (μg/g dry wt) for three pooled cohorts, 10 individual plants each, with a variance of 3–5% for each steroid.
[b] Control *N. tabacum*.
[c] Transgenic *N. tabacum* expressing a 3-hydroxysteroid oxidase gene.
[d] Percent of individual sterol within the summed sterol pool.
[e] Percent of individual 3-ketosteroid within the summed 3-ketosteroid pool.
[f] Percent of individual stanol within the summed stanol pool.
[g] Percent contribution of each steroid class to the total steroid pool.
[h] Not detected (less than 0.1 μg/g dry wt).

undetectable levels in control tobacco and therefore were not included in the calculation of the total steroid budget for control tissues.

The transgenic lines presented here are the same as those examined in Corbin et al. (2001), and while the morphology of the plants is similar, the reported steroid accumulation profiles differ. The discrepancy is likely due to a combination of factors, including the growth conditions to which the plants were subjected, changes in the accumulation patterns of steryl esters in the plants throughout development and the experimental design of the research. It is clear from current results that steroid in these plants maintain a developmental distribution pattern, suggesting that the difference in results observed between the two studies is an artifact of tissue selected for analysis. Corbin et al. (2001) reported steroid contents obtained from small samples from intact plants to provide snap-shots of 3-hydroxysteroid oxidase action in the plant, while the current study initiates a complete, organ-specific assessment of steroid accumulation profiles generated by 3-hydroxysteroid oxidase action throughout ontogeny. Considering the intents and designs of the two studies, it is not unexpected that the levels of steroid reported differ. Interestingly, no 3-ketosteroid was reported in previous work (Corbin et al., 2001), and while it is possible that the authors overlooked these steroid pools it is more likely that the tissue analyzed did not maintain obvious levels of 3-ketosteroid or the 3-ketosteroid maintained the form of a steryl ester, which were not closely examined in the previous study. A similar report examining the action of a 3-hydroxysteroid oxidase in transgenic *Glycine max* and *Brassica napus* by Venkatramesh et al. (2003) also reports the presence of stanol while not reporting the accumulation of 3-ketosteroid, suggesting the production and accumulation of this steroid may be transitory or developmental in nature.

In previous work with this transgenic system, steryl esters were not apparent (Corbin et al., 2001). While the appearance of steryl esters is expected in both control and transgenic tobacco (see Maillot-Vernier et al., 1991) the intermittent occurrence of these molecules in this transgenic system appears to be largely regulated by environmental conditions (unpublished observation – Grebenok). Recently in our laboratory, we have determined that steryl ester exceeding 30% of the total steroid pool in particular organs occurs in the transgenic plants grown under short day conditions (unpublished observations – Grebenok). The authors are currently undertaking a complete analysis of steryl ester accumulation and distribution in the control and transgenic lines grown under differing environmental conditions to further clarify the observed relationship. It is possible that steryl esters account for the differences observed between the free sterol pools reported by Corbin et al. (2001) and those reported here.

2.2. Steroid accumulation profile in control and transgenic tobacco expressing a 3-hydroxysteroid oxidase gene

The transgenic tobacco plants lack appreciable levels of campesterol (**4**), isofucosterol, and sitosterol (**7**), while stigmasterol (**10**) and cholesterol (**1**) are present in relative abundance (Tables 1 and 2). In control plants campesterol (**4**), sitosterol (**7**) and stigmasterol (**10**) are present in all tissues analyzed (Table 2). The dramatic reduction of isofucosterol, campesterol (**4**) and sitosterol (**7**) that we observe in the present transgenic system has not been previously reported in higher plants that typically contain these sterols. It is possible that isofucosterol, campesterol (**4**) and sitosterol (**7**) occur in each organ at levels below our limits of detection, and it is equally likely that isofucosterol, campesterol (**4**) and sitosterol (**7**) esters occur in specific organs, providing a pool of these sterols for various physiological and biochemical needs.

We report an accumulation in a combined 3-ketosteroid pool of approximately 13 mg of steroid per gram dry weight in the transgenic tobacco. Cholestan-3-one (**2**) is abundant in select organs, while campestan-3-one (**5**) and sitostan-3-one (**8**) accumulate in all tissues examined (Tables 2 and 3). The mass spectrum for each of the 3-ketosteroids was obtained and is consistent with previous studies (Noguchi et al., 1999). The prominent mass ions for cholestan-3-one (**2**) were as follows: *m*/*z* 386 [17%], 371 [7%], 231 [100%] and 217 [26%]. Campestan-3-one (**5**) maintains prominent mass ions of: *m*/*z* 400 [18%], 385 [8%], 231 [100%] and 217 [29%]. Sitostan-3-one (**8**) produces the following prominent mass ions: *m*/*z* 414 [20%], 399 [10%], 231 [100%] and 217 [28%]. We presume that the 3-ketosteroid intermediates are derived from the respective corresponding sterols. We surmise that the elevated levels of 3-ketosteroid in our transgenic tobacco system are accounted for by the enzymatic activity of the 3-hydroxysteroid oxidase supplementing endogenous enzyme action (e.g., the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (Motteran et al., 2001)), followed by conversion of the steroid intermediates to 3-ketosteroid by an endogenous enzyme similar to the 5α steroid reductase described by Noguchi et al. (1999) (Fig. 1). The elevated levels of 3-ketosteroid in the transgenic system can be explained if a regulatory feed-back system acts at the level of the 3-ketoreductase, with 3-ketosteroid, stanol, or some other downstream product of the pathway acting as the feed-back agent for carbon movement through the pathway. It should be noted in this context that 3-ketosteroid and stanol maintain similar distribution patterns throughout the plant. Alternatively, due to the exaggerated levels of 3-ketosteroid and stanol, these molecules may be localized to lipid droplets similar to that which was reported by Gondet et al. (1994), rendering a large portion of the steroid pool isolated from active metabolism. Steroids maintaining structures typical of early BR pathway intermediates, other than the 3-ketosteroids, were not detected at elevated levels in the transgenic plants.

We report the accumulation of 7.2 mg of total stanol pool per gram dry weight in our transgenic tobacco. Sitostanol (**9**), campestanol (**6**) and cholestanol (**3**) were found at substantial levels in each tissue examined. The accumulation pattern of sterol, 3-ketosteroid and stanol parallel each other with the highest levels of each steroid observed in the leaves forming the lower-mid section of the plant, which is consistent with sterol accumulation patterns in tobacco observed by Chappell et al. (1995). The occurrence of sterol, 3-ketosteroid and stanol all bearing the same side chain chemistries in each organ analyzed suggests an organ-specific production of stanol. However, due to the hyper-accumulation of sterol, 3-ketosteroid and stanol in the lower-mid section of the plant, the possible transport of the sterols, 3-ketosteroids and or stanols from other organs to this region of the plant cannot be discounted. Devarenne et al. (2002) established the activity of squalene synthase, a key regulatory enzyme in the sterol biosynthetic pathway, to be localized to the apical meristem in tobacco. This identified the apical meristem as a potential source of sterol for the remainder of the plant and suggests steroid transport from the meristem to the observed sites of accumulation. However, it is also accepted that each tobacco cell maintains the ability to produce its own sterol Chappell et al. (1995). Whether the observed steroid accumulation profile is a product of transport or localized synthesis and destruction, or a product of the combined metabolic events, cannot be discerned from the current data. However, several observations about this system provide evidence for factors potentially regulating the production of sterol in these transgenic plants. Clearly, the accumulation of 3-ketosteroid and stanol in mature tobacco does not elicit an inhibitory regulatory response at key regulatory enzymes in the sterol biosynthetic pathway, as they exceed sterol levels in the control lines by 3-fold. Whether this is due to their sequestration and removal from active metabolism (e.g., lipid droplets) or their chemical nature remains to be determined. The level of stigmasterol (**10**) present in the transgenic tobacco is comparable to the level of the mixed sterol pool observed in control plants, suggesting that the level of stigmasterol (**10**) is likely not driving enhanced steroid accumulation. However, it is possible that the increased movement of carbon through the sterol biosynthetic pathway is in direct response to the reduced pools of campesterol (**4**) and sitosterol (**7**) observed in the transgenic tissues. Regardless of the mechanism by which sterol accumulation is regulated within a given tissue, the expression of the 3-hydroxysteroid oxidase gene is driven by a 35S promoter, which

Table 2
Phytosteroid composition of leaves from control *N. tabacum* and transgenic *N. tabacum* expressing a 3-hydroxysteroid oxidase gene[a]

	Leaf number[b]																						
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Stem[e]
Control[c]																							
Isofucosterol	–	–	1	8	9	–	6	11	7	–	3	–	–	3	–	–	–	2	1	–	–	–	12
Cholesterol (**1**)	17	15	31	23	56	51	–	–	34	53	33	54	23	13	4	–	1	–	3	–	–	–	13
Campesterol (**4**)	35	33	54	42	54	94	70	82	63	74	49	90	54	53	28	26	14	32	18	–	–	–	73
Sitosterol (**7**)	15	6	1	54	23	28	58	23	45	54	21	89	58	–	–	–	73						
Stigmasterol (**10**)	75	84	154	177	181	143	208	209	144	203	154	128	143	121	93	55	101	84	53	–	–	–	141
Total	142	138	241	304	323	316	336	325	293	384	260	361	278	241	156	93	159	141	99	–	–	–	312
Line 500103[d,f]																							
Cholesterol (**1**)	2	27	24	–	–	11	6	5	–	–	68	–	84	7	–	–	57	–	–	–	–	–	8
Stigmasterol (**10**)	96	83	53	16	164	113	31	70	163	163	1099	966	113	760	596	104	453	165	160	52	119	94	396
Cholestan-3-one (**2**)	66	103	–	6	–	32	18	20	–	–	854	611	113	613	386	66	315	72	21	–	–	–	84
Campestan-3-one (**5**)	29	61	50	20	130	49	54	48	91	75	484	462	86	640	445	460	386	171	77	135	40	17	173
Sitostan-3-one (**8**)	129	70	40	–	149	90	56	64	139	188	979	861	101	652	563	681	428	113	101	143	51	97	252
Cholestanol (**3**)	7	57	46	4	27	5	8	7	27	32	324	232	91	402	14	66	45	95	15	30	19	28	137
Campestanol (**6**)	29	69	56	5	38	16	25	13	58	60	353	276	107	472	116	134	93	77	58	59	45	46	150
Sitostanol (**9**)	50	64	37	8	27	26	31	25	77	197	588	475	137	137	315	238	143	105	86	70	61	85	173
Total	408	534	306	59	535	342	229	252	555	715	4749	3883	832	3683	2435	1749	1920	798	518	489	335	367	1373

[a] Values shown are averages (μg/g dry wt) of three cohorts maintaining ten plants each. Variances in steroid titer of 3 to 5% for each organ is typical.

[b] Leaves were numbered beginning with the most distil leaf on the plant and numbered in a consecutive fashion to the apical meristem. Leaf one was omitted from each sample due to poor physical appearance.

[c] Control *N. tabacum* plants maintain 20 leaves.

[d] Transgenic *N. tabacum* expressing a 3-hydroxysteroid oxidase gene.

[e] Stem represents all aerial tissue of the plant minus leaves and petioles.

[f] Trace levels (less than 0.1 μg/g dry wt) of sitosterol or campesterol are not reported.

Table 3
Comparison of side chain chemistries from control and transgenic *N. tabacum* steroids[a]

	Reduced[c] side chain	Methylated[d] side chain	Ethylated[e] side chain
Control[b]			
Isofucosterol			63
Cholesterol (**1**)	424		
Campesterol (**4**)		1038	
Sitosterol (**7**)			734
Stigmasterol (**10**)			2651
Total	424(9)[g]	1038(21)[g]	3448(70)[g]
Line 500103[f]			
Cholesterol (**1**)	300		
Stigmasterol (**10**)			6031
Cholestan-3-one (**2**)	3380		
Campestan-3-one (**5**)		4339	
Sitostan-3-one (**8**)			5947
Cholestanol (**3**)	1719		
Campestanol (**6**)		2355	
Sitostanol (**9**)			3159
Total	5339(19)[h]	6694(25)[h]	15137(56)[h]

[a] Values shown are averages (μg/g dry wt) for three pooled cohorts, 10 individual plants each,with a variance of 3–5% for each steroid.
[b] Control *N. tabacum*.
[c] Steroid class without additional carbon at C24 of the steroid side chain.
[d] Steroid class maintaining an additional methyl group at C24 of the steroid side chain.
[e] Steroid class maintaining an additional ethyl group at C24 of the steroid side chain.
[f] Transgenic *N. tabacum* expressing a 3-hydroxysteroid oxidase gene.
[g] Percent contribution of each sterol class to the total sterol pool of the control plant.
[h] Percent contribution of each steroid class to the total steroid pool of the transgenic plant.

implies the constant presence of 3-hydroxysteroid oxidase in every leaf throughout development. Therefore, it is likely that the levels of 3-ketosteroid and stanol in each organ is a direct result of 3-hydroxysteroid oxidase action on the resident sterols in that tissue at each developmental stage. Low levels of sterol observed in the lower and apical tissues in both control and transgenic plants could arise from a number of processes, including export, degradation, and/or the down regulation of sterol synthesis in these tissues. Regardless of the cause, we predict that the low levels of sterol in a given tissue would result in low 3-ketosteroid and stanol levels for that given tissue in the transgenic plant.

2.3. The relative abundance of steroid accumulated in organs obtained from control and transgenic tobacco expressing 3-hydroxysteroid oxidase gene

Previous research by Cooke et al. (1994), Popp et al. (1995) and Grandmougin-Ferjani et al. (1997) demonstrated a functional association between sterol structure and membrane-associated enzyme action in artificial membranes. In addition, Schaller et al. (1998) and Corbin et al. (2001) observed that the relative abundance of sterol appears to play an important role in successful growth and development of tobacco. In control tissues a similar relative abundance of sterol is maintained in each organ, clearly demonstrating a consistent pattern of ethylated, methylated and chemically reduced side chain sterol accumulation. However, tissues from the transgenic plants fail to accumulate control levels of campesterol (**4**), isofucosterol and sitosterol (**7**), creating a condition in which the relative abundance of sterol is altered when compared to controls (Table 4). However, a more expected relationship between steroid accumulation and ontogeny becomes apparent when the sum of sterol, the respective 3-ketosteroid plus stanol is examined in light of side chain chemistry. The relative abundances of steroids in the transgenic plants are similar to the relative abundances of sterols found in comparable control tissues. It is possible that the 3-ketosteroid and or stanol can supplant sterols in physiological processes throughout the plant (e.g., bulk membrane functions), allowing the trace levels of sterols (e.g., campesterol (**4**) and sitosterol (**7**)) to be utilized in more specific biochemical processes throughout ontogeny. Behmer and Elias (2000) demonstrated that in grasshoppers given a low cholesterol (**1**) diet, cholesterol (**1**) is ''spared'' and sequestered to fulfill unique biochemical functions, while other sterols are used to fulfill bulk membrane roles. Furthermore, the use of stanol in the context of normal ontogeny of higher plants is consistent with the observations of Corbin et al. (2001) and Venkatramesh et al. (2003), that normal growth was apparent despite a reduction in overall sterol titer. In addition, Moreau et al. (2002) and others reported the accumulation of sitostanol (**9**) and campestanol (**6**) in certain tissues of cereal grains, implying physiological function for these steroids. The contribution of steryl-esters to the overall steroid accumulation profile has yet to be fully examined, so any conclusions regarding the functional replacement of isofucosterol, campesterol (**4**) and or sitosterol (**7**) by modified steroids in particular roles throughout the plant would be premature.

2.4. The modulation of photosynthetic electron transport capacity in chloroplasts obtained from transgenic plants expressing a 3-hydroxysteroid oxidase

In an effort to understand the effect of the localization of the 3-hydroxysteroid oxidase within the chloroplast on sub-cellular steroid accumulation we examined the steroid accumulation profiles of the chloroplast. We have observed a substantial (exceeding 30-fold) enhancement of steroid levels in intact chloroplasts isolated from the transgenic tobacco in comparison to control chloroplasts (unpublished observation – Grebenok). As a result, we compared photosynthetic electron transport rates in these

Table 4
The relative abundances of steroids in control and transgenic tobacco expressing a 3-hydroxysteroid oxidase gene[a]

	Leaf number[b]																						Stem[h]
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
Control[c]																							
Reduced[d]	0.2	0.2	0.2	0.1	0.3	0.3	–	–	0.2	0.2	0.2	0.3	0.1	0.1	–	–	–	–	–	–	–	–	0.1
Methylated[e]	0.4	0.4	0.4	0.2	0.3	0.6	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.2	0.4	0.1	0.3	0.2	–	–	–	0.3
Ethylated[f]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	–	–	–	1
Line 500103[g]																							
Reduced	0.3	0.9	0.5	0.2	0.1	0.2	0.3	0.2	0.1	0.1	0.5	0.4	0.8	0.7	0.3	0.2	0.5	0.4	0.1	0.1	0.1	0.1	0.3
Methylated	0.2	0.7	0.7	1	0.5	0.3	0.6	0.4	0.4	0.3	0.3	0.4	0.8	0.7	0.4	0.6	0.5	0.6	0.4	0.7	0.4	0.2	0.4
Ethylated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

[a] Steroid classes are represented as abundances relative to the dominant steroid class within the organ (methylated/ethylated and reduced/methylated).
[b] Leaves were numbered beginning with the most distil leaf on the plant and numbered in a consecutive fashion to the apical meristem. Leaf one was omitted from each sample due to poor appearance.
[c] Control *N. tabacum plants* maintain 20 leaves and a steroid pool composed entirely of sterol.
[d] Reduced steroids maintain a side chain free of C24 modification.
[e] Methylated steroids maintain a methyl addition at C24 of the steroid side chain.
[f] Ethylated steroids maintain an ethyl addition at C24 of the steroid side chain.
[g] Transgenic *N. tabacum* expressing a 3-hydroxysteroid oxidase gene maintains a steroid pool composed of sterols, 3-ketosteroids and stanols. The three classes of steroid are summed based upon side chain chemistry to calculate the relative abundances of each steroid class.
[h] Stem represents all aerial tissue of the plant minus leaves and petioles.

Table 5
Whole chain electron transport rates[a] of chloroplasts isolated from control and transgenic *N. tabacum*

Sample	Plant growth temperature (°C)[b]	Assay temperature (°C)		
		10	15	22
Control	10	49(6)	60(9)	113(7)
Line 500103	10	79(6)	120(6)	168(4)
Control	22	33(2)	83(3)	166(6)
Line 500103	22	60(9)	110(3)	172(6)

[a] Rates are means of at least 3 oxygen uptake measurements, expressed in micromoles O_2/mg chl/hr with SE shown in parentheses.
[b] Plants were maintained at the temperatures shown for 2 weeks prior to isolation of intact chloroplasts.

two sets of chloroplasts. Photosynthetic whole chain electron transport capacities of chloroplasts isolated from transgenic leaves maintain up to 2-fold higher rates when compared to control chloroplasts (Table 5). It is tempting to suggest that a modified steroid content of the thylakoid membranes of the transgenic tobacco assists in maintaining membrane fluidity or stability favorable for electron transport at low temperature, as increased stigmasterol (**10**) content in plasma membranes in oat shoots was shown to cause an increase in membrane fluidity (Cooke et al., 1994), and the prevention of transition to gel phase has been thought to combat chilling injury (Raison and Wright, 1983; Murata et al., 1992). However, a causal relationship between steroid accumulation in the thylakoid membranes of the transgenic tobacco and increased photosynthetic electron transport at low temperatures cannot be established at this time. Growth at 10 °C appeared to induce acclimation in both the transgenic and control material that resulted in higher rates of electron transport when measured at 10 °C compared to their respective 22 °C-grown plants. This acclimation appears to limit electron transport when measured at higher temperatures, but only in control chloroplasts.

It is odd that a chloroplast-targeted enzyme modifies products of a cytosolic pathway. However, Hartmann and Benveniste (1987) reported the occurrence of sterols in both thylakoid and envelope membranes of spinach chloroplasts, implying physiological roles for their localization to this organelle. Although a mechanism for the directed movement of sterol into the chloroplast has not been elucidated to date, Dormann and Benning (2002) have clearly defined a mechanism for the movement of phospholipids from the endoplasmic reticulum to the membranes of the chloroplast. Considering that it is generally accepted that sterol synthesis does not take place within the chloroplast (Rhomer, 1999), it is logical to predict that a mechanism for the movement of sterol into the chloroplast must exist. Whether this mechanism includes the dynamic flexibility to respond to changes in the subcellular environment, including the oxidation of sterols, is a focus of current research. A curiosity of the current results rests in the fact that the chloroplast-targeted enzyme facilitates such a marked change in the sterol budget of the entire plant. As was postulated by Venkatramesh et al. (2003), it is possible that the 3-hydroxysteroid oxidase enzyme lacks 100% retention within the chloroplasts or that degeneration of chloroplast integrity may allow for 3-hydroxysteroid oxidase action within the cytosol, generating the observed phenotype. Interestingly however, previous studies reveal that the expression of the 3-hydroxysteroid oxidase gene within the cytosol resulted in a sterile, stunted phenotype typical of a classical

Brassinosteroid mutant in transgenic tobacco (Noguchi et al., 1999; Corbin et al., 2001). The normal growth and ontogeny of the transgenic tobacco in the previous and current study implies that localization of the active 3-hydroxysteroid oxidase is largely not cytosolic. An alternative explanation for the current results asserts that particular levels of sterol, potentially including sitosterol (**7**) and campesterol (**4**), are essential for normal functions of the chloroplast and the oxidized steroid within the chloroplast does not function in the biochemistry driving the movement of sterol into the chloroplast. However, the factors controlling the distribution of the active enzyme throughout plant ontogeny need to be confirmed before the effects observed in the cytosolic and chloroplast steroid pools can be interpreted and explained.

In summary, we have provided further biochemical information on transgenic tobacco that contains a chloroplast-targeted 3-hydroxysteroid oxidase enzyme. The transgenic tobacco steroid pool is altered, containing sterol, 3-ketosteroid and stanol, but side chain chemistries of the collective steroid pool reflect those of control sterols. The plant distribution of steroid mirrors that of control sterols in which mature, fully expanded leaves maintain the highest levels of steroid. Our results confirm the work by Schaller et al. (1998) demonstrating that the relative abundance of steroid maintaining particular side chain chemistries is conserved during tobacco ontogeny. In addition it appears that campesterol (**4**) and sitosterol (**7**) are essential for normal ontogeny of tobacco as their oxidation correlates directly with the increased levels of steroid accumulated within the transgenic system. Lastly we have shown that the action of 3-hydroxysteroid oxidase modulates thylakoid membrane-associated biochemical processes, linking the modification of the sterol pool with a modulation in the capacity for photosynthetic electron transport. Combined with our previous studies, the present study clearly demonstrates that a chloroplast-targeted 3-hydroxysteroid oxidase drastically modifies the total steroid budget of tobacco without altering development while possibly enhancing some physiological processes.

3. Experimental

3.1. Tobacco transformation and plant growth

Tobacco plants expressing a 3-hydroxysteroid oxidase enzyme that had been transformed with a vector carrying the chloroplast-targeted 3-hydroxysteroid oxidase gene, pMON33814, were morphologically indistinguishable from control plants that were transformed with a control vector (Corbin et al., 2001). Sterol accumulation was examined in cohorts of plants representing three independent R2 lines, 50087, 50095 and 500103. All three lines demonstrate altered steroid metabolism, however, line 500103 consistently accumulated highest levels of steroid, therefore, line 500103 was chosen for further analysis. Control and transgenic plants were soil grown under artificial lighting conditions on a 16:8 light to dark regime at 23 °C in a controlled environment. Analyses were performed on cohorts of plants numbering at least 10, which were similar in size and leaf number. Three independent analyses of control and transgenic cohorts were averaged for the data presented. Leaves were numbered in a consecutive fashion, with the oldest leaf on the plant at the time of harvest designated as number one. Leaf number one was eliminated from analysis because it maintained a brown and senescing appearance. Cohorts were collected between 45 and 55 days post emergence, and the results of the analyses are presented as designated.

3.2. Steroid isolation and analysis

Extraction, isolation and characterization of free steroids were performed as previously described (Corbin et al., 2001). Lyophilized plant material was pulverized and extracted in ethanol for 24 h. Ethanol was reduced to dryness under a stream of nitrogen and resuspended in MeOH:water (7:3). The MeOH:water fraction was extracted by partitioning three times with equal volumes of water equilibrated hexane. The hexane phases were pooled and evaporated, with the obtained residue resuspended in a minimal volume of hexane (typically 100 μl). Sterols were analyzed by GC–MS using the following conditions: Inlet temp 250 °C, transfer line temp of 290 °C, and column oven temp programmed from 180 to 300 °C with the initial temp maintained for 1 min and the final temp for 20 min and a ramp rate of 10 °C/min. The column used was a glass capillary MS-5 column (30 m) (Restek) with a film thickness of 0.25 μm. Helium at a flow rate of 1.25 ml/min served as carrier gas. The Agilent 5973 mass selective detector maintained an ion source temp of 250 °C and a quadrapole temp of 180 °C. The structural identifications of cholestan-3-one (**2**), campestan-3-one (**5**) and sitostan-3-one (**8**) were accomplished by GC–MS with mass ratios in agreement with Noguchi et al. (1999).

3.3. Chloroplast isolation

Intact chloroplasts were obtained following the procedures of Melo et al. (1995). All steps were carried out at 4 °C and in dim room light. Freshly harvested and deveined leaves were placed in ice cold homogenization buffer described by Melo et al. (1995) and subsequently homogenized in a Waring blender. The crude homogenate was filtered through 8 layers of cheesecloth and centrifuged at 1000*g* for 2 min. The pellet was resuspended and washed 5 times in homogenization buffer followed each time by centrifugation at 1000*g*. The final pellet was

divided into aliquots and stored at −80 °C until used for electron transport assays and steroid analysis.

3.4. Whole chain electron transport and chlorophyll assays

Frozen chloroplasts were thawed, and uncoupled, whole chain electron transport was measured as light-induced oxygen uptake with a Clark type oxygen electrode (YSI) in a stirred, water-jacketed 1.75 ml chamber (Gilson), with temperature controlled by a circulating water bath. For each temperature, equilibration was for a minimum of 10 min prior to calibration and subsequent measurements. Two point calibration was performed at each temperature with air-saturated water and glucose/glucose oxidase. Data were collected and analyzed using a LabPro analog/digital converter with LoggerPro software (Vernier Software & Technology). Saturating light was provided by a 150 W halogen lamp source (Dolan-Jenner) directed through a fiber optic guide, an IR1-1 infrared filter (Dolan-Jenner) and a red cut-off filter (Corning 2-59). The assay buffer included 0.4M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 40 mM MOPS, pH 7.5, 30 mM methylamine-HCl, 0.1 mM methyl viologen, 0.4 mM KCN, 100 units superoxide dismutase (Sigma) and chloroplast sample at 5 μg chlorophyll.

Chlorophyll concentrations were determined following extraction into 80% acetone, according to Arnon (1949), using the extinction coefficients of MacKinney (1941).

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